Investigation of How Receptor Localization and Endocytosis Regulate CXCR4 Signaling and Post-Translational Modification

by

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Dedication

I would like to dedicate this thesis to all of the individuals who have helped and supported my education and research.

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I would like to acknowledge all of my mentors, collaborators, family, and friends for supporting me throughout my education and research. I would like to thank my committee members, Lois, Alexey, and Gary. I can recall specific examples where each of you were influential in advancing my research. Additionally, I would like to thank Alan Smrcka for being a mentor to me over the last year, challenging me to think about my research from a different perspective and significantly contributing to the direction of my current research. Lastly, I would like to thank my advisors, Santiago and Allen, for supporting my research and giving me the freedom to explore my own ideas. While not always fruitful, these experiences have truly shaped how I conduct research and think about science.

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Abstract

Due to their great specificity and regulatory role in nearly all cellular processes, G protein-coupled receptors (GPCR) are a common therapeutic target. Investigating the molecular mechanisms that regulate receptor activity is essential for understanding how cells sense and adapt to their environment. The traditional model for GPCR activation occurs at the plasma membrane. Upon agonist binding, receptors are quickly post-translationally modified and activate signal transduction cascades. Receptor post-translational modifications are believed to regulate signaling outcomes, and initiate receptor endocytosis and down regulation. Over the past decade, it has become increasingly apparent that GPCR localization is a master regulator of cell signaling. However, the molecular mechanisms involved in this process are poorly understood. Using CXC chemokine receptor 4 (CXCR4) as a model GPCR, my thesis work investigates how clathrin and clathrin-independent endocytic pathways and intracellular localization regulate CXCR4 signaling and post-translational modification.

In Chapter 2, I present data that supports an expanded role for endocytosis in regulating CXCR4 signaling and post-translational modification. Clathrin heavy chain knockdown resulted in increased steady state and agonist-induced ERK1/2 phosphorylation. Interestingly, CXCR4 lipid raft localization has been implicated as a positive regulator of ERK1/2 signaling. In support of this hypothesis, we found that lipid rafts were necessary for CXCR4-dependent ERK1/2 phosphorylation and clathrin knockdown increased CXCR4 colocalization with caveolin-1. During this work, we also identified a new antibody to study CXCR4 post-translational

modification and discovered that clathrin knockdown increases steady-state CXCR4 post-translational modification. Together, these results suggest that endocytic pathways compensate for one another and differentially modulate receptor signaling and post-translational modification.

In Chapter 3, we shifted our attention from understanding how plasma membrane microdomains differentially regulate CXCR4 biology to investigate how intracellular GPCRs contribute to signaling. While receptor activation is traditionally thought to occur at the plasma membrane, there is growing evidence that intracellular GPCR activation also contributes to cell signaling. Through this work we discovered a new mechanism of intracellular GPCR activation. We found that upon agonist stimulation, both plasma membrane and intracellular pools of CXCR4 are post-translationally modified in a β-arrestin-1-dependent mechanism. Interestingly, intracellular CXCR4 activation increased CXCL12-dependent *Egr1* transcription. Notably, these observations may explain that while CXCR4 overexpression is highly correlated with cancer metastasis and mortality, plasma membrane localization is not. Together these data support a model were a small initial pool of plasma membrane-localized GPCRs are capable of activating internal receptor-dependent signaling events.

Identification of protein-protein interactions is one of the first steps in identifying a molecular mechanism. However, due to difficulty isolating GPCR signaling complexes, many of the molecular players involved in these processes remain unknown. In Chapter 4, I share our progress using spatially resolved proteomics to identify new CXCR4 interacting partners and investigate how the CXCR4 interactome changes during endocytic trafficking. Finally in Chapter 5, I use my work as an example to illustrate the importance of understanding the assumptions of

biological measurements and highlight several cases where measurement assumptions influence result interpretation.

In summary, the presented data support an increased role for receptor localization as a master regulator of cell signaling. By improving our understanding of receptor regulation, I hope that this research can lead to the identification of new molecular mechanisms that regulate cell signaling and potentially therapeutic strategies that target aberrant GPCR function.

Chapter 1 Introduction

1.1 How do cells sense their environment?

The ability for a cell to sense the environment and appropriately respond is essential to life. Cell membrane receptors are one class of proteins used by cells to do so. Receptors are well suited for this task because of their structure. They have three major domains: an ectodomain that is extracellular and allows a receptor to bind molecules from the environment, a transmembrane domain that helps receptors to stably reside in the cell membrane barrier and serves as a signal transducer, and an intracellular domain that translates ligand binding into an actionable intracellular signal by changing receptor-protein interactions. Ligand-dependent receptor-protein interactions activate intracellular programs that allow cells to accurately respond to the extracellular stimuli. From receptors that respond to mechanical and electrical inputs to those sensitive to light or extracellular molecules and proteins, cells employ a diverse array of cell membrane receptors to sense and respond to their environment.

1.2 A brief history on cell membrane receptors

The idea that cells had cell surface (plasma membrane) molecules originated in the late 19th century from Paul Ehrlich[1]. Ehrlich hypothesized that mammalian cells had side chains on their surface that were responsible for sensing the extracellular environment. The "side chain theory" postulated the existence of receptors that bind antigens and identified these molecules as antibodies – which are analogous to what we know as B cell receptors today[1]. This idea was not

widely accepted. However, ideas from J.N. Langley at the turn of the 20th century built upon Ehrlich's hypothesis and postulated that receptors had two connected functions. Langley believed that receptors interacted with environmental signals and that upon binding, were able to activate intracellular effector molecules to alter cell function[2,3]. This idea was further developed by Henry Dale and Raymond Ahlquist who hypothesized the existence of different classes of receptors defined by their ability to differentially bind molecules[3–5]. Clark, Ariens, Stephenson, Black, and Furchgott later applied the law of mass action to describe receptor agonist binding as well as developed initial antagonists (β-blockers) to inhibit or modulate receptor function[6–8]. Since then, experimental advances using radiolabeled ligands as well as the purification and cloning of cell membrane receptors led to the universal consensus of the importance of this specialized class of proteins for cell physiology. By the early 21st century, through advancements in genetic editing, fluorescence microscopy, and modern cell and molecular biology techniques we had developed a foundational model for how cells use membrane receptors to sense the environment as well as an understanding that these activities are linked to cell function. However, how membrane receptor activation regulates diverse cellular function remains an active area of research. Today, cell membrane receptors represent approximately 4% of all translated proteins. The two major classes of ligand-dependent membrane receptors are receptor tyrosine kinases (RTK) and G protein-coupled receptors (GPCRs).

1.2.1 Receptor tyrosine kinases (RTKs)

RTKs were initially discovered by Levi-Montalini and Cohen when they were studying the effects of nerve growth factor (NGF) and epidermal growth factor (EGF) stimulation on cell growth[9–11]. These initial studies also identified receptor signaling as a potent cellular regulator as they found that compared to normal cells, cancer cells required less serum growth factors for cell

proliferation[12–14]. By this time, insulin was a proposed treatment for diabetes and radiolabeled ligand experiments quickly led to the identification of the insulin receptor[15]. Soon after, additional research identified that these receptors contained tyrosine kinase motifs and that receptor dimerization and clustering was important for function[16–19].

RTKs are single-pass membrane proteins that have an intracellular tyrosine kinase domain. Ligand binding activates intrinsic catalytic tyrosine kinase activity which leads to receptor phosphorylation[20]. While receptor dimerization is often driven by ligand binding, in some circumstances, RTKs oligomerization can occur independent of ligand and is necessary for ligand binding and kinase activity[21]. Phosphorylation of the C-terminal regulatory domain promotes recruitment of signaling proteins and endocytic adapters that facilitated downstream receptor signaling events and receptor internalization to intracellular vesicles.

Today, 58 human RTKs have been identified and shown to regulate a variety of cellular processes including cell proliferation, differentiation, and metabolism[20]. As a result, aberrant function is a hallmark of diseases such as cancer and diabetes. Epidermal growth factor receptor (EGFR) family members exemplify how both receptor overexpression and mutation lead to disease. EGFR family member, HER2, overexpression is found in approximately 30% of all breast cancers[22] and an activating EGFR mutation is associated with non-small cell lung cancer[23].

1.2.2 G protein-coupled receptors (GPCRs)

Defined by their seven transmembrane domain architecture[6], GPCRs are the most numerous and diverse class of cell membrane receptor[24]. Earliest evidence for the existence of GPCRs dates back approximately 1.2 billion years prior to the evolution of plants, fungi, and animals from a common ancestor[25]. Over time GPCR diversity has drastically expanded due to

gene, chromosome, and whole genome duplication events and led to their role in regulating nearly all cellular processes[25,26]. Over 800 GPCRs have been identified and are classified by sequence and structural similarity into 5 families: rhodopsin (family A), secretin (family B), glutamate (family C), frizzled/Taste2, and adhesion[27]. From regulation of immune response and neurotransmission, to cancer metastasis and pathogen invasion, GPCRs contribute to both normal and aberrant cellular functions[24,28]. GPCRs are known for tightly controlled and oftentimes tissue-specific expression profiles and ligand specificity. Consequently, these receptors have been targeted for a wide array of therapeutic applications from pain management (opioid receptors), cancer (chemokine receptors), viral infection (chemokine receptors), and cardiovascular disease (β-adrenergic receptors). A distinguishing feature from RTKs is that GPCRs themselves are not catalytically active and this property played an important role in their discovery.

While the discovery of cyclic AMP (cAMP) was an important initial step in identifying the mechanism of GPCR signaling[29], it wasn't until Rodbell and Gilman discovered that guanine nucleotide regulatory proteins were the intermediary effector molecules between activated receptors and adenylyl cyclase activity that the traditional mechanism of GPCR signaling was revealed[30,31]. Similarly to RTKs, radioligand binding assays were vital for early GPCR research and led to the proposition of the ternary complex model to explain β2-adrenergic receptor signaling[32]. This model postulated that the minimal system for receptor signaling consisted of a receptor, an agonist, and a G protein[32]. Purification and reconstitution of β2-adrenergic receptor on phospholipid vesicles confirmed this model and for the first time established that ligand-induced cAMP production was dependent on GPCR and G protein (Gαs) activation[33–36]. While receptor purification was a major advancement, cloning of the first GPCRs in the 1980s led to

the discovery of their conserved seven transmembrane structure and resemblance to rhodopsin[37]. Mutagenesis studies and generation of chimeric receptors by swapping receptor extracellular and C-terminal motifs contributed to our early understanding of how GPCRs coupled to G proteins as well as provided a connection between receptor sequence/structure and signaling activity – i.e. ligand binding and cAMP activity[38,39]. At this point, researchers began to investigate how GPCR signaling activities were regulated.

Tight regulation of receptor signaling is essential as cellular resources are limiting and hyperactivation can lead to a costly disproportionate response. Protein post-translational modification (PTM) is one way that cells regulate activity at the protein level. PTMs are small molecules (phosphate, methyl groups etc.) or small proteins (ubiquitin) that are enzymatically added to proteins to modulate protein structure, disrupt or facilitate protein-protein interactions, and/or function as a tag for protein degradation or localization. This mechanism is fast, reversible, occurs with great precision, and is used ubiquitously to regulate protein function.

Receptor phosphorylation was initially identified from an observation that β2-adrenergic receptor SDS-PAGE migration was altered post receptor stimulus and was subsequently found to regulate receptor signaling[40]. This led to the discovery of G protein coupled receptor kinases (GRKs) and characterization of their role in agonist-dependent GPCR phosphorylation[41–43]. At the time, it was known that rhodopsin was also phosphorylated and that a 48 kDa protein associated with phosphorylated receptors and regulated rhodopsin-transducin (transducin is a photoreceptor G protein) interactions[44]. This protein became known as arrestin and soon after related proteins, β-arrestins, were discovered to bind to phosphorylated GPCRs and regulate activity[45,46]. In the decades of research that followed, β-arrestins and GRKs have been shown to be master regulators for GPCR desensitization and signaling[6].

Unlike RTKs, GPCR function is not tied to intrinsic enzymatic activity but through coupling to small heterotrimeric GTPases, known as G proteins. Ligand binding stabilizes GPCR confirmations that promote G protein guanidine nucleotide exchange activity, which results in the exchange of GDP for GTP. This exchange results in the disassociation/activation of the G protein subunits from the activated GPCR. The heterotrimeric G protein subunits are α, β, and γ. The Gβ and Gγ subunits form a stable dimeric complex referred to as Gβγ. There are 16 Gα, 5 Gβ, and 12 Gγ G protein subunits that differentially interact with each other and each class of GPCRs[47]. This accounts for a massive number of potential combinations and consequently signaling diversity. The Gα subunit is the GTPase and is activated by receptor stimulus[48]. There are 4 Gα subfamilies that have different signaling implications. Gαs subunits activate adenylate cyclase while Gαi are inhibitory[48]. Gαq activates phospholipase C and $G\alpha_{12/13}$ regulates the actin cytoskeleton by directly activating RhoGEFs[48,49]. Upon Gα GTP exchange, the Gβγ dimer is released from the trimeric complex and interacts with a variety of downstream signaling molecules – Gβγ itself is not catalytic [50]. Early work illustrated that the Gβγ subunits are necessary for GPCR signaling as Gβγ prenylation helps membrane trafficking for Gα subunits[50]. Interestingly, structural studies using GPCR peptide mimics have also shown that Gβγ directly interacts with GPCRs[50]. In addition to mediating GPCR-dependent Gα interactions, Gβγ regulates a myriad of downstream signaling events itself. While initially shown to be important for regulating ion channel activation[51], Gβγ activation has also been linked to ERK1/2 activation[50]. Interestingly, most Gβγ-dependent signaling appears to arise from Gαi-associated GPCRs as Gβγ signaling processes are not universally activated by all GPCRs[50]. One of the major downstream implications of Gβγ activation is the recruitment and activation of GRKs to the plasma membrane to phosphorylate activated GPCRs[52,53]. GRK-mediated GPCR

phosphorylation serves 2 purposes: to facilitate the binding of adaptor proteins such as β-arrestins and to initiate receptor internalization by endocytosis. While β-arrestin binding was originally thought to arrest G protein signaling and prevent activated receptors from interacting with additional G proteins, recently G protein-independent β-arrestin signaling events have been reported suggesting a larger role for β-arrestins in regulating GPCR signaling[54].

1.2.3 Chemokine Receptors and CXCR4

Chemokine receptors are 50 rhodopsin-like family GPCRs that bind small, secreted proteins known as chemokines[55,56]. There are two groups of chemokine receptors –atypical and typical. Atypical chemokine receptors are believed to be involved in G protein-independent chemokine scavenging and not contribute to canonical G protein-dependent signaling[57]. There are four types of typical chemokine receptors and are defined by the localization of their N-terminal cysteine residues: CXC, CC, C, and CX3C[55,56]. These receptors primarily couple to Gαi, pertussis sensitive Gα subunits, which inhibit adenylate cyclase activity. However they have been shown to bind to the other Gα subunits as well including Gαq and $G\alpha_{12/13}$[58]. Chemokine receptors are primarily expressed in immune cells such as leukocytes and are known for their important role in regulating immune cell migration[57].

CXC chemokine receptor 4 (CXCR4) was initially discovered in leukocytes[59] and signaling activity is important for a variety of biological processes including immune cell homeostasis, embryonic development, and cell migration[60–65]. It is expressed in multiple different cell types including, leukocytes, hematopoietic progenitor and stem cells, lymphoid tissues such as bone marrow and lymph nodes as well as endothelial and epithelial cells[66]. CXCR4 signaling is activated by agonist (CXCL12 also known as SDF-1) binding and depending on CXCL12 isoform and/or CXCR4 C-terminal tail PTM, regulates cell proliferation

and growth, chromatin remodeling, or cell migration and chemotaxis[60,61]. Additionally, while CXCR4 can bind extracellular ubiquitin[67], macrophage migration inhibitory factor (MIF)[68] and HIV envelope protein gp120, CXCL12 is its only known chemokine ligand[58]. Conversely, CXCL12 also binds to CXCR7 – also known as atypical chemokine receptor 3 (ACKR3) – which has been shown to scavenge CXCL12 but not activate G protein signaling[58,69]. In contrast to other chemokine receptors, knockout of CXCR4 or CXCL12 leads to perinatal death in mice[58,62]. Interestingly CXCR7 knockout is also lethal and leads to aberrant cardiac development in rodents[58]. CXCL12 has greater that 90% nucleotide and protein sequence identity in humans and mice[70] and similar protein orthologs in other vertebrates such as Xenopus and zebrafish[71,72]. Together these observations suggest that there is significant pressure to retain the CXCR4/CXCL12 receptor pair during evolution and highlights the importance of the CXCR4/CXCL12/CXCR7 signaling axis.

Additionally, aberrant CXCR4 expression can lead to multiple pathologies including cancer and rheumatoid arthritis[58]. CXCR4 expression is dysregulated in 23 cancers and has been shown to increase cancer cell metastasis towards CXCL12 expressing cells[73–76]. Additionally, meta-analysis studies of breast and lung cancer patients have shown that CXCR4 overexpression is highly correlated with poor survival outcomes[77,78]. To date, efforts to pharmacologically target CXCR4 have been largely unsuccessful[79]. Plerixafor (AMD3100) is the only FDA-approved CXCR4 antagonist [80]. Plerixafor blocks CXCL12-mediated retention of hematopoietic stem cells in bone marrow, mobilizing them into the bloodstream for collection and autologous transplantation in non-Hodgkin's lymphoma and multiple myeloma patients[80]. Unfortunately, tumor cell mobilization is a potential side effect of this treatment and thus application of this drug is limited. Consequentially, CXCR4 drug development research has recently shifted away

from receptor antagonism to the development of bias-agonists and/or inhibiting downstream "pro-metastasis" pathways. CXCR4 is also well known for its role as a co-receptor for HIV and inhibition of CXCR4 has been investigated as treatment strategy to limit HIV infection[81]. Interestingly, WHIM syndrome (Warts, Hypogammaglobulinemia, Infection, and Myelokathexis) is the only known immunological disease directly resulting from CXCR4 mutagenesis[82,83]. WHIM syndrome arises from a deletion of the final 19 C-terminal residues of CXCR4 and leads to aberrant receptor activation and attenuated internalization[82,83].

CXCR4 signaling is largely thought to be G protein-dependent and the receptor has been shown to couple to multiple different Gα subunits – Gαi, Gαq, and $G\alpha_{12/13}$[58] – to activate the phosphatidylinositol-3 kinase pathway (PI3K/AKT) and the mitogen-activated protein kinase (MAPK/ERK1/2) signaling pathways as well as induce intracellular calcium mobilization[84]. It has also been associated with G protein-independent JAK-STAT signaling [58]. Similarly to other GPCRs, CXCR4 forms both homo- and heterodimers with other receptors [66,85,86]. This can occur either pre- or post-CXCL12 binding[85,86]. Interestingly, CXCL12 has also been shown to oligomerize at high concentrations which may contribute to agonist-dependent CXCR4 oligomerization[84]. It is thought that CXCR4 and/or CXCL12 oligomerization leads to distinct signaling outcomes[84,87] including potentially G protein-independent pathways; however this remains poorly understood [84].

1.4 The cell signaling bottleneck

While extracellular signals, receptors that detect them, and cellular responses are diverse, intermediary signaling hubs are often limited and conserved. This effectively creates a bottleneck effect during the signal transduction step. Consider the following example with Protein Kinase B (also known as AKT) signaling. AKT is a serine/threonine kinase and a centralized signaling hub

downstream of an array of signaling pathways that plays an important role in regulating cellular decision-making[88]. While mechanosensitve proteins such as integrins activate AKT, it is also activated by a multitude of ligand-activated receptors – such as GPCRs, RTKs and B cell receptor (BCR) – that bind fundamentally distinct ligands. Interestingly, while all of these receptor-signaling pathways converge on a signaling hub, the transcriptional outcomes are at least equally diverse as the extracellular inputs (Figure 1-1). From an evolutionary perspective, this is advantageous as reusing signaling machinery for multiple purposes preserves cellular resources. However, how cells are able to maintain input information diversity during this process remains poorly understood. There are several potential hypotheses to explain how multicellular organisms overcome this bottleneck effect: cell type specificity, ligand accessibility, and spatiotemporal signaling.

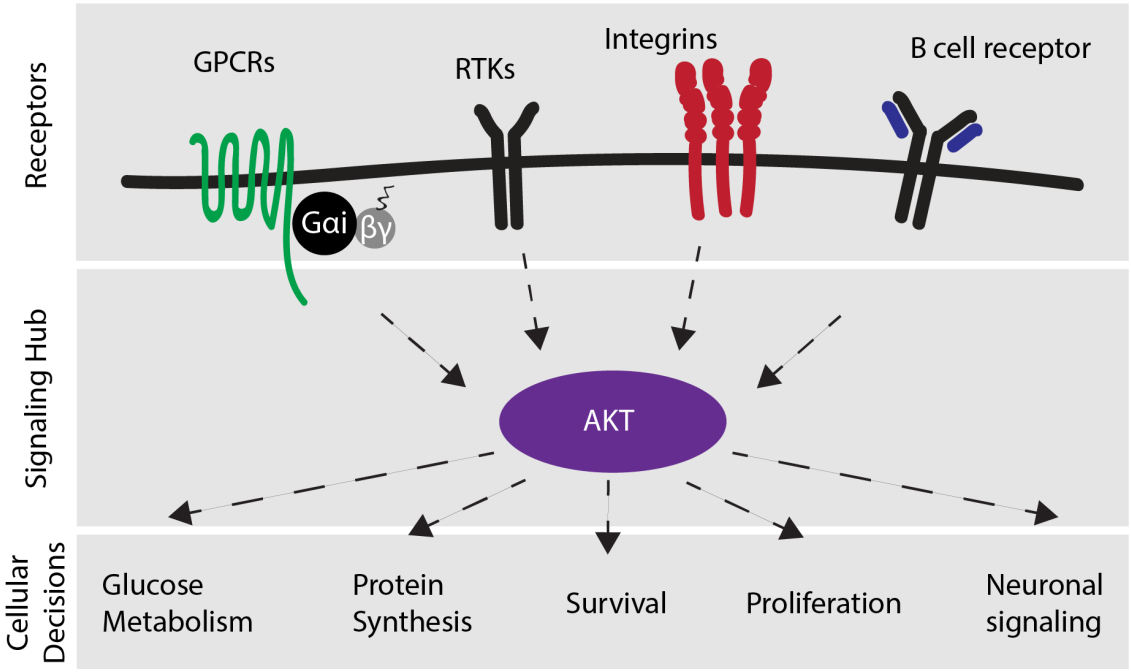


Figure 1-1 The signaling bottleneck dilemma

AKT is activated by a variety of different receptors to elicit distinct cellular responses.

1.4.1 Cell type specificity hypothesis

One mechanism that could resolve the cell signaling bottleneck is that receptor expression, intermediary signaling proteins, and/or transcriptional programs are cell type specific. The logic for this is that depending on cell type, cells only express a subset of all receptors and therefore relieve potential mixed signaling effects via a single centralized signaling hub. One of the reasons why cell membrane receptors are great drug targets is that their expression is oftentimes cell type specific. This is advantageous from a disease treatment standpoint as it limits unintended negative drug effects on non-target cells. Therefore, it is possible that by restricting expression of receptors that signal via the same signaling hub proteins, cells do not have to worry about deciphering different signals.

Cell type specific responses can also occur at the level of transcription. In this scenario, a single cell membrane receptor may activate different transcriptional programs depending on the cell type. This has been observed with CXCL12 stimulation. Researchers found that CXCL12 stimulation had profoundly different transcriptional responses in N15C6 (non-transformed prostate epithelial cells) and LNCaP (transformed prostate epithelial cells) cell lines [89]. This could be possible due to the differential expression of signaling co-factors such as transcription factors in different tissues as well as differential chromatin architecture that permit tissue-type specific transcriptional programming. Development is a common example of this as receptor expression or functional downstream signaling implications often evolve during differentiation.

1.4.2 Ligand accessibility hypothesis

A second mechanism that multicellular organism could use to overcome the cell signaling bottleneck is ligand accessibility. In a multicellular organism, extracellular signaling molecules

often originate at different parts of the organism. Therefore, if cells located in a different area rarely receive a signal then expressing multiple receptors that activate different transcriptional programs via a conserved signaling hub likely will not be an issue. This is apparent during development. A fundamental idea in embryogenesis is the ability for cells to differentially differentiate in space and time. Morphogen (ligand) gradients play an essential role in regulating this process as they provide cells with instructions of how and when to induce a specific and often dose-dependent transcriptional response to drive differentiation. Well-studied extracellular gradients include WNT, hedgehog, FGF and TGF-β ligands. While in some cases morphogen gradients have been shown to freely diffuse, oftentimes cells actively regulate this process either by endocytic uptake of morphogens[90] or the use of extracellular proteins such as heparin sulfate proteoglycans (HSPGs) to antagonize morphogen diffusion[91]. Interestingly, chemokines such as CXCL12 have been shown to interact with HSPGs to define ligand gradients[92,93]. Morphogen gradients have also been implicated in cell polarity in both embryogenesis[94,95] and chemotaxis which impacts cancer metastasis and other diseases[96].

1.4.3 Spatiotemporal hypothesis

Given that individual cells often express upwards of 100 different GPCRs[97], a third mechanism that cells use to overcome the cell signaling bottleneck is spatiotemporal signaling. This idea has rapidly gained acceptance in the field over the last decade as an increasing number of examples of spatiotemporal signaling have been described. In this mechanism, the result of the signaling event is dependent on the timing and location of the signal. For example, a receptor signaling at the plasma membrane may lead to a different signaling outcome than the same receptor when it signals from an intracellular compartment. Spatiotemporal GPCR signaling can

be divided into 3 major categories: plasma membrane microdomain specific, endocytic trafficking dependent, and independent GPCR signaling.

1.5 Plasma membrane microdomain dependent GPCR signaling

The plasma membrane is a highly heterogeneous environment composed of complex arrangements of lipids and proteins and over the past two decades the importance of different microdomains in regulating receptor signaling has become increasingly apparent[98,99]. To explore plasma membrane heterogeneity and its contribution is signaling regulation, researcher have developed a variety of tools and techniques to study membrane organization using fluorescent molecular probes that partition to areas of ordered or disordered membrane[100] and pharmacological sequestering agents of plasma membrane cholesterol to modulate plasma membrane microdomain composition.

Ordered membrane domains are often referred to as lipid rafts and have been implicated as an essential regulator of CXCR4 signaling. Early work showed that CXCR4 localizes to cholesterol-rich regions of the cell membrane and that this localization is important for HIV endocytosis into cells[101]. Localization in lipid rafts also impacts CXCR4 signaling. Malik et al. reported that CXCR4 localization in cholesterol-rich microdomains is required for ERK1/2 signaling[102] and lipid composition has also been implicated as a key regulator for CXCL12-dependent cell migration[102–104]. The importance of ordered microdomains has been also observed with other GPCRs as abolishment of lipid rafts using cholesterol-sequestering agents also reduces NH1 and Serotonin 1A receptor signaling[102,105,106].

Lipid rafts have also been shown to organize receptor signaling effector molecules such as G proteins and endocytic proteins[98]. Interestingly, β2 adrenergic receptor has been shown to differentially traffic to plasma membrane microdomains depending on whether the receptor was

recycled or newly synthesized via the biosynthetic pathway[107,108]. Researchers found that while β2 adrenergic receptors initially coupled to Gαs, after receptor recycling these receptors associated with Gαi in cardiomyocytes and speculated that this was due to enrichment of specific G protein subunits in different plasma membrane microdomains[108]. GPCRs can either traffic directly to lipid rafts or diffuse to lipid rafts upon ligand stimulus. GnRH receptor has been shown to almost exclusively reside in ordered membrane microdomains irrespective of ligand stimulus in specific cell lines[109]. On the other hand, the Veatch lab discovered that upon receptor stimulus, B cell receptors (BCR) cluster in ordered lipid microdomains and propose a model where these higher ordered structures are capable of sorting important regulatory effectors for BCR signaling[100,110]. Lipid rafts have also been shown to regulate receptor endocytic trafficking and consequently signaling[111]. Kinin B1 receptor translocation between ordered and disordered membrane domains has been shown to modulate its accessibility to different endocytic pathways[111]. Interestingly, aging disorders have also been linked to changes in membrane composition (specifically sphingosine kinase function) and GPCR signaling[111]. Together these data support a model were partitioning of receptors and downstream effector molecules in ordered vs. disordered plasma membrane microdomains allow cells to differentiate signaling response based on receptor localization.

1.6 Endocytic trafficking dependent GPCR signaling

While receptor endocytosis was initially thought to be solely involved with desensitization, over the last decade, it has become increasingly clear that endocytosis has a larger role in not only quenching, but also potentiating receptor signaling. Endocytic pathways are commonly divided into two major categories based on their dependency on clathrin: clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE). CME is the best-

characterized pathway and is well documented to play a major role in regulating GPCR signaling and internalization including CXCR4[61,102,112–114]. Comparatively, little is known about CIE, but it is becoming increasingly evident that these pathways also play a critical role in receptor regulation[115,116]. There are many types of CIE including caveolin-, flotillin-, and RhoA-dependent endocytosis[117]. Interestingly, significantly less is known about how the dynamic interplay between different endocytic pathways as well as how cell membrane heterogeneity regulates receptor biology.

Several groups have illustrated that transforming growth factor β (TGFβ) receptor and EGFR are internalized by both CME and CIE pathways and that the balance between these endocytic pathways is dependent on ligand concentration[115,116,118,119]. Initial evidence for this was the presence of internalized TGFβ receptors in both caveolin- (CIE) and EEA1 (early endosome antigen 1)- (CME) positive vesicles[118]. Interestingly, researchers found that CME internalization led to sustained TGFβ signaling. In contrast, CIE internalization increased Smad7-Smurf2 bound TGFβ receptor complexes and was associated with receptor turnover[118]. Others have investigated ligand-free internalization and have similarly shown that EGFR internalizes through clathrin-independent mechanisms upon UV radiation and oxidative stress [120,121]. In both cases (EGF and TGFβ receptors), differential receptor PTM was responsible for modulating the balance of CME- and CIE-dependent internalization[116,120,121]. Interestingly, CME and CIE internalization has also been observed with GPCRs[122–124]. While it is known that CME plays an important role in CXCR4 internalization and signaling[61,114,125], there is evidence to suggest CIE-dependent CXCR4 regulation. In contrast to an early report that did not find CXCR4 colocalization with caveolar (type of CIE) protein, cavin-1, positive vesicles[125], recent research has shown that

CXCR4 caveolar localization (membrane) is critical for CXCL12-induced phospho-ERK1/2 activation[102]. G proteins have also been shown to localize to CIE pits, including caveolae [126].

Endocytic machinery proteins themselves have also been implicated as important regulators of receptor signaling. Garay et al. found that knockdown of clathrin significantly reduced EGF-induced AKT phosphorylation and that this was independent of receptor internalization as perturbation of dynamin 2 did not affect EGF-induced AKT phosphorylation[127]. Others have postulated that specialized signaling endocytic pits exist. Rosselli-Murai et al. discovered that EGFR signaling was primarily initiated in short-lived clathrin-coated pits (CCPs) and that shifting the relative abundance of these pits by depleting tumor suppressor PTEN influenced EGFR signaling[128]. Together these data support an expanded role for endocytosis and endocytic proteins themselves as important regulators of signaling and not only as a mechanism to reduce plasma membrane localization and receptor desensitization.

In addition to regulating receptor signaling at the plasma membrane, receptor internalization has also been shown to be essential for complete signaling for some GPCRs. Initial evidence that endocytosis was required for complete GPCR signaling was facilitated by the use of cAMP biosensors that revealed sustained cAMP signaling post-receptor internalization[129,130]. This led to a new model for GPCR signaling and hypothesis that receptor signaling occurrs in two steps: first at the plasma membrane and secondly post-internalization at intracellular compartments such as endosomes. The development of receptor nanobodies – genetically encoded, fluorescently tagged small antibody fragments that recognize activated receptor conformations – confirmed this hypothesis and showed that β2 adrenergic receptors were indeed activated at endosomes to elicit a second wave of signaling[131]. Researchers discovered that endosomal GPCR activation was distinct from plasma membrane pools and

resulted in distinct downstream signaling consequences[132]. For β2 adrenergic receptor endosomal signaling was necessary for *PCK1* transcription[133] and alpha arrestin ARRDC3 was shown to regulate endosomal residence time for β2 adrenergic receptors to regulate this signaling response[134]. Since then endosomal signaling has also been reported for other GPCRs including CXCR4[135–138]. English et al. reported that blocking CXCR4 internalization significantly decreased CXCL12-dependent AKT signaling and concluded that this was due to the fact that endosomal signaling was necessary for complete AKT phosphorylation[135]. Endocytosis and endosomal signaling has also been linked to action potential firing. Researchers found that dopamine receptors (D1R) internalized much quicker than previously anticipated and that inhibition of endocytosis ablated agonist-dependent action potential firing in neurons[139]. Sustained agonist-induced luteinizing hormone receptor (LHR) and β1 adrenergic receptor ERK1/2 signaling was found to require both receptor internalization and correct targeting to endosomal compartments in a GIPC (Gai-interacting protein C-terminus)-dependent mechanism[97,140]. This finding highlights that, similarly to plasma membrane microdomains, there is compartmental bias at intracellular endosomes[97]. In addition to endosomes, GPCR signaling has also been shown at other intracellular endocytic compartments including late endosomes and has been shown to impact WNT signaling[141,142].

The role of β-arrestins in regulating localized GPCR signaling events remains unclear. While it was initially thought that G protein and β-arrestin-GPCR binding were mutually exclusive, recent biochemical and structural evidence suggest that β-arrestins, activated GPCRs, and G proteins can form stable mega-signaling complexes at intracellular compartments[143–146]. Using single particle electron microscopy, researchers were recently able to visualize a single GPCR in complex with β-arrestins and Gβγ. Furthermore, the researchers found that GPCR-β-

arrestin coupling conformation dictated downstream functions[147]. In cells, using parathyroid hormone receptor (PTHR) as a model, the Vilardaga group discovered that Gβγ and β-arrestins form functional intracellular/endosomal-signaling complexes[143,144]. Additionally, they recently revealed that Gβγ exchange between activated β2 adrenergic receptors and PTHR promoted sustained endosomal PTHR cAMP signaling[143].

1.7: Endocytic trafficking-independent GPCR signaling

GPCRs have also been shown to signal from intracellular compartments such as the nuclear membrane, endoplasmic reticulum (ER) and Golgi independent of endocytosis. While nuclear GPCR localization is well established, its role in receptor signaling remains poorly understood[148]. Multiple GPCRs have been shown to localize to the nuclear membrane including CXCR4[148], metabotropic glutamate receptors[149], and β-adrenergic receptors[150]. In fact, some GPCRs even contain nuclear localization sequences[148]. Researchers have postulated that nuclear GPCR localization are physiologically relevant as nuclear localization is conserved in both *C. elegans* and mammals[151] CXCR4 nuclear localization is correlated with metastatic potential in renal cell carcinoma (RCC) where increased CXCR4 association with HIF-1a has been shown to promote HIF-1a target gene transcription[148]. Interestingly, others have shown that many of the canonical GPCR signaling proteins also reside at nuclear membranes including G proteins, GRKs, adenylate cyclase, and β-arrestin-1[151] suggesting that nuclear GPCRs may be signaling-competent. In fact, researchers were able to activate β-adrenergic receptor agonist-dependent adenylate cyclase signaling using isolated nuclei from rat and mouse cardiomyocytes[150]. However, one outstanding question in this field is how nuclear GPCRs are activated in cells and whether this is ligand-dependent.

GPCRs have also been shown to localize at the endoplasmic reticulum (ER). GPR30, an estrogen receptor, is an example of ER-localized GPCR signaling. Upon estrogen stimulus, GPR30 has been shown to functionally regulate intracellular calcium mobilization and nuclear phosphatidylinositol 3,4,5-triphosphate synthesis[152]. Research investigating GABA B receptor subunits provides further evidence of ER-localized GPCR signaling. Without GABA B2, GABA B1 receptor subunits are often retained in the ER. While in some cell types GABA B2 is necessary for GABA B1 function, GABAB1 has much broader expression profile in the central nervous system compared to GABA B2 suggesting that intracellular GABA B1 are functional and physiologically relevant[151].

Many GPCRs have also been shown to localize to the Golgi and have functional implications[153–156]. δ opioid receptor Golgi localization in neurons has been reported for decades[155,157–159] and provided initial evidence for the importance of intracellular GPCR localization. There are two hypotheses for how Golgi localized receptors exist: endocytic trafficking of receptors to the Golgi post receptor internalization or active retention of newly synthesized receptors. Endocytic trafficking-dependent Golgi localization was recently reported for TSH receptors where trafficking to the Golgi post-receptor internalization was shown to induce specific Gogli-localized Gαs signaling[160]. Work from the Puthenveedu group recently illustrated that newly synthesized δ opioid receptors are localized at the Golgi and that their plasma membrane trafficking was regulated by kinase-phosphatase pair PI3K and PTEN competition[155,161]. COPII vesicles appear to be involved in receptor retrograde transport and Golgi retention[155,161]. Interestingly, increasing δ opioid plasma membrane localization sensitized mice to previously ineffective opioid agonist (SCN80) for pain management[155,161]. The physiological role of Golgi-localized δ opioid receptor in neurons remains elusive; however,

membrane permeable small molecule ligands have been shown to activate Golgi-localized δ opioid receptors[162] and others have postulated that this intracellular pool might be important for quick replenishment of plasma membrane receptors post-stimulus[156]. Additional work has revealed that specific C-terminal motifs R-X-R and K-X-K present on multiple GPCR C-terminal tails are involved in regulating GPCR-Golgi retention. Interestingly, CXCR4 also has a C-terminal K-X-K and may play a role in poor CXCR4 membrane localization in some cell types and tumor models [148,163] .

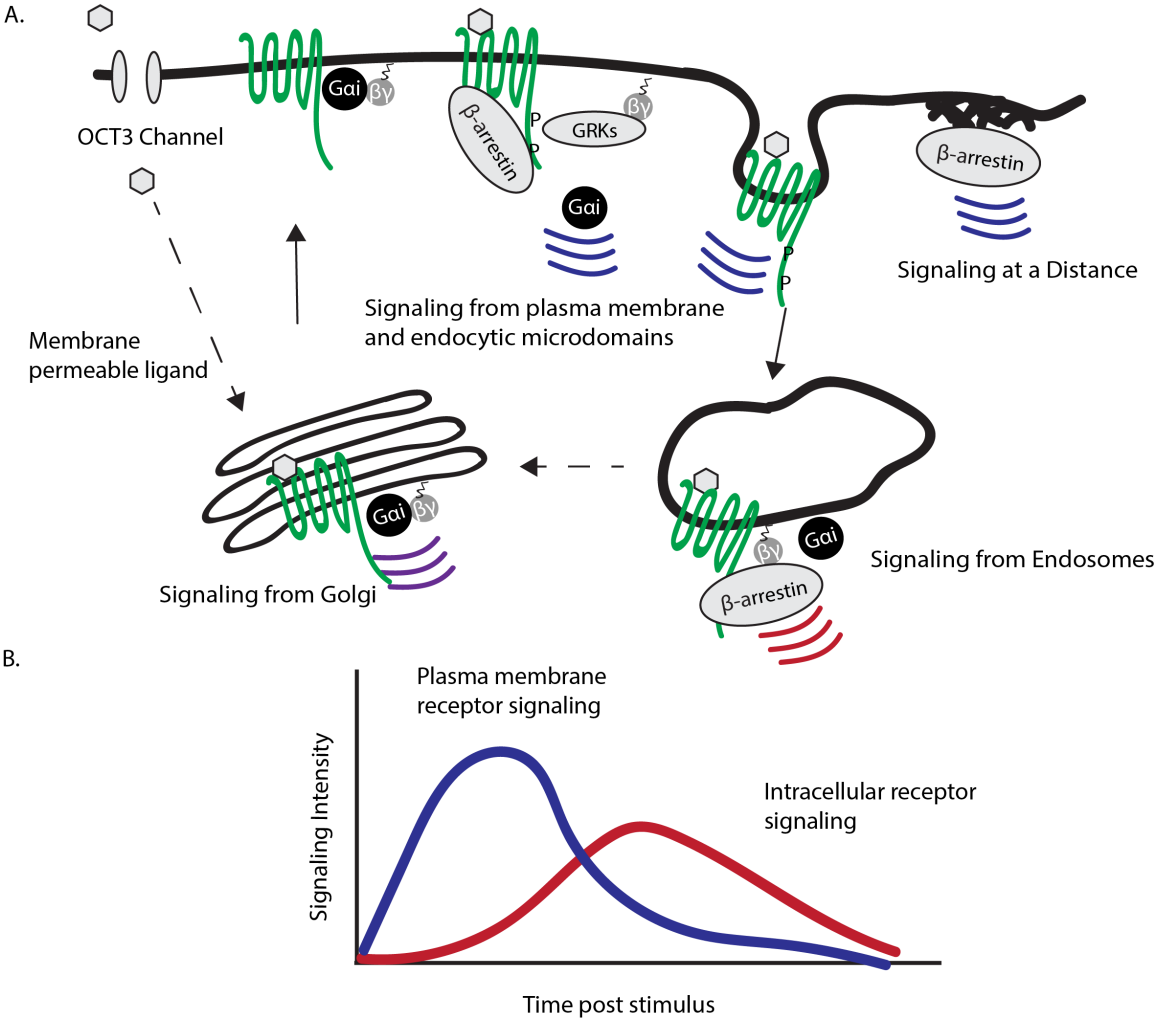


Figure 1-2 Overview of localized GPCR signaling within cells

(A) Schematic of GPCR activation at different plasma membrane and/or endocytic microdomains and intracellular compartments. Activation of Golgi-localized GPCRs has been shown to occur either by direct activation by membrane permeable ligands (OCT3 cation channel) or during endocytic trafficking from endosomes (TSH receptor trafficking). G proteins and β-arrestins are involved in propagating signaling at different locations. (B) There are multiple waves of receptor signaling events based on the location of the receptor. Plasma membrane-restricted events are thought to induce an initial fast signaling response (red lines) whereas intracellular signaling is believed to represent a second more attenuated wave (blue line). Interestingly, signaling from the Golgi can in theory occur at either time as it can occur either by direct ligand binding or during endocytic trafficking. Regardless, recent research supports that distinct downstream transcriptional programs are activated depending on where receptors are activated.

Golgi-localized GPCR signaling has also been suggested to play a role in cardiovascular function. β1 adrenergic receptor was recently shown to be present at the Golgi and be activated by dobutamine[153]. Researchers found that β1 adrenergic receptor signaling activity was dependent on a cation transporter OCT3 which actively pumps extracellular small molecule ligands into the cell where they are able to activate the Golgi-localized receptors[153]. G proteins have also been shown to localize to the Golgi to induce PI4P hydrolysis by PLCε[154]. Researchers found that selective inhibition of G protein signaling at the Golgi or prevention of norepinephrine transport via OCT3 blocked norepinephrine-dependent cardiac myocyte hypertrophy[154,164]. Together these data are summarized in Figure 1-2 and illustrate the importance and complexity of GPCR localization in regulating cell decision-making.

1.8 Dissertation outline

Using CXCR4 as a model GPCR, in the following chapters I will investigate how endocytosis and receptor localization regulate CXCR4 signaling and post-translational modification. In Chapter 2, I will present data that supports an expanded role for clathrin and clathrin-independent endocytosis in regulating CXCR4 signaling as well as identify a new antibody to study CXCR4. Next in Chapter 3, I will present work that supports a new mechanism for intracellular GPCR activation that is independent of receptor endocytosis and membrane permeable ligands. Afterwards, I will describe our progress using spatially resolved APEX2 proteomics to study how the CXCR4 protein interactome changes during endocytic trafficking in Chapter 4. In Chapter 5, I will deliberate several biometrology insights that I have learned while carrying out my cell biology research that raises questions regarding how we evaluate and interpret receptor-signaling data. Finally, Chapter 6 summarizes important findings from the work and explores future directions for localized GPCR signaling.

Chapter 2 Endocytosis Regulates CXCR4 Signaling and Post-Translational Modification

This chapter was published in the Frontiers in Cell and Developmental Biology special edition on membrane trafficking (2019). Author Contributions: M.S.D. designed research, analyzed data, and wrote the manuscript. L.K.R contributed some reagents. A.P.L. and S.S. provided experimental advice, edited the manuscript, and provided funding.

2.1 Abstract

Recent research has implicated endocytic pathways as important regulators of receptor signaling. However, the role of endocytosis in regulating chemokine CXC receptor 4 (CXCR4) signaling remains largely unknown. In the present work we systematically investigate the impact of clathrin knockdown on CXCR4 internalization, signaling, and receptor post-translational modification. Inhibition of clathrin-mediated endocytosis (CME) significantly reduced CXCR4 internalization. In contrast to other receptors, clathrin knockdown increased CXCL12-dependent ERK1/2 signaling. Simultaneous inhibition of CME and lipid raft disruption abrogated this increase in ERK1/2 phosphorylation suggesting that endocytic pathway compensation can influence signaling outcomes. Interestingly, using an antibody sensitive to CXCR4 post-translational modification, we also found that our ability to detect CXCR4 was drastically reduced upon clathrin knockdown. We hypothesize that this effect was due to differences in receptor post-translational modification as total CXCR4 protein and mRNA levels were unchanged. Lastly, we show that clathrin knockdown reduced CXCL12-dependent cell migration

irrespective of an observed increase in ERK1/2 phosphorylation. Altogether, this work supports a complex model by which modulation of endocytosis affects not only receptor signaling and internalization but also receptor post-translational modification.

2.2 Introduction

Tight regulation of how cells interact with their environment and process extracellular information is essential for survival. Consequentially, mammalian cells have evolved several tightly regulated yet partially redundant pathways to control cell communication[165–167]. While endocytic pathways are predominantly known for their role in receptor internalization and desensitization, recent technological advancements have allowed researchers to interrogate endocytic dynamics in the context of cognate receptor-cargo pairs and systems-level studies have increased our understanding of endocytic pathways functioning a master regulators of receptor signaling, recycling and degradation[168–173].

G protein-coupled receptors (GPCRs) play an important role for both essential and pathogenic biological processes and are a common cargo of endocytic pits. CXC chemokine receptor 4 (CXCR4) is a type 1 GPCR important for a variety of biological processes including immune cell homeostasis, embryonic development, and cell migration[174–178]. CXCR4 signaling is activated by agonist C-X-C ligand 12 (CXCL12) binding[179]. Depending on CXCL12 isoform and/or CXCR4 C-terminal tail post-translational modifications (PTM), CXCR4-CXCL12 signaling regulates cell proliferation and growth, chromatin remodeling, or cell migration and chemotaxis[174,179–181]. CXCR4 expression is deregulated in 23 cancers and has been shown to increase cancer cell metastasis towards CXCL12 expressing cells[182,183]. Aside from being the most commonly deregulated GPCR found in cancer, CXCR4 was also identified as an HIV co-receptor[184]. Additionally, WHIM syndrome (Warts, Hypogammaglobulinemia, Infection, and

Myelokathexis) is the only known immunological disease directly results from aberrant CXCR4 function, and it has been attributed to a deletion of C-terminal residues of CXCR4 that prevents proper receptor internalization[185].

Receptor tyrosine kinases and GPCRs such as CXCR4 commonly activate the ERK1/2 signaling pathway in an agonist-dependent manner[180,186,187]. ERK1/2 are serine/threonine protein kinase members in the MAPK signaling cascade and are involved in regulating numerous biological processes including cell migration and chemotaxis[188–190]. While the precise mechanism by which CXCL12 activates the MAPK cascade is unclear, the non-visual arrestins (beta 1 and 2) and CXCR4 PTMs have been implicated as key regulators of this process[180,191,192]. Furthermore, multiple lines of evidence suggest tight spatial and temporal control is necessary for proper ERK1/2 activation[193]. While the importance of spatiotemporal control and compartmentalization of receptor signaling have recently been shown to play a significant role in regulating receptor signaling, the molecular mechanisms that regulate this process remain largely unknown.

Recently, endocytosis and receptor localization in membrane microdomains have been implicated as key regulators of agonist-induced receptor signaling[172,193–195]. It was shown that drug perturbation as well as genetic silencing of clathrin heavy chain (CHC) significantly decreased EGF-induced EGFR internalization and AKT signaling and that these effects are rescued by ERBB2 expression in HeLa cells[172]. Interestingly, EGFR surface expression and phosphorylation state was unaffected by clathrin inhibition[172]. Additionally, recent work from our group suggested that a specific subset of clathrin-coated pits (CCPs) are specialized for signaling and concluded that clathrin acts as a scaffold for EGF-induced AKT phosphorylation at the cell membrane[173]. Clathrin-independent endocytosis and lipid rafts have also been implicated

as essential regulators of receptor signaling[193,195,196]. In particular, disruption of caveolae by depleting cholesterol or caveolin-1 genetic silencing significantly reduced CXCL12-induced ERK1/2 signaling[193]. Interestingly, this effect appeared to be CXCR4-specific as EGFR activation was unaffected by cholesterol depletion[193]. Altogether, a model emerges in which endocytosis plays an essential role in ensuring complete receptor signaling pathway activation. While it is known that caveolin-1 plays an important role in CXCR4 signaling and CXCR4 is believed to primarily internalize *via* CME, it remains unclear how CME regulates CXCR4 signaling, PTMs, and protein levels.

In this study, we investigated the effects of clathrin genetic silencing on CXCR4 internalization, signaling, receptor protein levels, and PTM. We found that CHC knockdown significantly decreased CXCL12-induced CXCR4 internalization. In contrast to decreased ERK1/2 activation upon caveolin-1 knockdown, we observed an increased in ERK1/2 activation upon CHC knockdown. Using an antibody sensitive to CXCR4 PTM, we observed that increased signaling potential coincided with an increase in CXCR4 PTM, while total CXCR4 protein and mRNA levels were unaffected by clathrin knockdown. Interestingly, we also discovered that clathrin knockdown significantly impaired CXCL12-dependent cell migration irrespective of the observed increase in ERK1/2 phosphorylation. Altogether, our data support a more complex model in which clathrin is an important regulator of receptor signaling, internalization, and PTM.

2.3 Materials and Methods

Cell Culture

HeLa Cells were originally obtained from ATCC. Retinal pigment epithelial (RPE) cells were obtained from Dr. Sandra Schmid at UT Southwestern and all stable cell lines were derived directly from this line. HeLa cells were cultured in DMEM media (Corning) supplemented with

10% FBS while RPE cell lines were cultured in DMEM/F12 media (Corning) supplemented with 10% FBS.

Stable Cell Lines

Stable RPE cell lines expressing FLAG-CXCR4 WT (+/- c-terminal myc tag) receptors were generated using lentiviral transduction produced from the pLVX vector. Lentiviruses were generated in our lab by co-transfecting HEK293T cells (ATCC) with the transfer plasmid, psPAX2, and pMD2.G lentiviral envelope and packaging plasmids. Cell supernatant containing mature lentiviral particles was collected 4 days after transfection. RPE cells were transduced in DMEM/F12 media supplemented with FBS and 10 μg/mL polybrene and stable cell lines were generated through puromycin selection (3 μg/mL).

Cell Signaling, Cycloheximide Experiments, and Western Blotting

Cells were plated onto 12 well plates (Cell Treat) 24 hrs before each signaling experiment. Prior to each experiment cells were wash 1x with PBS and serum-starved for 4 hrs. Cells were stimulated with ligand (specified in figure legends) (R&D Systems) diluted in serum-free media for 0, 5, or 20 min as indicated in each figure legend. For clathrin knockdown experiments, cells were transduced with pKLO.1 clathrin heavy chain (CHC 17) (sequence 1: Sigma TRCN0000342755, CCGGCGGTTGCTCTTGTTACGGATACTCGAGTATCCGTAACAAGAGCAACCGTTTTT G (2755), sequence 2: Sigma TRCN0000007981, CCGGCGTGTTCTTGTAACCTTTATTCTCGAGAATAAAGGTTACAAGAACACGTTTTT (7981) or scramble non-targeting control Sigma-SCH002) shRNA containing lentiviral particles

5 days prior to the signaling experiment. 72 hrs post transduction, cells were selected using 2 µg/mL (HeLa) or 3 µg/mL puromycin (RPE).

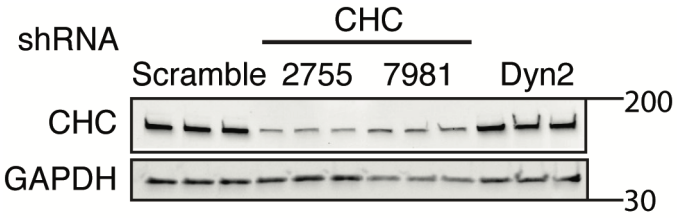


Figure 2-1 Clathrin heavy chain knockdown efficiency

Representative western blot of clathrin heavy chain shRNA knockdown efficiency.

Knockdown efficiencies ranged from 55-85% for all experiments (Figure 2-1). Cells were plated 24 hrs prior to each signaling experiment as described above. After each signaling experiment, cells were washed with PBS 1x and lysed using RIPA buffer (Pierce) supplemented with protease (EDTA Peirce Cocktail and phosphatase inhibitor cocktails (HALT Phosphatase Inhibitor) for 10 min on ice. Afterwards, cells were scraped, lysate added to 1.5 mL tubes, and centrifuged at 4 ^{o}C for 45 min at 16,100 g. Approximately 10-50 µg of protein were loaded for SDS-PAGE gels (BioRad) analysis depending on the abundance of the target protein. PDVF 0.2 µm membranes (Thermo Fisher Scientific) were used for all western blotting experiments and transferred using the iBlot transfer system suitable for mixed range proteins. Blots were blocked using 5% BSA in TBS not supplemented with Tween for 1 hr rocking at room temperature. Blots were then incubated overnight with primary antibodies diluted in 5% BSA supplemented with 1% Tween 20 in TBS (TBST). Blots were washed 3x for 5 minutes with TBST after which they were incubated with secondary antibodies (Table 2-1). All blots were imaged using the LiCor Odyssey Imaging System. Bands were quantified using the LiCor Image Analysis Software in accordance with manufacturer guidelines. We established that our knockdowns did not

significantly alter GAPDH expression using the REVERT total Protein Stain and therefore used GAPDH as a loading control and for normalization of all experiments. As previously described, all signaling experiments were additionally normalized to the scramble control at the 5-min stimulus time point[172]. For the cycloheximide (CHX) experiments, RPE cells overexpressing CXCR4 were grown as described above and treated with cycloheximide (50 μM) described in the figure legend. The exact western blot protocol used for signaling assays was used for CHX experiments as well. All statistics were conducted using paired 2 tailed *t*-test (* represents $p < 0.05$).

Immunofluorescence Assays

Cells were plated onto 6-well plates on glass coverslips 24 hrs prior to each experiment. Cells were washed 1x with PBS and serum-starved for 4 hrs as described above. Cells were stimulated as specified in each figure legend and immediately washed with cold PBS 2x and fixed in 4% paraformaldehyde for 15 min on ice. Cells were permeabilized for 5 min at room temperature with 0.2% Triton-X100 diluted in PBS and then blocked with 2.5% BSA in PBS for 1 hr. Cells were incubated with primary antibody diluted in 2.5% BSA and incubated overnight at 4 ^{o}C (Table 2-1). Slides were wash 3x for 5 min each with PBS and incubated with secondary antibodies diluted in 2.5% BSA for 1 hr at room temperature (Table 1). Cells were washed with PBS 3x, 5 min per wash and incubated with DAPI (Table 1) diluted in PBS for 5 min at room temperature. Afterwards, cells were washed with PBS and mounted onto glass slides with Fluoromount-G (Invitrogen). Slides were imaged by spinning disk confocal microscopy, TIRF microscopy, or epifluorescence (using a laser-based system). Different experimental samples were imaged using the same imaging settings each day and antibody controls (secondary and

non-permeabilized samples) were included in each experiment to account for background staining.

Internalization Assays

Cells were plated onto 10 cm dishes 24 hrs prior to each experiment, washed and serum-starved as described above. Afterwards, cells were non-enzymatically disassociated from each plate using 50 μM EDTA in Ca^{2+}-free PBS. Cells were pelleted by centrifugation (500 g for 10 min) and resuspended in serum-free media on ice. Cells were transferred into 1.5 ml eppendorf tube and treated with either a vehicle or ligand and transferred to a hot plate at 37 ^{o}C for a time course (described in figure legends). Immediately afterwards, cells were transferred back on ice, pelleted (centrifuged 3,000g for 3 min), and resuspended in 4% paraformaldehyde for 10 min on ice. Cells were pelleted and incubated with conjugated antibody (Table 2-1) diluted in 2.5% BSA for 1.5 hrs on ice. Afterwards, cells were washed 1x with PBS and 10,000 cells were analyzed by the Guava EasyCyte Flow Cytometer and its accompanied software. Internalization was quantified as previously described[197]. All statistics were conducted using paired 2 tailed *t*-test (* represents $p < 0.05$).

RT-qPCR Assays

Scramble- or clathrin-silenced HeLa cells were plated onto 12 well dishes at similar confluency 24 hr prior to each experiment and subsequently serum-starved or treated with fresh 10% FBS containing medium for 4 hr. RNA was extracted using the RNAeasy kit (BioRad) and 1 μg of cDNA prepared in accordance with the iScripts cDNA synthesis protocol (BioRad). qPCR assays were conducted using SYBR Green (BioRad) per BioRad protocol instructions. Primers used to

quantify *gapdh* (Fw-GAGTCAACGGATTTGGTCGT, Rev-CTTGATTTTGGAGGGATCTCGC), *actin* (Fw-CATGTACGTTGCTATCCAGGC, Rev-CTCCTTAATGTCACGCACGAT), *chc* (Fw-ACAGAGACACAACCCATTGTTT, Rev-CGGTGGTGCGGTATAACCAT), *cxcr4* (Fw-CCTATGCAAGGCAGTCCATGT, Rev-GGTAGCGGTCCAGACTGATGA), and *egfr* (Fw-AGGCACGAGTAACAAGCTCAC, Rev-ATGAGGACATAACCAGCCACC). Relative transcript levels were quantified using the $\Delta\Delta C_t$ method as previously described. Briefly, the change in C(t) values between scramble and clathrin knockdown samples were determined. Afterwards the change between the deltas of paired scramble and clathrin knockdowns was calculated (i.e. $\Delta\Delta C_t$) and relative transcript levels was determined by calculating $2^{-\Delta\Delta Ct}$. Results were collected in duplicate from 4 independent experiments; $\log_2$ transformed, and plotted mean +/- standard deviation.

Migration Assays

HeLa cells expressing either scramble or CHC shRNA were plated onto 24 well plates at 100% confluence. After 24 hr, cells were serum-starved (DMEM (-) FBS) for 4 hr and a vertical scratch was created using a 200 µl pipet tip. Afterwards, cells were treated with either complete media (10% FBS + DMEM) or CXCL12 media (25 nM CXCL12 + DMEM). Cell migration was monitored, imaged and quantified after 20 hr using phase contrast microscopy on a Cytation 5 automated plate reader at 4x magnification. Migration was quantified by measuring the area remaining between the cell borders by using ImageJ as previously described[173]. Results were collected from 3 independent experiments and are plotted mean +/- standard deviation. All statistics were conducted using paired 2 tailed *t*-test (* represents $p < 0.05$).

Table 2-1 Reagents

Reagent	Supplier	Dilution	Assay
ms-FLAG-647	Genscript	1:1000	FC, IF
Gt-anti-rb Dylight 800	Invitrogen	1:10000	WB
Gt-anti-ms Dylight 680	Invitrogen	1:10000	WB
Human CXCR4 R-PE	Invitrogen	1:500	FC
ms-GAPDH	Santa Cruz	1:1000	WB
rb-Phospho-pERK1/2	CST	1:2000	WB
ms-Total ERK1/2	CST	1:1000	WB
rb-CXCR4 UMB2	Abcam	1:1000	WB, IF
Rb-EEA1	CST	1:500	IF
Gt-anti-rb-AF488	Invitrogen	1:1000	IF
Rb-Caveolin-1	CST	1:500	IF
Ms-EGFR (A-10)	Santa Cruz	1:1000	WB

2.4 Results

CXCR4 overexpression in retinal pigment epithelial (RPE) cells recapitulates endogenous CXCR4 internalization and signaling in HeLa cells.

To study the effects of CXCR4 overexpression without background from endogenous receptors and other chemokine receptors responsive to CXCL12 (*e.g.,* CXCR7), we used both HeLa and an exogenous CXCR4 overexpression cell line model. To limit background from endogenous CXCR4 and CXCR7, we overexpressed CXCR4 in retinal pigment epithelial cells

(RPE) because this cell line has very low CXCR4 expression [198]. As expected, CXCL12 stimulus rapidly induced both ERK1/2 phosphorylation in both HeLa and RPE cells stably overexpressing CXCR4 as early as the 5 minutes time point (Figure 2-2A). Likewise, agonist-induced receptor internalization was not significantly different between HeLa and RPE CXCR4 (Figure 2-2B). Lastly, to ensure that the overexpressed CXCR4 construct localized and trafficked properly in RPE cells, we found that CXCR4 colocalized with known early endosome marker EEA1 20 min-post CXCL12 addition, as expected (Figure 2-2C).

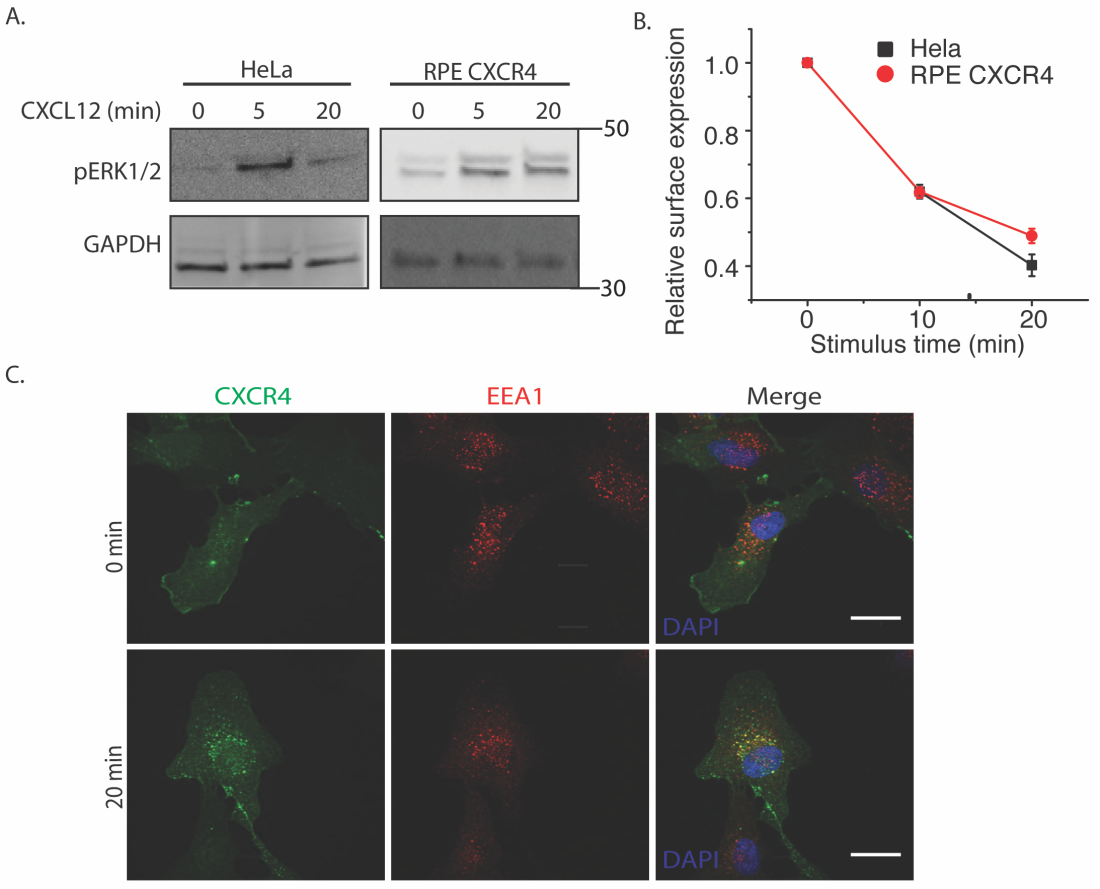


Figure 2-2 Overexpressed CXCR4 in RPE cells recapitulate endogenous CXCR4 signaling and internalization dynamics.

(A) Representative western blot of CXCL12-induced ERK1/2 phosphorylation in HeLa and RPE cells overexpressing CXCR4. **(B)** Flow cytometry analysis of CXCR4 internalization in HeLa (endogenous) and RPE CXCR4 (overexpressed receptor). Relative surface expression was

calculated by taking the mean fluorescence between a paired stimulus and vehicle control at each timepoint. The mean of 4 independent experiments is plotted +/- SEM. **(C)** Confocal microscopy images of CXCR4 internalization labeled by FLAG antibody in RPE cells and an endosomal marker EEA1 antibody before and after 25 nM of CXCL12 treatment. Scale bars are 10 μm.

Clathrin silencing decreases CXCR4 internalization and increases CXCL12-induced ERK1/2 phosphorylation.

Having established an experimental model to study CXCR4 in RPE cells, we next examined the effect of CHC knockdown on CXCR4 internalization and signaling. It has previously been hypothesized that CXCR4 is primarily internalized by CME[199]. To test this hypothesis, we used shRNA to reduce functional clathrin triskelia and measured CXCL12-induced receptor internalization by flow cytometry. Consistent with this hypothesis, CXCR4 internalization was significantly attenuated, both in rate and in final level (Figure 2-3A). However, CXCR4 surface expression was unchanged by clathrin knockdown (Figure 2-3B). Next we investigated the effect of clathrin knockdown on CXCL12-CXCR4-mediated ERK1/2 signaling. In accordance with previous literature, we hypothesized that clathrin knockdown would reduce CXCL12-induced signaling. Surprisingly, clathrin knockdown significantly increased CXCL12-induced ERK1/2 phosphorylation both pre- and post-agonist addition (Figure 2-3C-D). To establish that these effects were not artifacts of receptor overexpression, we confirmed these observations in HeLa cells (Figure 2-4). Previous reports have indicated that caveolae/lipid rafts are essential for complete CXCL12-mediated ERK1/2 phosphorylation[193]. Consistent with this result, we found that inhibition of caveolae/lipid raft (using cholesterol depleting agent and caveolae inhibitor nystatin) reduced CXCL12-mediated ERK1/2

phosphorylation irrespective of clathrin knockdown (Figure 2-3C-D). As expected, total ERK1/2 levels were unchanged upon clathrin knockdown (Figure 2-3E-F). It has previously been reported that upon receptor stimulus, CXCR4 colocalizes with both adapter protein 2 (AP2) and caveolin-1[193]. Therefore, we hypothesized that clathrin knockdown may increase agonist-induced CXCR4 colocalization with caveolin-1. While not statistically significant, a slight increase in CXCR4 colocalization with caveolin-1 was observed upon clathrin knockdown after 5-minute agonist addition (Figure 2-5A-B). In contrast to previous work, CXCR4 colocalization with caveolin-1 was limited irrespective of clathrin knockdown. However, others have shown that CXCR4 localizatizes to lipid rafts more generally[200,201]. Thus, it is plausible that clathrin knockdown may shift CXCR4 localization to both caveolin-1 positive and negative lipid rafts. Together, these observations support a model where endocytic pathway redundancy may play a role in modulating receptor internalization and signaling. Interestingly, a statistically significant increase in ERK1/2 phosphorylation was also observed at the 0-min time point (Figure 2-3D) in RPE cells overexpressing CXCR4. We believe that this might be due to ineffective clearance of activated CXCR4 during serum starvation as receptor internalization is significantly reduced upon clathrin knockdown (Figure 2-3A). An increase in ERK1/2 phosphorylation at the 0-min time point was not observed with HeLa cells with clathrin knockdown. We believe that this discrepancy is likely due to cell type specificity or difference in signal intensity and consequently detection sensitivity.

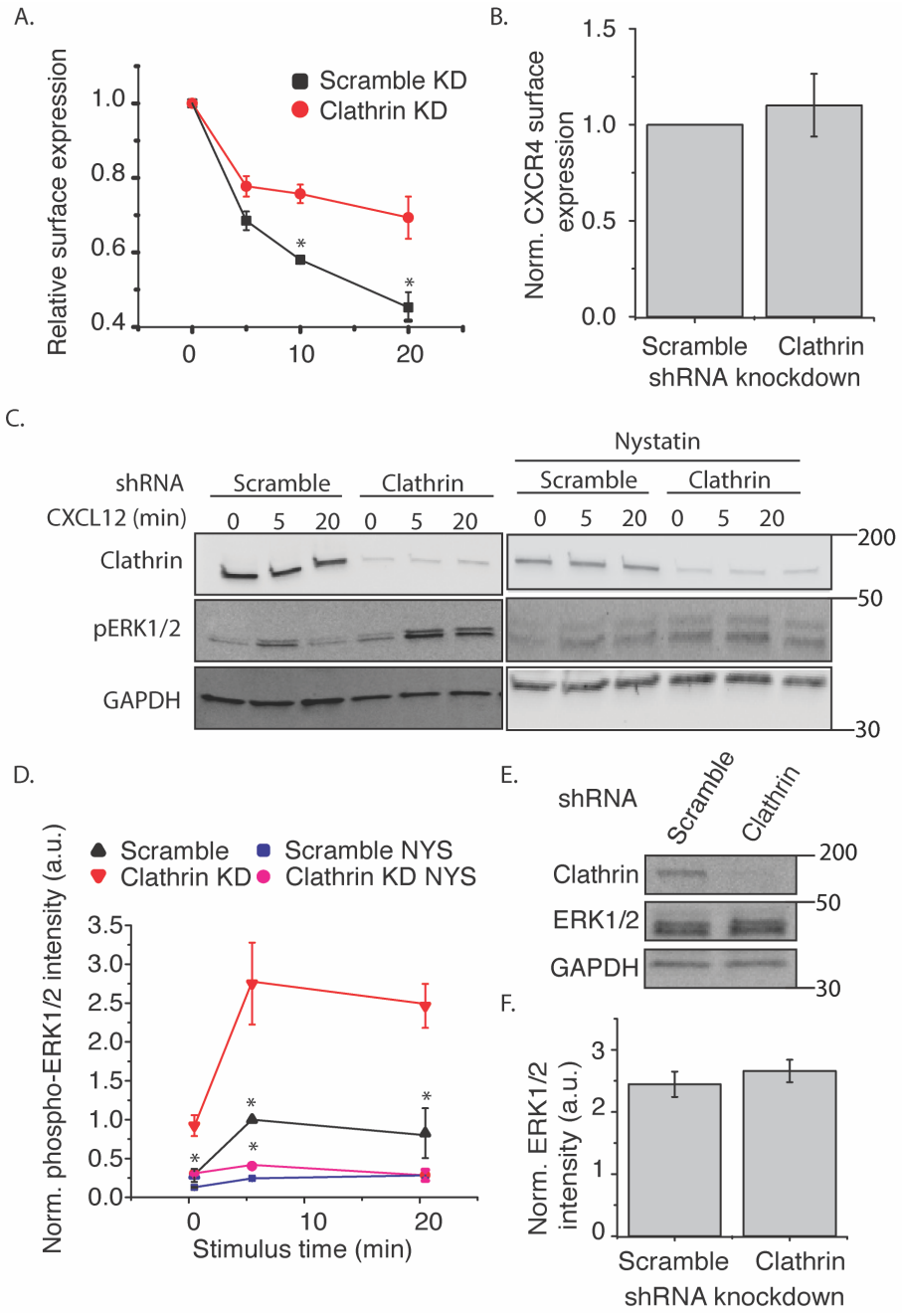


Figure 2-3 Clathrin inhibition reduces agonist-induced CXCR4 internalization and increases ERK1/2 phosphorylation.

(A) Flow cytometry analysis of CXCL12-induced CXCR4 internalization upon scramble or clathrin knockdown (shRNA 2755 or 7981). Relative surface expression was calculated by taking the mean fluorescence between a paired stimulus and vehicle control at each time. The mean of 4 independent experiments is plotted +/- SEM. **(B)** Quantification of relative CXCR4 surface expression upon clathrin knockdown (shRNA 2755 or 7981). CXCR4 surface expression was detected using the FLAG antibody in RPE cells overexpressing CXCR4 and measured by

flow cytometry (n = 4, normalized mean +/- SEM). **(C-D)** Representative western blots and quantification of CXCL12-induced ERK1/2 phosphorylation upon scramble or clathrin knockdown +/- nystatin (NYS) treatment (50 μM, 30 min pretreatment). For all experiments, RPE cells overexpressing CXCR4 were transduced with either scramble or clathrin heavy chain shRNA (shRNA 2755) and stimulated with 25 nM CXCL12 for the labeled time course. Signaling experiments were conducted in pairs. Western blot bands were normalized to GAPDH as well as to 5-min stimulus in the scramble condition. The mean of 4 independent experiments is plotted +/- SEM. **(E-F)** Representative western blot and quantification showing that relative ERK1/2 levels are unchanged upon clathrin (shRNA 2755) knockdown. Total ERK1/2 expression was normalized to GAPDH. The mean of 4 independent experiments is plotted +/- SEM. (* denotes statistical significance $p < 0.05$).

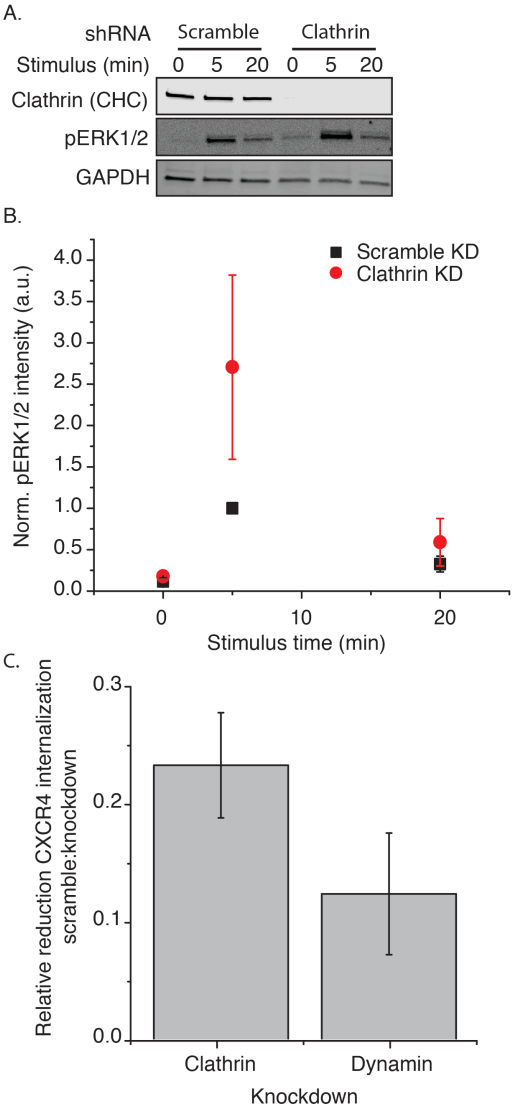


Figure 2-4 Clathrin knockdown reduces CXCR4 internalization and increases ERK1/2 phosphorylation in HeLa Cells.

(A) Representative western blot showing the effects of clathrin knockdown (shRNA 2755) in ERK1/2 phosphorylation in HeLa cells. **(B)** Quantification of relative CXCL12-induced ERK1/2 phosphorylation in HeLa cells. Relative ERK1/2 phosphorylation was normalized to ERK1/2 phosphorylated at 5 min post-CXCL12 stimulus of the scramble knockdown condition. **(C)** Relative reduction of CXCR4 internalization upon clathrin knockdown (shRNA 2755 or 7981) by flow cytometry analysis (n = 3, mean +/- SEM, * denotes statistical significance $p < 0.05$).

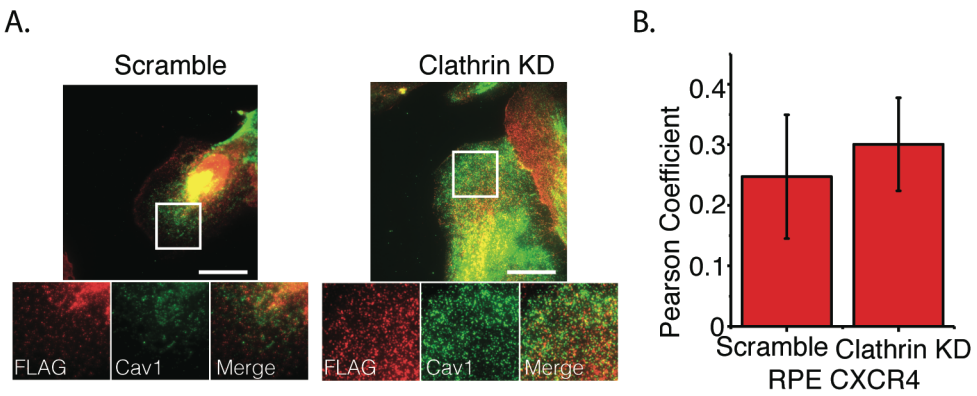


Figure 2-5 CXCR4 and caveolin-1 colocalization upon clathrin knockdown.

(A) Representative images of CXCR4 caveolin-1 colocalization in RPE cells stably overexpressing CXCR4 (scale bar 10 µm) +/- scramble or clathrin knockdown (shRNA 7981). Cells were serum-starved for 4 hrs and stimulated with 25 nM CXCL12 for 5 min. After immunofluorescence labeling (FLAG and caveolin-1 antibodies), cells were imaged by TIRF microscopy. **(B)** Quantification of CXCR4-caveolin-1 colocalization in cropped images (Pearson correlation coefficient, n = 6, mean +/- SEM) is plotted. While an increase in colocalization is observed with clathrin knockdown, it is not statistically significant.

The CXCR4 UMB2 antibody immunoblotting is a robust method to detect changes in CXCR4 PTM.

To further investigate the mechanism by which clathrin knockdown increased CXCR4 signaling, we tested whether clathrin knockdown influenced CXCR4 protein expression. Upon conducting these experiments, we noticed that the CXCR4 UMB2 antibody differentially detected CXCR4 post receptor stimulus (Figure 2-6A). To validate that the decrease in receptor western blot intensity was not due to rapid receptor degradation, we co-labeled overexpressed FLAG-CXCR4 with FLAG and UMB2 antibodies pre- and post-CXCL12 stimulus. Consistent with our hypothesis, the FLAG antibody robustly detected CXCR4 pre- and post-CXCL12 stimulus while the UMB2 antibody signal was significantly reduced post-stimulus (Figure 2-6B).

To confirm that the UMB2 disappearance was dependent on CXCL12, we stimulated cells with EGF and monitored relative CXCR4 levels. As expected, while CXCL12 addition quickly led to the disappearance of the UMB2 CXCR4 band, EGF stimulus did not affect CXCR4 detection (Figure 2-6C). To exclude that CXCR4 was moving to an insoluble compartment post-stimulus, we generated a stable cell line expressing a c-terminally tagged myc CXCR4 construct (CXCR4-myc) (Figure 2-6D). As expected, while the CXCR4 UMB2 antibody's ability to detect CXCR4 post-stimulus was diminished, the myc antibody staining remained constant (Figure 2-6E), and hence we do not believe the receptors were degraded. Since the cytosolic c-terminal tail of CXCR4 is rapidly post-translationally modified by phosphorylation and ubiquitination upon CXCL12 addition, our results suggest that the UMB2 antibody is sensitive to changes in CXCR4 PTM. Equipped with this new tool, we asked whether overexpressed CXCR4 had different PTM kinetics than endogenous CXCR4, and observed no significant changes in CXCR4 PTM kinetics between HeLa and RPE CXCR4 cells (Figure 2-6F).

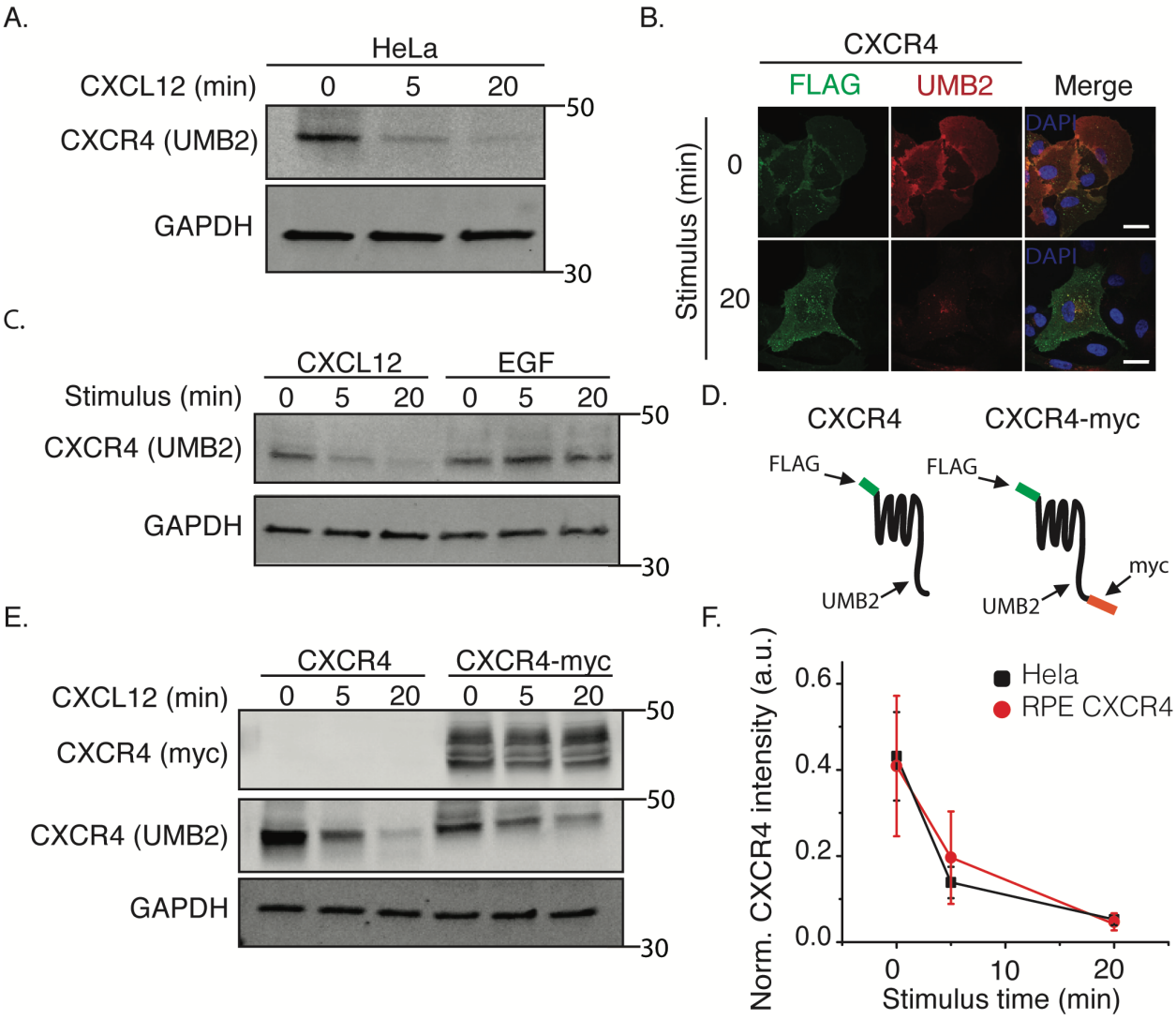


Figure 2-6 The UMB2 monoclonal CXCR4 antibody detects changes in agonist-induced CXCR4 PTMs.

(A) Representative western blot of CXCR4 detection upon CXCL12 addition in HeLa cells. Cells were stimulated with 25 nM for the described time course and CXCR4 protein levels were quantified using the UMB2 monoclonal antibody and normalized to GAPDH. **(B)** Confocal microscopy images of overexpressed FLAG-tagged CXCR4 in RPE cells. CXCR4 was labeled using both FLAG and UMB2 antibodies pre- and post-CXCL12 addition (25 nM). Scale bars are 10 μm). **(C)** Reduced detection of CXCR4 using the UMB2 antibody is ligand-specific. RPE cells overexpressing CXCR4 were treated with either 25 nM CXCL12 or 20 nM EGF for the described time course. **(D)** Schematic of CXCR4 and CXCR4-myc overexpression constructs illustrating where the different antibodies used bind to CXCR4. **(E)** RPE cells overexpressing

CXCR4-myc were treated with 25 nM CXCL12 or 20 nM EGF for the described time course and CXCR4 was detected by western blotting using the myc or UMB2 antibody**. (F)** Western blot quantification of CXCR4 detection by UMB2 antibody in HeLa and RPE CXCR4 cells. CXCR4 intensity was normalized to GAPDH and plotted mean +/- SEM from 4 independent experiments.

Clathrin inhibition increases CXCR4 PTMs.

Using the UMB2 antibody, we observed that clathrin knockdown significantly reduced CXCR4 detection in both HeLa and RPE cells overexpressing CXCR4 (Figure 2-7A-C). To determine whether this was due to a change in receptor expression or PTM state, we used flow cytometry and qPCR to assess total endogenous CXCR4 protein and mRNA levels upon clathrin knockdown. Interestingly, clathrin inhibition did not affect total endogenous CXCR4 protein (Figure 2-7D) or mRNA levels in HeLa cells (Figure 2-8A-B). Cycloheximide chase experiments in RPE cells overexpressing c-terminal myc tagged CXCR4 further confirmed that clathrin knockdown did not lead to a change in CXCR4 degradation kinetics (Figure 2-8C-D) and additionally total CXCR4 labeling using the myc antibody confirmed that CXCR4 total protein levels were unchanged in the overexpression CXCR4 model as well (Figure 2-8E). To test whether this phenomenon was specific to CXCR4 or more broadly applicable to other receptors, we measured EGFR protein levels using an antibody (A-10 clone) not expected to be sensitive to receptor PTM[202]. Interestingly, clathrin knockdown significantly reduced EGFR detection in both HeLa and RPE cells as well. EGFR mRNA levels were also unchanged (Figure 2-8C-D). Since EGFR is internalized by both CME and clathrin-independent internalization[196,203,204], it is possible that upon clathrin knockdown EGFR internalization is compensated by clathrin-independent internalization and consequentially leads to increased

receptor degradation as previously reported. Reliable quantification of changes of CXCR4 PTM kinetics was not possible for the clathrin knockdown condition in HeLa cells due to the limited linear detection range of fluorescent imagers since these cells had a drastically lower initial CXCR4 detection intensity. Consequentially, we explored whether clathrin knockdown had any effect on CXCR4 PTM kinetics in the overexpression context. As previously reported with EGFR[172], we did not see a significant change in agonist-induced CXCR4 PTM kinetics upon clathrin knockdown in RPE cells overexpressing CXCR4 (Figure 2-7E).

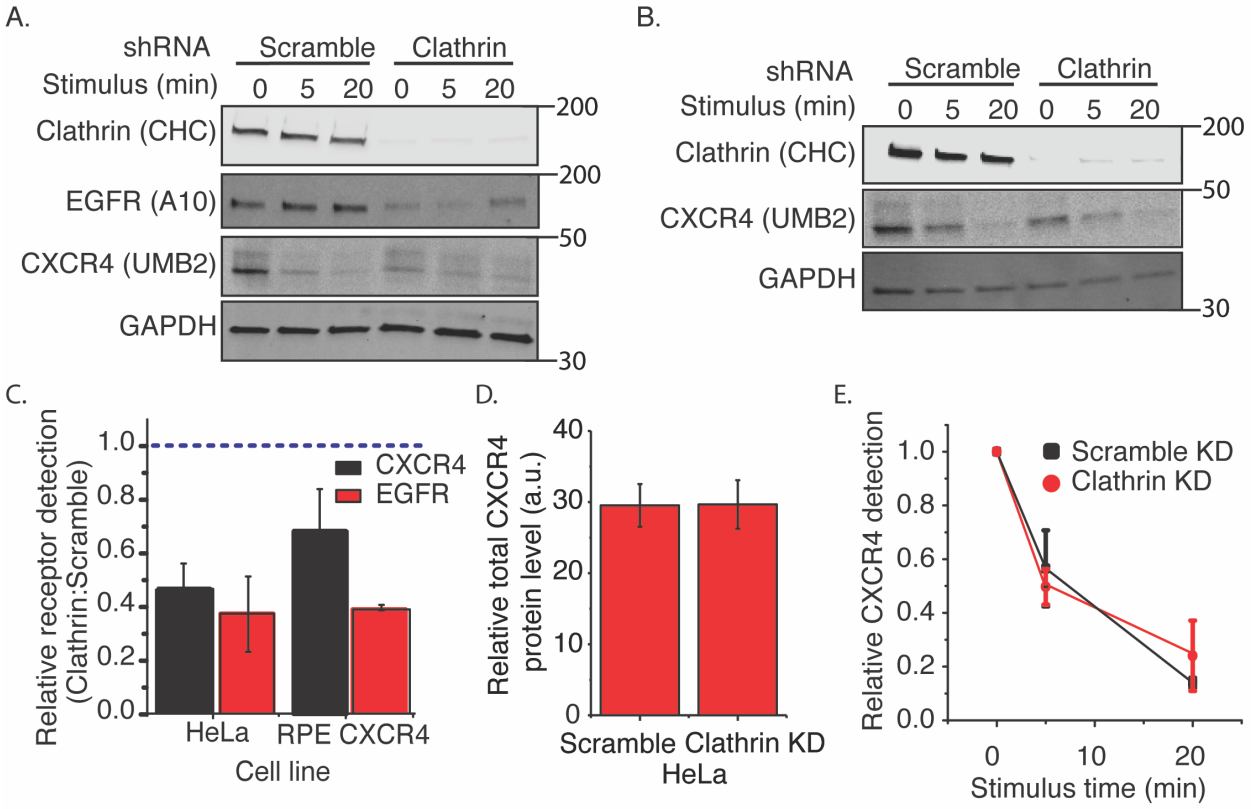


Figure 2-7 Clathrin knockdown increases CXCR4 PTM.

(**A-B**) Representative western blots showing the effect of clathrin knockdown on CXCR4 and EGFR protein levels in **(A)** HeLa or **(B)** RPE CXCR4 cells. **(C)** Quantification of western blot analysis of CXCR4 and EGFR protein levels upon clathrin knockdown. Relative receptor detection was calculated by taking the ratio of normalized receptor intensity (receptor:GAPDH) between scramble and clathrin shRNA transduced cells (0 min time point). Cells were transduced

with scramble or clathrin shRNA (2755) for 5 days and serum-starved for 4 hrs prior to each signaling/receptor detection experiment. Quantification is mean +/- SEM from 4 independent experiments **(D)** Relative detection of CXCR4 by UMB2 with and without clathrin knockdown in RPE cells overexpressing CXCR4. Relative CXCR4 detection was calculated upon receptor stimulus (25 nM CXCL12) for scramble and clathrin knockdown cells. CXCR4 detection was normalized to the 0 min time point and plotted as mean +/- SEM (n = 4). Similar analysis was not carried out for HeLa cells as the signal was outside of the linear range of detection (see **(A)**). **(E)** Total endogenous CXCR4 detection in HeLa cells. Cells were infected with either clathrin or scramble shRNA and serum-starved for 4 hr. After permeabilization and immunofluorescence labeling of endogenous CXCR4, total CXCR4 protein level was measured by flow cytometry. Mean +/- SEM is plotted from 3 independent experiments.

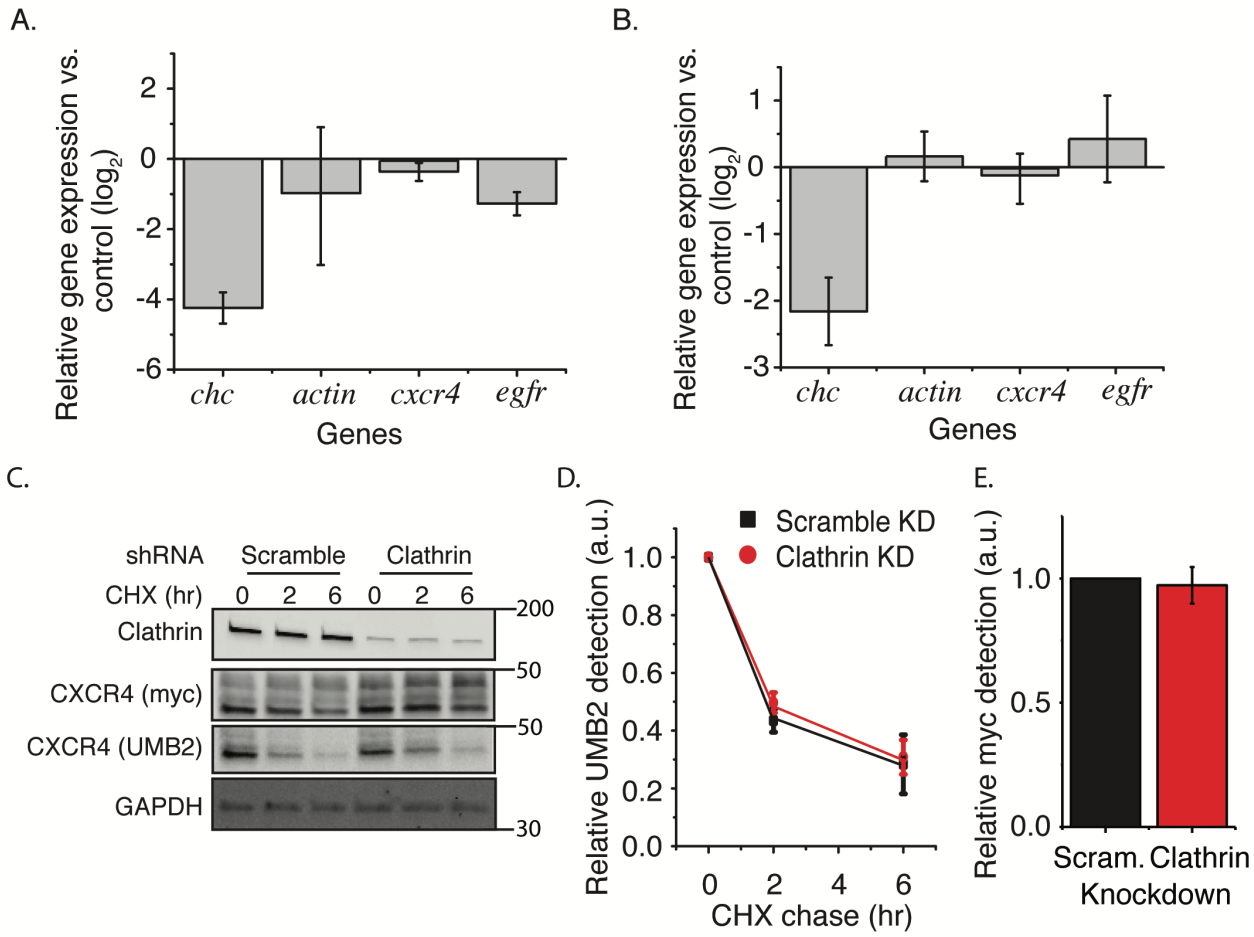


Figure 2-8 Clathrin knockdown does not change overexpressed CXCR4 degradation kinetics in RPE cells or mRNA levels in HeLa cells.

(A-B) RT-qPCR quantification of *clathrin heavy chain* (*chc*), *actin*, *cxcr4*, and *egfr* transcript levels upon clathrin knockdown (shRNA 2755) in **(A)** serum-free or **(B)** serum conditions. Relative transcript levels were calculated using the $\Delta\Delta C_t$ method and normalized to *gapdh*. The mean relative gene expression (log_2 transformed) +/- SD is plotted. Data was collected with duplicate technical replicates from 4 biologically independent experiments. **(C)** A representative western blot showing the effects of clathrin knockdown (shRNA 2755) on overexpressed CXCR4-myc protein degradation kinetics. In serum-supplemented media, cells were treated with 50 µM cycloheximide for the described time course and CXCR4 was detected using UMB2 and myc antibodies. **(D)** Relative UMB2 detection is plotted with mean +/- SEM from 3 independent experiments upon scramble or clathrin knockdown. Relative normalized CXCR4 detection was calculated by taking the ratio of GAPDH normalized UMB2 detection to the initial time point.

No statistical significance was observed. Of note, CXCR4-myc constructs did not have as large of a decrease in UMB2 detection upon clathrin knockdown. This is potentially due to the fact that these experiments were conducted in serum or potential inhibition of receptor PTMs by myc addition to the receptor C-terminus. **(E)** Quantification of relative myc antibody detection (i.e. total CXCR4) upon scramble or clathrin knockdown (shRNA 2755). Total CXC4 protein levels were unchanged with clathrin knockdown. Data is plotted with mean +/- SEM from 3 independent experiments upon clathrin or scramble shRNA knockdown.

Clathrin knockdown decreases CXCL12-dependent and independent cell migration in HeLa cells.

To investigate the implication of CXCL12-induced increase of ERK1/2 phosphorylation in clathrin-silenced cell on cell migration, we used a wound-healing assay. Compared to scramble controls, irrespective of the type of ligand (CXCL12 *vs.* FBS), clathrin knockdown significantly reduced cell migration in HeLa cells (Figure 2-9). This result corroborated a previous report where compared to a wildtype receptor, a mutant CXCR4 has indistinguishable ERK1/2 phosphorylation yet has significantly reduced chemotaxis[205]. Since EGF- and CXCL12-mediated ERK1/2 phosphorylation have been shown to occur at distinct membrane micro-domains[193], we investigated how EGF-dependent cell migration was affected by clathrin knockdown. Similar to CXCL12, clathrin knockdown reduced EGF-induced cell migration (Figure 2-10). This suggests that while CXCL12 and EGF signaling mechanisms are distinct and differentially regulated by clathrin, the effects on cell migration are comparable.

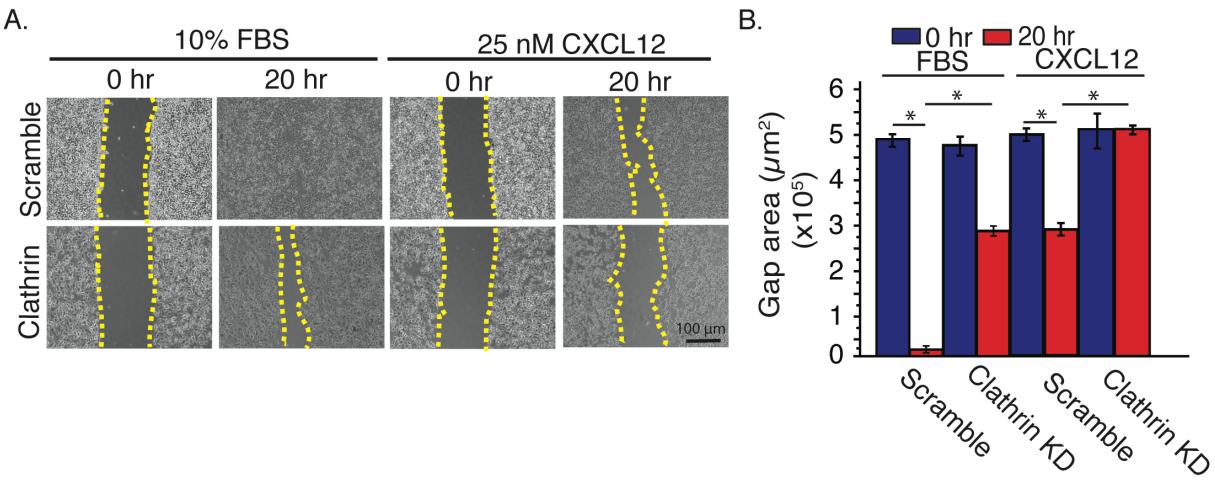


Figure 2-9 Clathrin knockdown decreased CXCL12-dependent and independent cell migration in HeLa cells.

(A) Representative images of a scratch assay time course. HeLa cells transduced with scramble or clathrin shRNA (shRNA 2755 or 7981) (day 5) were serum-starved for 4 hrs and treated with medium containing 10% FBS or 25 nM CXCL12 in serum-free medium. A line was scratched on each plate and relative cell migration was measured by phase contrast microscopy at 0 hr or 20 hr post scratching. Cell boundaries used to calculate cell migration are outlined in yellow dotted line. **(B)** Quantification of gap area (between dotted yellow boundaries) under each condition. Mean gap area +/- SEM is plotted from 4 independent experiments (* denotes statistical significance $p < 0.05$).

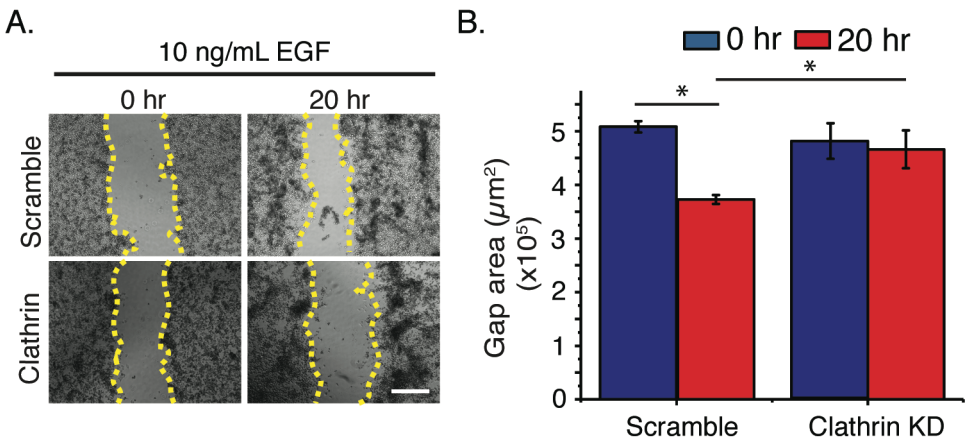


Figure 2-10 Clathrin knockdown decreases EGF-dependent HeLa cell migration.

(A) Representative images of a scratch assay time course. Five days post transduction with either scramble or clathrin (shRNA 2755 or 7981) knockdown HeLa cells were serum-starved for 4 hrs and treated with medium containing 10 ng/mL EGF in serum-free medium. Using a p200 pipet tip, a line was scratched on each plate and relative cell migration was measured by phase contrast microscopy at 0 hr or 20 hr post scratching. Cell boundaries used to calculate cell migration are outlined by the yellow dotted line. **(B)** Quantification of gap area (between dotted yellow boundaries) under each condition. Mean gap area +/- SEM is plotted from 3 independent experiments (* denotes statistical significance $p < 0.05$).

2.5 Discussion

The expanded role of endocytosis in not only regulating receptor internalization but as a signaling platform and master regulator of protein expression is becoming increasingly clear[206]. Here we provide new evidence of how endocytic proteins regulate CXCR4 internalization, signaling, and receptor PTM level and highlight the redundancy of endocytic pathways.

Redundancy of endocytic pathways is not a new idea and work in this area has been pioneered with EGFR[203]. Interestingly, we found that while clathrin knockdown increased CXCR4 PTM, this was not the case for EGFR as the antibody used to measure total EGFR has

been previously shown to not be sensitive to EGFR PTMs[202] and previous research suggests that EGFR PTMs are unaffected by clathrin inhibition[172]. Additionally, while EGFR is preferentially internalized by CME at increasing high ligand levels, at times it is also internalized by clathrin-independent mechanisms[203]. It was further reported that the endocytic mechanism of EGFR internalization predestined receptors for either receptor recycling or degradation in a PTM specific manner[196,203,204]. Consequently, it is possible that clathin knockdown leads to an increase in clathrin-independent EGFR internalization and subsequent degradation and provides additional evidence that clathrin inhibition differentially impacts receptors such as EGFR and CXCR4.

Upon clathrin knockdown, we observed an increase in CXCR4 PTM pre-stimulus. However, total as well as surface receptor levels remained unchanged. Since clathrin is involved in multiple aspects of endocytic trafficking (Golgi transport as well as endocytosis), it is possible that clathrin knockdown may lead to changes in CXCR4 localization in plasma membrane micro-domains or intracellular compartments that are responsible for the observed change in receptor PTM. Even though CXCR4 is both phosphorylated and ubiquitinated, we did not discern which modification or combination of modifications were responsible for these observations and this remains to be a subject of further investigation.

Interestingly, increased CXCR4 PTM increased and correlated with CXCL12-dependent ERK1/2 phosphorylation, further implicating compensatory mechanisms of CXCR4 regulation upon clathrin knockdown. It was previously reported that CXCL12-induced ERK1/2 phosphorylation occurs independently of CXCR4 internalization and that CXCR4 localization in cholesterol rich lipid rafts is essential for complete ERK1/2 phosphorylation and that this effect was not observed with EGFR receptor[193]. Indeed, transient silencing of the caveolae resident

protein caveolin-1 was reported to lead to an approximately 50% reduction in CXCL12-induced ERK1/2 phosphorylation[193]. Together, these results provide additional evidence that modulation of endocytic dynamics regulates receptor biology differently. For CXCR4, clathrin is a negative regulator of ERK1/2 phosphorylation and increased receptor PTMs, whereas for EGFR dependent ERK1/2 signaling is unaffected and total protein levels are reduced during extended periods of clathrin knockdown.

Interestingly, beta-arrestin (1 and 2) knockdown phenocopies ERK1/2 phosphorylation that we observed with clathrin knockdown[193]. Together these results suggest a potential shared mechanism by which clathrin and beta-arrestins are negative regulators of CXCR4-dependent ERK1/2 signaling and a compensatory mechanism by which cells increase CXCR4 localization in lipid rafts (caveolin-1 positive and/or negative) and become increasingly capable for CXCL12-induced ERK1/2 phosphorylation. Additionally, while AP2 inhibition has been shown to decrease CXCR4 internalization, it has not been shown to affect ERK1/2 signaling[193]. Coupled with our results, this provides evidence for a model where AP2 is important for CXCR4 signaling but an independent adapter protein is important for mediating CXCL12-dependent ERK1/2 phosphorylation. Unfortunately, co-knockdown experiments of clathrin and caveolin-1 resulted in cells that were not viable. However, consistent with the model, it is possible that clathrin knockdown increases clathrin-independent internalization of CXCR4 (clathrin knockdown slightly increased CXCR4/caveolin-1 colocalization) and that unlike EGFR, this mechanism of internalization does not lead to increased receptor degradation.

Surprisingly, a significant increase in ERK1/2 phosphorylation was observed at the 0-minute time point in CXCR4 RPE cells upon clathrin knockdown. We speculate that this observation could be due to the inability of clathrin knockdown cells to efficiently clear activated

receptor during serum starvation. This is supported by the observation that clathrin inhibition drastically reduced CXCR4 internalization. Interestingly, elevated ERK1/2 phosphorylation did not lead to increased cell migration. Clathrin silencing nearly ablated cell migration independent of agonist and our observation that clathrin knockdown increases ERK1/2 signaling suggests that CXCL12-induced ERK1/2 signaling and cell migration are decoupled or that there may be additional roles that clathrin plays in cell migration. While clathrin inhibition has not been previously associated with a change in ERK1/2 signaling, previous studies with CXCR4 have focused on transient pharmacological inhibition of CME (30 min timescale)[171], whereas in this study shRNAs were used to specifically knockdown clathrin heavy chain over the course of 3-5 days) and consequently provides additional evidence for cellular compensation. Additionally, this effect appears to be different in HL-60 derived neutrophils where inhibition of clathrin abrogated ERK1/2 signaling and polarization[207]. This could be due to a strong dependence of signaling on the clathrin scaffold for a professional migratory cell as well as differences between pharmacological *vs.* genetic perturbations. Our present findings are supported by the work of Mines *et al.* that used a lysine triple mutant CXCR4[205]. They showed that this mutant CXCR4 had significantly reduced chemotactic potential independent of ERK1/2 phosphorylation. Together with the results presented, this evidence supports a model in which CXCL12-induced chemotaxis and cell migration is decoupled from CXCL12-induced ERK1/2 phosphorylation. We additionally investigated how EGF-dependent cell migration was affected upon clathrin knockdown. Irrespective of ligand, clathrin knockdown significantly reduced cell migration. This suggests that while CXCL12 and EGF signaling mechanisms are distinct (different membrane localization) and differentially regulated by clathrin, the effects on cell migration are similar.

Additionally, it is likely that clathrin is impacting cell migration *via* an ERK1/2-independent mechanism.

In addition to advancing our knowledge of how endocytosis modulates CXCR4 biology, we also report that the UMB2 antibody differentially detects CXCR4 post receptor stimulus. This effect is agonist specific, independent of receptor internalization, and not due to differential protein extraction. While not directly tested in this work, and since the UMB2 antibody binds to the c-terminus of the receptor, we believe that the observed effect is due to changes in CXCR4 PTM. As CXCR4 is rapidly post-translationally modified upon receptor-ligand binding, it is plausible that these modifications interfere with antibody binding. In agreement with previous work with EGFR, our results support that clathrin silencing does not modulate receptor PTM kinetics[172].

While our study along with a growing number of others supports a dynamic model of compensatory endocytic mechanism working together to precisely regulate receptor biology, additional research is necessary to determine whether these observations are direct effects of endocytic adapter protein-GPCR interactions or secondary effects of modulating this essential cellular process. Additionally, due to the extraordinary cell type and tissue specificity of GPCR expression, it is necessary to interpret these experiments within this context and it will be important to determine whether these observations are tissue- and/or GPCR-specific or more broadly applicable to other receptors. Together with knowledge that endocytic dynamics are modulated by cancer[173,208], further mechanistic investigations of how CME and clathrin-independent endocytosis regulate receptor biology is likely to reveal novel signaling mechanism that may provide new therapeutic strategies to selectively target pathogenic GPCR signaling such as CXCR4 in metastatic cancers.

2.6 Acknowledgements

MSD conceived, designed, carried out experiments, analyzed data, and wrote the manuscript. LR-M contributed to reagent generation, data acquisition, and data interpretation. SS contributed to data interpretation. AL conceived experiments and edited the manuscript. The work is supported in part by Pardee Foundation and a gift from Kendall and Susan Warren. MSD thanks support from the National Science Foundation GRFP.

Chapter 3 β-arrestin Regulates Communication Between Plasma Membrane and Intracellular GPCRs to Control Signaling

This chapter is currently in submission and posted on bioRxiv. Author Contributions: M.S.D., A.P.L., and A.V.S. designed experiments. M.S.D analyzed data, and wrote the manuscript. A.V.S contributed some reagents. A.P.L., A.S.V., and S.S. provided experimental advice, edited the manuscript, and provided funding.

3.1 Abstract

It has become increasingly apparent that G protein-coupled receptor (GPCR) localization is a master regulator of cell signaling. However, the molecular mechanisms involved in this process are not well understood. To date, observations of intracellular GPCR activation can be organized into two categories: a dependence on OCT3 cationic channel-permeable ligands or the necessity of endocytic trafficking. Using CXC chemokine receptor 4 (CXCR4) as a model, we identified a third mechanism of intracellular GPCR signaling. We show that independent of membrane permeable ligands and endocytosis, upon stimulation, plasma membrane and internal pools of CXCR4 are post-translationally modified and collectively regulate *EGR1* transcription. We found that β-arrestin-1 (arrestin 2) is necessary to mediate communication between plasma membrane and internal pools of CXCR4. Notably, these observations may explain that while CXCR4 overexpression is highly correlated with cancer metastasis and mortality, plasma membrane localization is not. Together these data support a model were a small initial pool of

plasma membrane-localized GPCRs are capable of activating internal receptor-dependent signaling events.

3.2 Introduction

While extracellular inputs, cell membrane receptors, and resulting transcriptional programs are diverse, many receptor-signaling events converge to a reduced number of signaling hubs. Cellular mechanisms that mediate this process as well as strategies to control these actions remain outstanding questions. Over the last decade, we have learned that GPCR spatiotemporal signaling is one mechanism used by cells to translate diverse environmental information into actionable intracellular decisions while using seemingly redundant signaling cascades[54]. Extensive research has illustrated that GPCRs elicit distinct signaling events at different plasma membrane micro-domains as well as endocytic compartments that are important for cell physiology and disease pathogenesis[54,100,131,135,153,154,209–211]. These studies support a model where the location, in addition to magnitude, of a signaling event is important for cellular decision-making. Others have shown that GPCR site-specific post-translational modifications (PTMs) modulate adaptor protein recruitment, GPCR localization, and consequently receptor signaling events[60,61,212]. Together these observations motivated us to reexamine some confounding observations pertaining to the relationship of receptor localization, PTM, and signaling for CXCR4.

CXCR4 is a type 1 GPCR that regulates a variety of biological processes such as cell migration, embryogenesis, and immune cell homeostasis[60,62–65]. It is deregulated in 23 different cancers and overexpression is often correlated with metastasis and mortality[74,75,163,213,214]. However, surprisingly, plasma membrane expression is not correlated with metastasis[163] and in some cancer tumor specimens as well as cell culture models, samples with poor CXCR4 plasma

membrane localization remain responsive to CXCR4 agonist[215–219]. CXCR4 is activated by a highly receptor-specific 8 kDa chemokine, CXCL12[84,220,221]. Unlike β-adrenergic receptors which have been shown to be activated at intracellular compartments in an OCT3 cationic transporter-dependent mechanism[153,154], endocytic-independent internalization of CXCL12 is unlikely due to its size. Given that receptor activation is dependent on ligand binding or transactivation by another receptor[222,223], the aforementioned observations are confounding, as cells with low plasma membrane CXCR4 remain highly responsive to CXCL12. There are two potential explanations for this observation. Firstly, this could be due to spare receptors on the plasma membrane as it is well established that only a limited number of plasma membrane receptor contribute to signaling[224]. Alternatively, this could be due to activation of intracellular pools of receptors.

3.3 Materials and Methods

Table 3-1 Reagents

Inhibitors	**PN**	**Supplier**	**Working Concentration**
Gallein	3090/50	R&D Systems	10 μM
Dynasore	324410	Sigma	100 μM
MTA	D5011	Thermo Fisher	200 μM
Antibodies			
ms-FLAG-647	A01811-100	Genscript	1:1000 (FC/IF)
rb-MYC	A190-105A	Bethyl	1:5000 (WB)

		Laboratories	
rb-CXCR4 (UMB2)	Ab124824	Abcam	1:2000 (WB), 1:1000 (FC/IF)
Rb-phospho-ERK1/2	4370S	Cell Signaling Technologies	1:2000 (WB)
ms-total-ERK1/2	4696S	Cell Signaling Technologies	1:1000 (WB)
rb-phospho-AKT S473	4060S	Cell Signaling Technologies	1:2000 (WB)
rb-total AKT	C67E7	Cell Signaling Technologies	1:1000 (WB)
rb-GM130	12480S	Cell Signaling Technologies	1:1000 (IF)
ms-GAPDH	sc-47724	Santa Cruz Biotechnology	1:1000 (WB)
STREP-568	S11226	Thermo Fisher	1:5000 (WB)
ms-CXCR4-APC (12G5)	FAB170A	R&D Systems	1:200 – 1:2000 (FC)
rb-β-Arrestin-1	30036S	Cell Signaling Technologies	1:1000 (WB)
gt-anti-rb Dylight 800	SA5-35571	Thermo Fisher	1:5000 (WB)
gt-anti-ms Dylight	35518	Thermo Fisher	1:5000 (WB)

680			
gt-anti-rb-488-AlexaFluor-Plus	A32731	Thermo Fisher	1:1000 (FC/IF)
Gt-anti-rb-555	84541	Thermo Fisher	1:1000 (IF)
Gt-anti-rb-Alexafluor-Plus	A32733	Thermo Fisher	1:1000 (IF)
Gt-anti-ms-488	A10680	Thermo Fisher	1:1000 (IF)
Gt-anti-ms-647	A21235	Thermo Fisher	1:1000 (IF)
Biologics			
CXCL12	350-NS-050	R&D Systems	12.5 nM
Pronase	10165921001	Sigma	0.1% Solution
Lambda Phosphatase	sc-200312A	Santa Cruz Biotechnology	Manual instructions
Others			
Streptavidin agarose	20361	Thermo Fisher	
DAPI	D9542	Sigma	1µg /mL
NewBlot PVDF Stripping buffer	928-40032	LiCor	Manual instructions
PVDF 0.22 µm membranes	IB401001	Thermo Fisher	NA
NHS-Sulfo-LC-biotin	21335	Thermo Fisher	1 mg/mL

ITAQ Universal SYBR Green	1725121	BioRad	Manual instructions
iScript cDNA Synthesis Kit	1706891	RioRad	Manual instructions
Quick-RNA miniprep	R1054	Zymo Research	Manual instructions

Equipment

LiCor Odessey CLX & SA Imagers

Azure Sapphire 4 laser Imager

BioRad RT-qPCR ThermoCycler

Cell culture

HeLa cells were originally obtained from ATCC. HeLa cells were cultured in DMEM media (Corning) supplemented with 10% FBS (Corning). Retinal pigment epithelial (RPE) cells were a gift from Dr. Sandra Schmid at UT Southwestern. All stable cell lines were directly derived from this RPE line. RPE cell lines were cultured in DMEM/F12 media (Corning) supplemented with HEPES, glutamate and 10% FBS (Corning). HEK293T cells were obtained from ATCC and grown in DMEM (Corning) media supplemented with 10% FBS.

DNA constructs and stable cell lines

WT CXCR4 was generated as previously described[225]. K3R and K3R/Q mutant receptors were generated by PCR mutagenesis of WT CXCR4 in the pLVX plasmid using the NEB Quick-

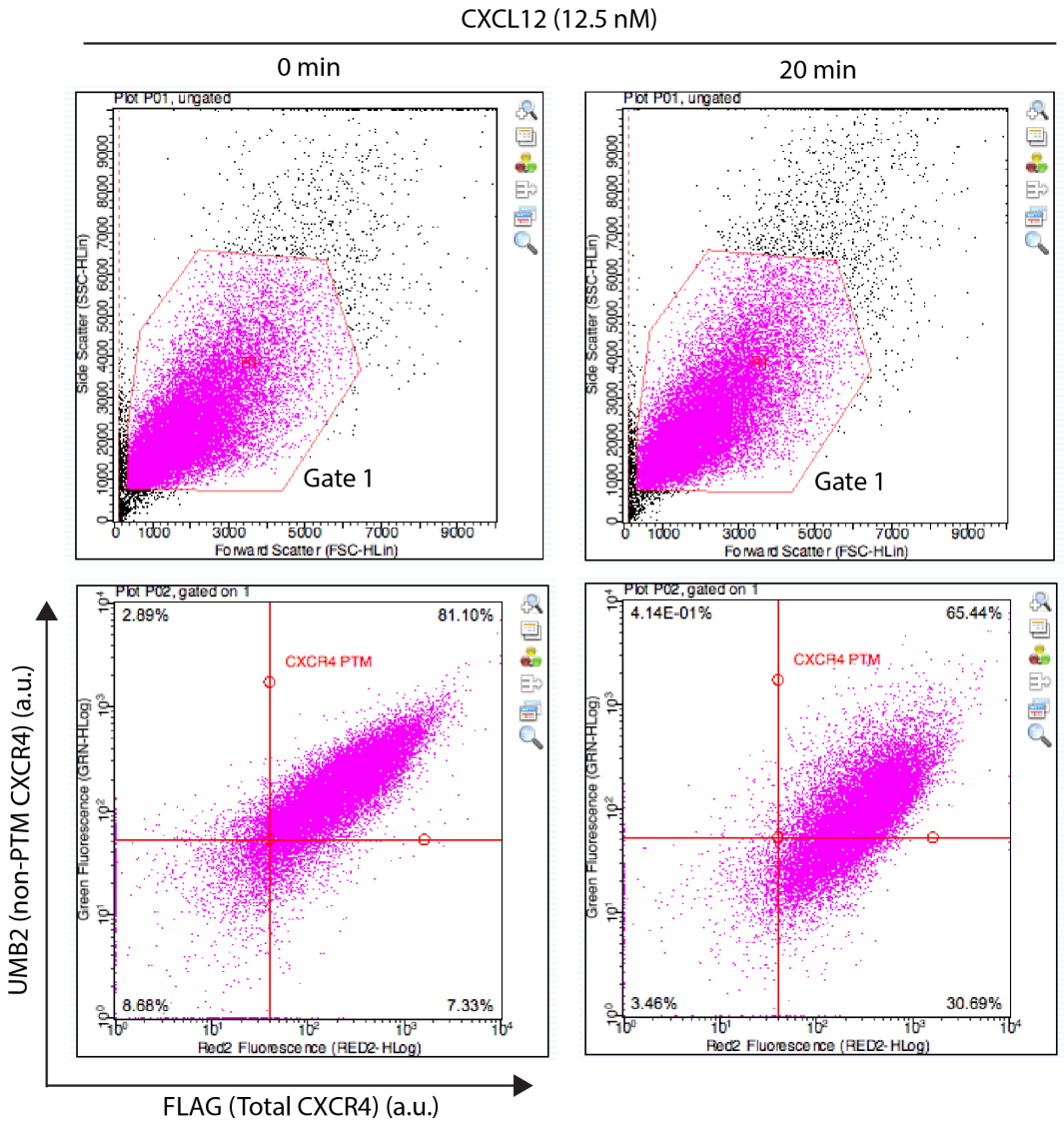
change mutagenesis kit. The low plasma membrane CXCR4 construct was generated by PCR amplification (excluding the 5' plasma membrane HA localization peptide) and restriction enzyme cloning using the *BsrRI* and *EcoRI* restriction enzymes. All CXCR4 constructs had an N-terminal FLAG tag and C-terminal MYC tag for easy antibody detection. Stable cell lines expressing WT and mutant CXCR4 receptors were generated by lentiviral transduction. Lentiviruses (shRNA and CXCR4 constructs for stable cell lines) were generated by co-transfecting HEK293T cells with the pLVX transfer plasmid, psPAX2, and pMD2.G lentiviral envelope and packaging plasmids. To generate stable cell lines, supernatant media containing mature lentiviral particles was collected 4 days post transfection and added to RPE cells, and cells stably expressing the constructs were generated via puromycin selection (3 μg/mL). All transfections were conducted using Lipofectamine 2000 (Life technologies).

Flow cytometry experiments

Flow cytometry experiments for plasma membrane receptor labeling were conducted as previously described[225]. For intracellular staining, cells were first disassociated using 50 μM EDTA in Ca^{2+}-free PBS and fix for 10 min in 4% paraformaldehyde at room temperature. Afterwards, cells were permeabilized using 0.2% Triton-X 100 for 10 min at room temperature. Intracellular targets were labeled with primary antibodies for 1 hr at room temperature after which cells were washed with PBS and incubated with secondary antibodies for 1 hr at room temperature – see Table 3-1 for antibody specifics. Afterwards, cells were washed 1x with PBS and 25,000 events were analyzed by the Guava EasyCyte flow cytometer for each experimental condition. When co-staining, compensation was conducted post experiment using controls with either 488 or 640 fluorescence alone. After fluorescence compensation, the median fluorescence

was calculated for each channel and sample as well as for no stain and RPE WT controls (not expressing CXCR4). As previously described, median control sample fluorescence was subtracted from each sample and data was normalized and plotted as described in each figure legend[225]. An example of flow cytometry raw data, gating strategy, and compensation controls are shown in Figure 3-1.

a


CXCL12 (12.5 nM)
0 min
20 min
Plot P01, ungated
Side Scatter (SSC-HLin)
Forward Scatter (FSC-HLin)
Gate 1
Plot P02, gated on 1
2.89%
81.10%
8.66%
7.33%
4.14E-01%
65.44%
3.46%
30.69%
CXCR4 PTM
Green Fluorescence (GRN-HLog)
Red2 Fluorescence (RED2-HLog)
UMB2 (non-PTM CXCR4) (a.u.)
FLAG (Total CXCR4) (a.u.)

b

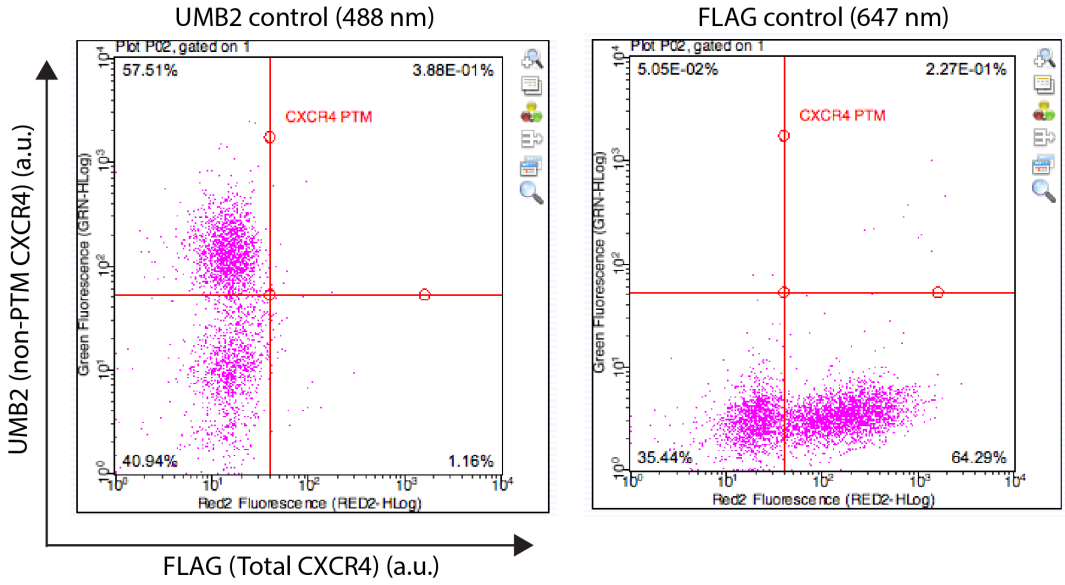

UMB2 control (488 nm)
FLAG control (647 nm)
Plot P02, gated on 1
57.51%
3.88E-01%
40.94%
1.16%
5.05E-02%
2.27E-01%
35.44%
64.29%
CXCR4 PTM
Green Fluorescence (GRN-HLog)
Red2 Fluorescence (RED2-HLog)
UMB2 (non-PTM CXCR4) (a.u.)
FLAG (Total CXCR4) (a.u.)

Figure 3-1 Representation of flow cytometry data and gating strategy.

(**A**) Representative SSC and FSC for flow cytometry analysis. Gate 1 is defined by the red polygon. Two samples are shown from the same experiment investigating UMB2 detection. The second row of plots correspond to the UMB2 and FLAG detection of CXCR4 in RPE cells overexpressing CXCR4 during the CXCL12 stimulus time course. Quadrants are shown to illustrate that while total CXCR4 detection remains constant, UMB2 detection decreases upon CXCL12 stimulation. X and Y axis are labeled accordingly. (**B**) Control experiments used for fluorophore compensation and deciding background fluorescence for quantification. With the exception of compensation controls (b) where only 5000 events were recorded, 25,000 events were collected in all experiments. All analyses were repeated for a minimum of 3 independent biological replicates and is stated in figure legends.

Cell signaling, shRNA and inhibitor experiments

Cells were seeded in 12 well plates 24 hr prior to each signaling experiment achieving 70-80% confluence at the experimentation time. Cells were serum-starved in DMEM/F12 media without FBS for 4 hr prior to each signaling experiment. For inhibitor experiments (Gallein, Dynasore) cells were pretreated for 30 min with the respective inhibitors and throughout the signaling experiment. For shRNA experiments, cells were transduced with either scramble or β-arrestin-1 or 2 shRNA (Table 3-2) for 3 days. shRNA knockdown levels are shown in Figure 3-2. shRNA lentiviral particles were generated as described above. Afterwards cells were stimulated with 12.5 nM CXCL12 (R&D Systems) for the labeled time course. Samples were washed with PBS 1x and lysed using RIPA buffer supplemented with protease inhibitors (EDTA-free Peirce protease inhibitor cocktail) and phosphatase inhibitors (HALT Phosphatase Inhibitor). For Lambda Phosphatase experiments, phosphatase inhibitors were excluded in the lysis buffer.

After incubating cells with lysis buffer for 10 min on ice, lysates were collected and centrifuged at 16,000 *g* for 45 min at 4 °C. Afterwards lysates were immediately stored at -20 °C or processed for immunoprecipitation or western blotting.

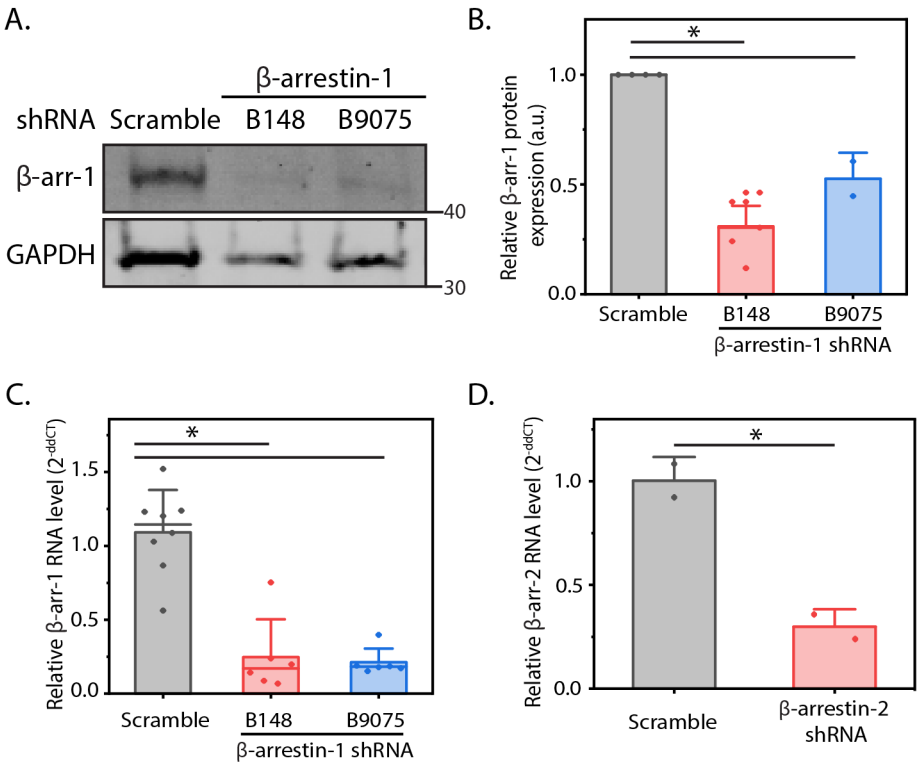


Figure 3-2 β-arrestin knockdown confirmation

(A) Representative western blot illustrating relative β-arrestin-1 knockdown with two different shRNAs. **(B)** Quantification of β-arrestin-1 knockdown efficiency by western blot. Protein knockdown was calculated by dividing normalized β-arrestin-1 detection with β-arrestin-1 shRNA with scramble shRNA. **(C-D)** Relative β-arrestin-1 and β-arrestin-2 transcript levels were calculated using the ΔΔct method normalized to GAPDH and the scramble shRNA control. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course. Individual data points from each experiment are plotted; mean, SD, median line. Statistical significance (*) denotes $p < 0.05$.

Table 3-2 shRNA constructs

shRNA (pKLO1 vector)	Sequence
Scramble (non-targeting)	Sigma-SCH002
β-arrestin-1	
TRCN0000230148	CCGGAGATCTCAGTGCGCCAGTATGCTCGAGCATACTGGCGCACTGAGATCTTTTTTG
TRCN0000219075	GTACCGGACACAAATGATGACGACATTGCTCGAGCAATGTCGTCATCATTTGTGTTTTTTTG
β-arrestin-2	
TRCN0000280686	CCGGGATACCAACTATGCCACAGATCTCGAGATCTGTGGCATAGTTGGTATCTTTTTG
TRCN0000280619	CCGGGCTAAATCACTAGAAGAGAAACTCGAGTTTCTCTTCTAGTGATTTAGCTTTTTG

Immunoprecipitation

For plasma membrane biotinylation experiments, biotinylated plasma membrane proteins were isolated from WCL using high capacity streptavidin agarose beads (Table 3.1). Approximately 35 µl of bead slurry was added to 350 µl WCL and incubated overnight (~ 18 hr) rotating at 4 °C. Afterwards, samples were pelleted (centrifuged for 3 min at 2,000 *g*) and internal (non-plasma membrane) proteins collected by removing the supernatant. To prevent potential biotinylated protein contamination, only 200 µl of supernatant was removed. Afterwards, beads were washed 3 times with RIPA buffer containing protease inhibitors. After the final wash, all buffer was removed.

Western blotting and data analysis

Prior to western blotting, samples were incubated with Laminelli buffer supplemented with β-mercaptoethanol (loading buffer). For surface biotinylation samples, β-mercaptoethanol concentration was increased 2-fold and samples were incubated at room temperature in the loading buffer for 30 min prior to western blotting to denature proteins from beads. Samples were run on SDS-PAGE 4-20% BioRad gels (15 well/15 µl or 10 well/50 µl gels). For all signaling experiments, 12.5 µl of lysate was loaded while for surface biotinylation assays, 35 µl of lysate was loaded. SDS-PAGE gels were run at constant 140 V for approximately 60 min. Afterwards, proteins were transferred to PVDF membranes using the iBlot transfer systems (mixed range proteins 7 min setting) and membrane incubated in blocking solution (1% BSA in TBST) rocking for 1 hr at room temperature. Afterwards, blots were incubated with their respective antibodies (Table 3-1) overnight at 4°C. Prior to secondary labeling, blots were wash 3x for 5 min per wash with TBST. Blots were then incubated with the corresponding secondary antibody (Table 3-1) for 1 hr at room temperature. Blots were then washed with TBST as described above. Western blots were dried and imaged using a LiCor Odessey SA, LiCor CLX, or Azure Biosystems Sapphire System. Data was analyzed using the LiCor image studio software to calculate band intensity as previously described[225]. Specific normalization procedures for each experiment are described in the respective figure legends. All statistics were calculated using two tailed t-tests.

RT-qPCR experiments

Cells were seeded in 6 well plates 24 hr prior to each signaling experiment achieving 70-80% confluence at the experimentation time. Cells were serum-starved in DMEM/F12 media for 4 hr prior to each signaling experiment. Afterwards, cells were stimulated with 12.5 nM CXCL12 (R&D Systems) for the respective time courses shown in the figure legends. RNA was extracted using the Zymogen RNA extraction kit (R1054) and 1 μM cDNA was synthesized using the iScript synthesis kit (BioRad). qPCR assays were conducted using SYBR Green (BioRad) per BioRad protocol instructions using 12.5 ng of cDNA for each well. Samples were run in duplicate and primers used in this study are shown in Table 3-3. Samples were run on the BioRad CFX thermocycler and data was quantified using the ΔΔCT method as previously described[225].

Table 3-3 qPCR Primers

Primers	**Forward**	**Reverse**
GAPDH	GAGTCAACGGATTTGGTCGT	CTTGATTTTGGAGGGATCTCGC
EGR1	GGTCAGTGGCCTAGTGAGC	GTGCCGCTGAGTAAATGGGA
β-arrestin-1	ATCCCTCCAAACCTTCCATG	TGACCAGACGCACAGATTTC
β-arrestin-2	AAGTGTCCTGTGGCTCAA	TTGGTGTCCTCGTGCTTG

Immunofluorescence Assays

Cells were seeded in 6-well plates on glass coverslips 24 hr prior to each experiment and serum-starved for 4 hr as described above. Cells were stimulated as specified in each figure legend and

immediately washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized for 10 min with 0.2% Triton-X100 diluted in PBS and subsequently blocked with 2.5% BSA diluted in PBS (blocking solution) for 1 hr. Cells were incubated with primary antibody diluted in blocking solution and incubated overnight at 4 ^{o}C (Table 1). Slides were wash 3x for 5 min each with PBS and incubated with secondary antibodies diluted in blocking solution for 1 hr at room temperature (Table 1). Cells were washed with PBS 3x, 5 min per wash and incubated with DAPI (Table 1) diluted in PBS for 10 min at room temperature. Afterwards, cells were washed with PBS and mounted onto glass slides using Fluoromount G. Slides were imaged by spinning disk confocal microscopy as specified in the figure legends. Different experimental samples were imaged using the same imaging settings each day.

3.4 Results

We began first by investigating the role of CXCR4 localization on receptor signaling and PTM. To do so, we needed a strategy to robustly detect CXCR4 PTM as well as a method to modulate receptor localization. We previously established the use of a monoclonal CXCR4 antibody (UMB2) as a robust tool to study CXCR4 PTM[225]. This commercially available antibody is raised against the C-terminus of the receptor, and upon CXCL12 stimulus quickly loses its ability to detect CXCR4 due to receptor PTM[225]. To attempt to identify the specific PTMs responsible, we treated lysates from WT and ubiquitination mutant receptor expressing cells with phosphatase. While phosphatase treatment ablated AKT S473 phosphorylation, no change in UMB2 detection was observed (Figure 3-3A, B). Since it has also been reported that CXCR4 is methylated at C-terminal arginine residues[226,227], we tested whether CXCR4

methylation was responsible for the agonist-dependent loss in UMB2 detection. Similar to phosphatase treatment, protein methylation inhibition did not affect UMB2 detection (Figure 3-3C). Together these results suggest that the agonist-dependent reduction in UMB2 antibody detection is likely due to a combination of CXCR4 PTMs.

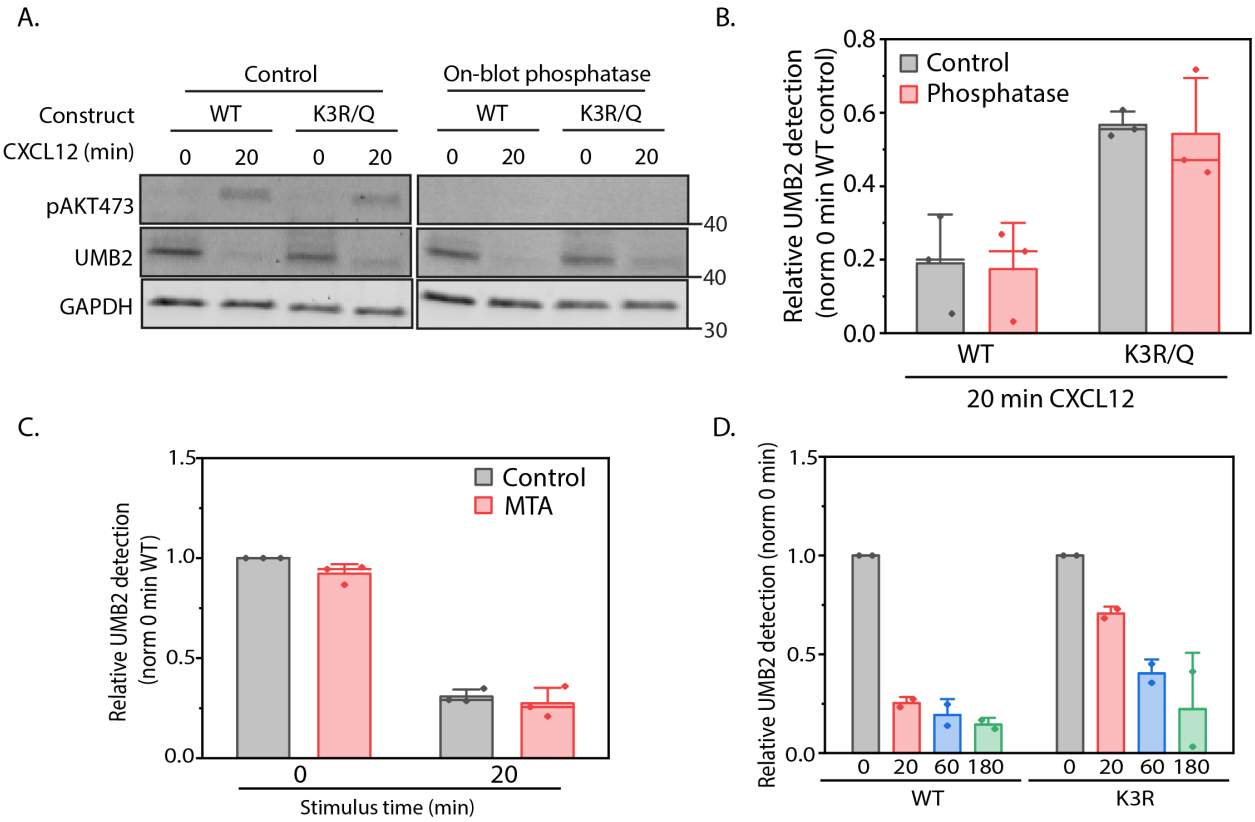


Figure 3-3 UMB2 antibody characterization

(A) Representative western blot of on-blot phosphatase experiment with WT and K3R/Q mutant CXCR4 constructs. The K3R/Q mutant receptor is unable to be ubiquitinated at the canonical C-terminal CXCR4 ubiquitination sites. The on-blot phosphatase blot was incubated with Lambda Phosphatase to remove phosphate groups from phosphorylated protein residues. Phospho-AKT S473 probing was included as a phosphatase treatment positive control. **(B)** Quantification of on-blot phosphatase western blotting experiments. Data was normalized by dividing the UMB2 detection at 20 min by the 0 min time point for either the WT or K3R/Q receptor. **(C)** Flow cytometry analysis of arginine protein methylation activity on CXCR4 UMB2 detection. As

illustrated, cells were treated with methylthioadenosine (MTA 200 μM), an arginine protein methyltransferase inhibitor. UMB2 detection was normalized to total CXCR4 fluorescence and secondly to 0 min control detection. **(D)** Flow cytometry analysis of UMB2 antibody detection for an extended CXCL12-stimulus time course using WT or K3R mutant CXCR4. UMB2 detection was normalized to total CXCR4 fluorescence and secondly to 0 min detection for either WT or K3R mutant receptor. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course. Individual data points from each experiment are plotted; mean, SD, median line.

To manipulate receptor localization, we generated several mutant receptors that modulate the steady-state distribution of CXCR4 within retinal pigment epithelial (RPE) cells (Figure 3-4A). We chose RPE cells to study CXCR4 overexpression because they do not have appreciable endogenous CXCR4 expression, are unresponsive to CXCL12, and were previously established as a cell culture model to study CXCR4 biology[225]. We found that by mutating C-terminal lysine residues to arginine (K3R), CXCR4 plasma membrane localization was reduced by more than 50% (Figure 3-4B, C). While the CXCR4 K3R mutant has been previously used to study CXCR4 degradation[114,228], we found that these mutations caused a drastic change in the spatial distribution of CXCR4. This was due to the unintended creation of an R-X-R motif, which has been shown to increase GPCR retention in the Golgi[155,161,229]. Indeed, mutating a single residue in the R-X-R motif (i.e., K3R/Q) restored receptor plasma membrane localization to near WT levels (Figure 3-4B). Interestingly, while total CXCR4 expression was unchanged by the K3R mutant, the K3R/Q mutant had slightly higher expression compared to WT receptor (Figure 3-4C). In accordance with previous literature, K3R mutant receptors partially colocalized with a Golgi compartment marker (Figure 3-4D)[161,229]. We also noticed a steady-state population of WT CXCR4 retained at the Golgi (Figure 3-4D). Non-plasma membrane localized CXCR4 has been

previously reported[230] and could potentially be due to the presence of a K-X-K motif, which has also been implicated in Golgi protein retention[231–233] or receptor overexpression. It is important to point out that while we observed partial CXCR4 colocalization with the Golgi, it is evident from our microscopy results that CXCR4 is present at other intracellular compartments as well (Figure 3-4D).

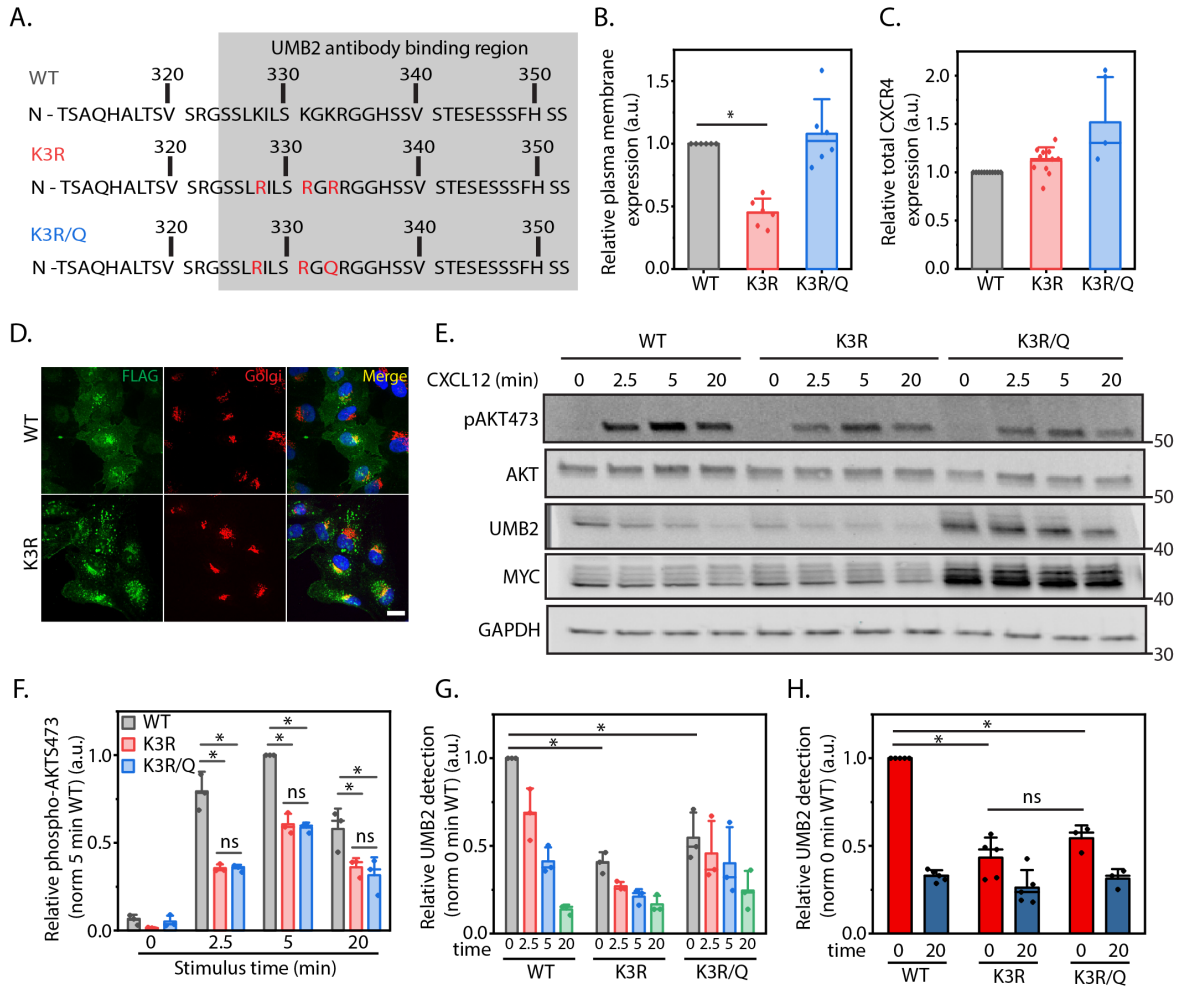


Figure 3-4 CXCL12-depedent AKT S473 phosphorylation and CXCR4 PTM are independent of CXCR4 localization

(A) Illustration of CXCR4 mutant receptor constructs. The gray box denotes the binding region of the UMB2 antibody that is sensitive to CXCR4 PTM. **(B)** Flow cytometry analysis of overexpressed WT and mutant receptor plasma membrane localization in RPE cells. Data was

normalized to total receptor and WT plasma membrane expression. **(C)** Flow cytometry analysis of WT and mutant receptor total expression. Individual data points were normalized to WT CXCR4 expression. **(D)** Representative microscopy images illustrating the distribution of WT and K3R CXCR4 localization within RPE cells overexpressing each construct. CXCR4 was labeled with a FLAG antibody and the Golgi was detected using a GM130 antibody. Scale bar is 10 μm. Images were captured using 60x magnification on a spinning disk confocal microscope. **(E)** Representative western blot illustrating CXCL12-induced (12.5 nM) AKT S473 phosphorylation and CXCR4 PTM for WT and mutant receptors. Total CXCR4 was detected using a MYC antibody and unmodified CXCR4 by UMB2. **(F)** Western blot quantification of AKT S473 phosphorylation for WT and mutant CXCR4. Relative AKT phosphorylation was calculated by normalizing phospho-AKT to total AKT band intensity and secondly to the 5 min control time point. **(G)** Western blot quantification of CXCR4 PTM (i.e. UMB2 detection). A decrease in UMB2 detection is correlated to increased CXCR4 PTM. CXCR4 PTM was calculated by dividing the UMB2 intensity by the MYC intensity (total CXCR4) and secondly to the 0 min time point for the WT receptor. **(H)** Flow cytometry analysis of agonist-dependent WT and mutant CXCR4 PTM. Relative UMB2 detection was determined by dividing median UMB2 detection by total CXCR4 fluorescence and normalized to 0 min WT CXCR4. All experiments were conducted a minimum of 3 times in RPE cells overexpressing WT or mutant CXCR4. Individual data points from each experiment are plotted; mean, standard deviation (SD), median line. Statistical significance (*) denotes $p < 0.05$.

Having established methods to modulate receptor localization and monitor PTM, we proceeded to investigate the role of CXCR4 plasma membrane localization on CXCL12-dependent AKT S473 phosphorylation. Since CXCL12 is not membrane permeable, we hypothesized that plasma membrane localization is essential for CXCR4 signaling and that reducing receptor plasma membrane expression would decrease CXCL12-dependent AKT phosphorylation. Compared to WT, both mutants had significantly reduced CXCL12-dependent AKT phosphorylation (Figure 3-4E). This was expected as mutating biologically relevant

residues may affect G protein coupling to CXCR4. However, surprisingly, there was no difference in AKT phosphorylation between high (K3R/Q) and low (K3R) plasma membrane localized mutant receptors (Figure 3-4E, F). Additionally, while not explicitly investigated, earlier studies using the K3R mutant also reported that CXCL12-induced ERK1/2 phosphorylation was similar between WT and K3R mutant receptor expressing cells[228]. Since receptor PTM plays a role in mediating receptor signaling, we investigated the effect of receptor localization on agonist-induced CXCR4 PTM. Although UMB2 detection continued to decrease over a course of three hours (Figure 3-3D), here we focused on the UMB2 detection in the first 20 minutes post stimulus since we were interested in how early CXCR4 PTM regulates cell signaling. We hypothesized that receptor plasma membrane localization is essential for agonist-dependent PTM as agonist-induced receptor PTM is believed to require ligand binding. Surprisingly, irrespective of plasma membrane localization, mutant receptor PTMs were similar (Figure 3-4E, G, H). Relative to total receptor expression, initial detection using the UMB2 antibody for both mutant receptors was also reduced (Figure 3-4E, G, H). We believe this is because these mutations occur in the UMB2 antibody-binding region of the receptor (Figure 3-4A). Alternatively, this could suggest a difference in steady-state mutant CXCR4 PTM.

Intrigued by the observation of a similar degree of CXCR4 PTM despite vastly different plasma membrane localization, we wondered whether internal (non-plasma membrane) pools of CXCR4 could be post-translationally modified in response to agonist stimulation and contribute to signaling. To investigate this, we examined the localization of CXCR4 PTM during receptor signaling. As previously observed[225], upon CXCL12 addition UMB2 detection was drastically reduced at both plasma membrane and intracellular compartments, suggesting that both plasma membrane and internal pools of CXCR4 are post-translationally modified (Figure 3-5A). To test

this directly, we developed an assay to selectively isolate plasma membrane proteins from whole cell lysate. We used a membrane-impermeable promiscuous biotin molecule to selectively label and immunoprecipitate plasma membrane proteins with accessible extracellular domains (Figure 3-5B). Receptor internalization was blocked throughout these experiments to keep plasma membrane and internal receptor pools distinct. We hypothesized that only plasma membrane receptors would be post-translationally modified, as internal pools of receptors are inaccessible to ligand and endocytosis of plasma membrane receptors is blocked. Surprisingly, we found that both surface and internal pools of receptors were post-translationally modified after ligand addition (Figure 3-5C, D). To ensure that the labeling strategy was working as expected, we probed for GAPDH and biotinylated proteins and showed enrichment in expected localizations (Figure 3-5C). To further examine intracellular CXCR4 PTM, we utilized blocking antibodies to effectively tune plasma membrane activity of endogenous CXCR4 in HeLa cells by varying the concentration of blocking antibodies (Figure 3-5E). While the blocking antibody effectively reduced steady-state non-post-translationally modified CXCR4 (Figure 3-5F), agonist addition had no effect on CXCR4 PTM after 20 min stimulus irrespective of CXCR4 plasma membrane expression level (Figure 3-5G). Together these data support a model where internal pools of CXCR4 are post-translationally modified in response to CXCL12. Additionally, plasma membrane proteins are required for this process as removal of plasma membrane extracellular motifs using protease treatment completely ablated CXCL12-dependent CXCR4 PTM (Figure 3-6).

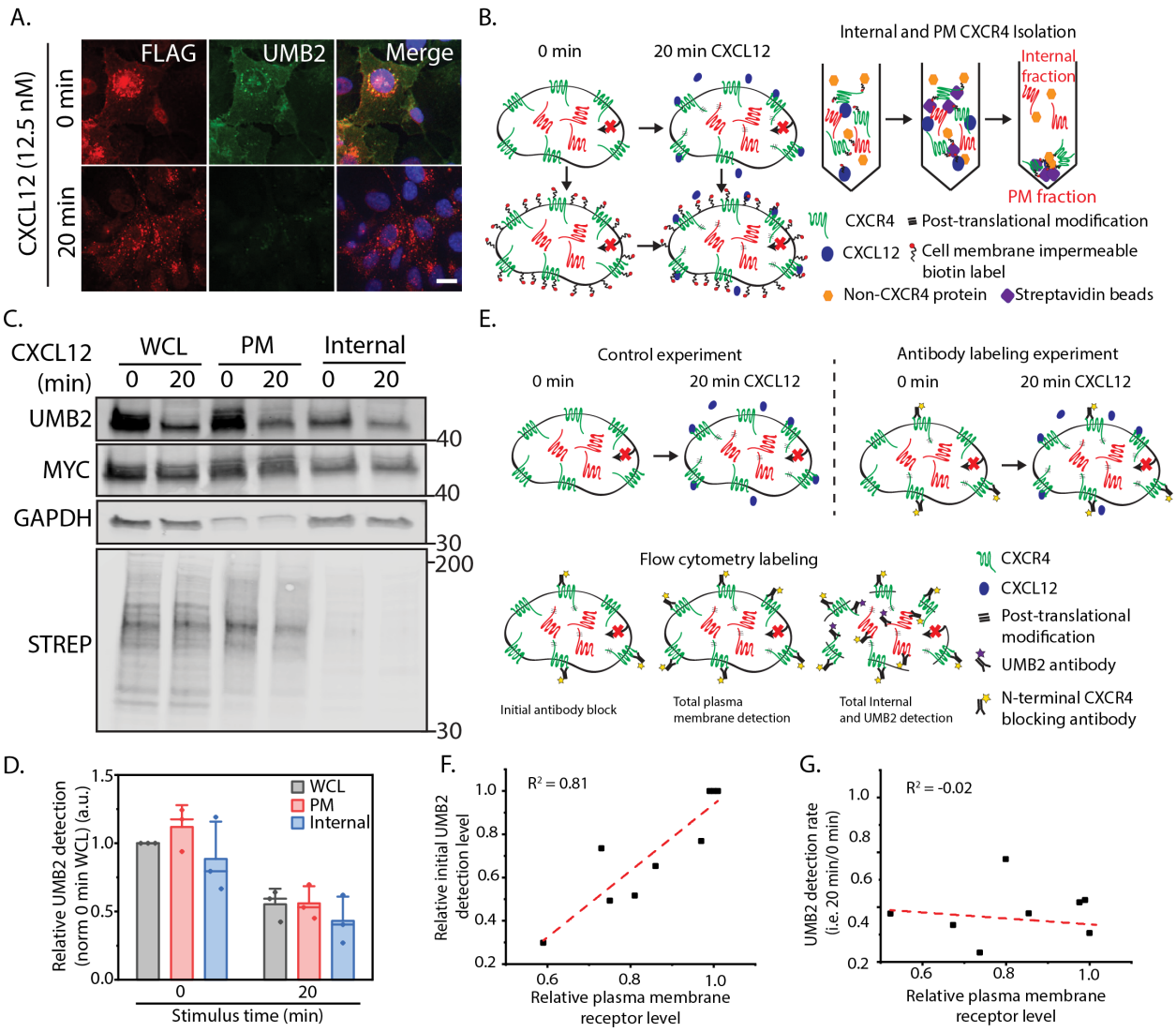


Figure 3-5 Plasma membrane and internal pools of CXCR4 are post-translationally modified upon receptor stimulus

(A) Representative microscopy images of total and non-post-translationally modified CXCR4 pre and 20 min post CXCL12 (12.5 nM) stimulation. Total CXCR4 is detected by FLAG antibody and non-PTM CXCR4 by UMB2. Images were captured using 60x magnification on a spinning disk confocal microscope. Scale bar is 10 µm. **(B)** Plasma membrane and internal

CXCR4 isolation assay schematic. Receptor internalization was blocked using Dynasore (100 μM) throughout the experiment. At the completion of CXCL12 stimulation, plasma membrane proteins from control and CXCL12-treated samples were covalently labeled using promiscuous, membrane impermeable NHS-sulfo-biotin. Afterwards, plasma membrane proteins were isolated from whole cell lysate (WCL) by immunoprecipitation and WCL, plasma membrane, and internal pools of CXCR4 were analyzed for PTM by western blot. **(C)** Representative western blot showing CXCL12-dependent (12.5 nM) CXCR4 PTMs of WCL, plasma membrane, and internal CXCR4. STREP and GAPDH were used as experimental validation. **(D)** Quantification of CXCR4 PTMs at plasma membrane and internal locations. CXCR4 PTMs were calculated by dividing UMB2 detection (non-PTM CXCR4) by MYC intensity (total CXCR4) and normalized to the 0 min WCL sample. **(E)** Experimental schematic for antibody blocking experiment. Incubating cells with different concentrations of CXCR4 antibody reduced plasma membrane-localized, ligand-accessible, CXCR4. Afterwards, total plasma membrane, total CXCR4 and PTM CXCR4 were quantified by flow cytometry. Experiments were conducted in HeLa cells stimulated with or without 12.5 nM CXCL12. CXCR4 was blocked using various dilutions of a 12G5 allophycocyanin-conjugated CXCR4 antibody. Receptor internalization was blocked throughout all experiments using Dynasore (100 μM). **(F)** Initial antibody block reduced relative CXCR4 expression and basal UMB2 detection. **(G)** Relative UMB2 detection 20 min post CXCL12 stimulus plotted against relative CXCR4 plasma membrane expression. R^2 values are shown for each experiment. All experiments were conducted in RPE cells overexpressing CXCR4 unless noted. A minimum of 3 independent replicates were conducted for all experiments and individual data points from each experiment are plotted; mean, SD, median line.

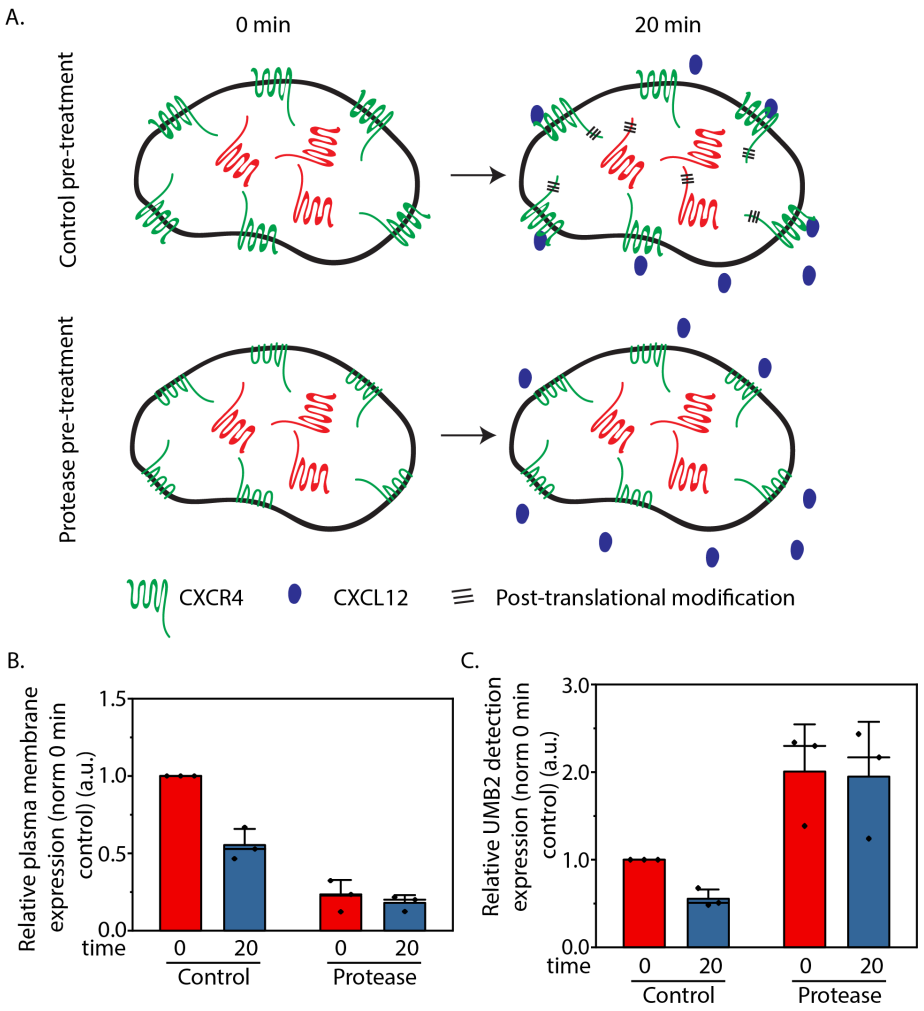


Figure 3-6 Functional plasma membrane proteins are required for CXCR4 PTM

(A) Experimental design schematic for the protease cleavage assay. Cells were pretreated with a protease (pronase 0.11% diluted in PBS) to remove extracellular motifs of transmembrane plasma membrane proteins. Afterwards, cells were stimulated with CXCL12 and plasma membrane CXCR4 and UMB2 detection was measured by flow cytometry. **(B)** Flow cytometry analysis of protease treatment on functional CXCR4 plasma membrane localization. Plasma membrane expression was measured using a FLAG antibody that detected an N-terminal FLAG-tagged CXCR4 and normalized to total CXCR4 expression. Data was normalized to the 0 min control sample. **(C)** Flow cytometry analysis of protease treatment on CXCR4 UMB2 detection. Relative UMB2 detection was calculated by dividing UMB2 signal by total CXCR4 signal (FLAG antibody) and then normalized to the 0 min control sample. An increase in UMB2 detection in the protease treatment sample is expected as the detectable total CXCR4 population is reduced due to protease cleavage of the N-terminal CXCR4 FLAG tag. All experiments were

conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course. Individual data points from each experiment are plotted; mean, SD, median line.

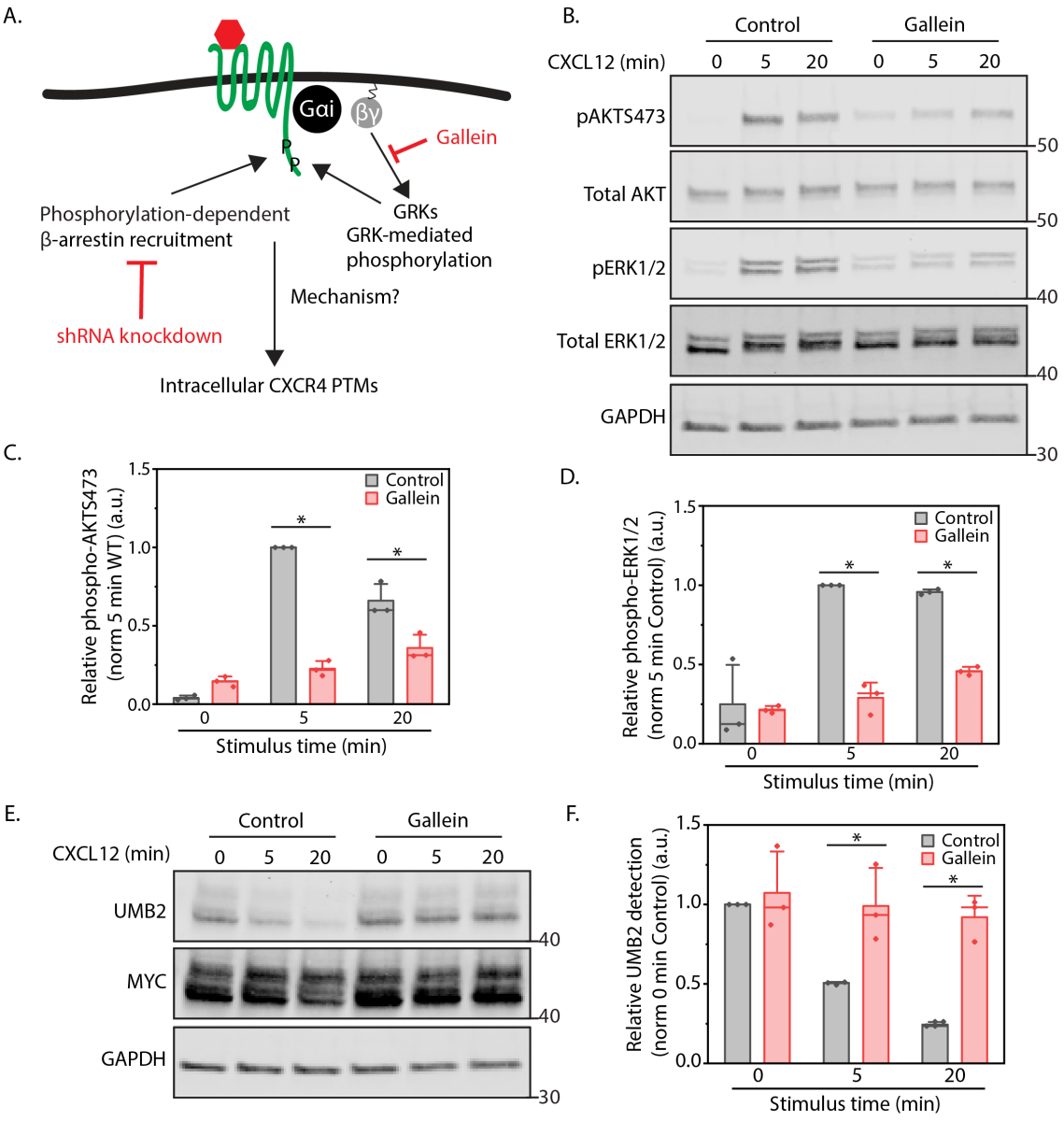


Figure 3-7 $G_{\beta\gamma}$ signaling is essential for CXCR4 signaling and PTM.

(A) Illustration of the current model of GPCR desensitization. Perturbations used to antagonize different components of the pathway are highlighted in red. **(B)** Representative western blot illustrating the effects of $G_{\beta\gamma}$ inhibition (Gallein, 10 μM) treatment on CXCL12-induced AKT

S473 and ERK1/2 phosphorylation. Cells were pretreated with Gallein for 30 min prior to and throughout each signaling time course. (**C-D)** Western Blot quantification of AKT S473 and ERK1/2 phosphorylation after $G_{\beta\gamma}$ inhibition. Relative signaling protein phosphorylation was calculated by dividing the phosphorylated protein detection by total signaling protein detection and then normalized to the 5 min time point of the control sample. **(E)** Representative western blot illustrating the effect of $G_{\beta\gamma}$ inhibition (Gallein 10 μM) on CXCR4 PTM. Cells were pretreated with Gallein for 30 min prior to and throughout the signaling time course. **(F)** Western blot quantification of CXCR4 UMB2 detection (i.e. PTM) upon $G_{\beta\gamma}$ inhibition. CXCR4 PTM was calculated by dividing UMB2 detection (non-PTM CXCR4) by MYC intensity (total CXCR4) and normalized to the 0 min control sample. For all experiments a minimum of 3 independent replicates were performed. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course. Individual data points from each experiment are plotted; mean, SD, median line. Statistical significance (*) denotes $p < 0.05$.

Our findings so far suggest that a signaling cascade may be responsible for intracellular communication between plasma membrane and internal receptor pools. Next, we focused on identifying the proteins responsible for agonist-dependent internal CXCR4 PTM. G proteins are master regulators for GPCR signaling and recent studies have revealed tight spatiotemporal regulation of G protein signaling events[154,210,234]. Upon ligand binding, G protein $G_{\alpha i}$ and $G_{\beta\gamma}$ subunits are released from the GPCR due to guanidine exchange factor activity. $G_{\beta\gamma}$ activates GPCR kinases (GRKs), which quickly phosphorylate the C-terminus of activated receptors leading to β-arrestin recruitment (Figure 3-7A)[235]. Therefore, we hypothesized that $G_{\beta\gamma}$ inhibition would reduce CXCL12-induced signaling and consequentially CXCR4 PTM. Indeed, pharmacological inhibition of $G_{\beta\gamma}$ signaling significantly reduced both ERK1/2 and AKT phosphorylation (Figure 3-7B-D). Additionally, $G_{\beta\gamma}$ inhibition completely ablated CXCL12-dependent CXCR4 PTM (Figure 3-7E, F).

Since $G_{\beta\gamma}$ activation leads to GPCR phosphorylation and β-arrestin recruitment, we decided to investigate whether β-arrestins were involved in regulating internal CXCR4 PTM. β-arrestins have been previously implicated as potent messenger molecules. Coined signaling at a distance, work from the von Zastrow group proposed a new model for β-arrestin-dependent MAPK signaling in which β-arrestin-2, activated by stimulated GPCRs on the plasma membrane, traffics to nearby clathrin-coated structures to initiate localized MAPK signaling[211,236]. β-arrestin-1 and 2 are not equal, and significant research has revealed potential site-specific PTM and kinase phosphorylation-specific recruitment to CXCR4 as well as other GPCRs[61]. We hypothesized that β-arrestin 1 or 2 are important for communication between plasma membrane and internal CXCR4 (Figure 3-7A). β-arrestin-1 knockdown led to a reduction in agonist-dependent CXCR4 PTMs while β-arrestin-2 knockdown had no effect (Figure 2-8). β-arrestin-1 knockdown did not affect ERK1/2 phosphorylation but led to a slight increase in AKT phosphorylation (Figure 3-8B-E). This is potentially due to a failure to arrest G protein signaling. Intrigued by the potential new role of β-arrestin-1 in regulating the communication between plasma membrane and internal pools of receptors, we used the plasma membrane biotinylation assay to determine which CXCR4 population is regulated by β-arrestin-1. While β-arrestin-1 knockdown did not affect plasma membrane-localized CXCR4 PTM, internal CXCR4 PTM was reduced (Figure 3-8F-H). Together these data support a mechanism by which $G_{\beta\gamma}$ and β-arrestin-1 work together to regulate communication between plasma membrane and internal pools of CXCR4.

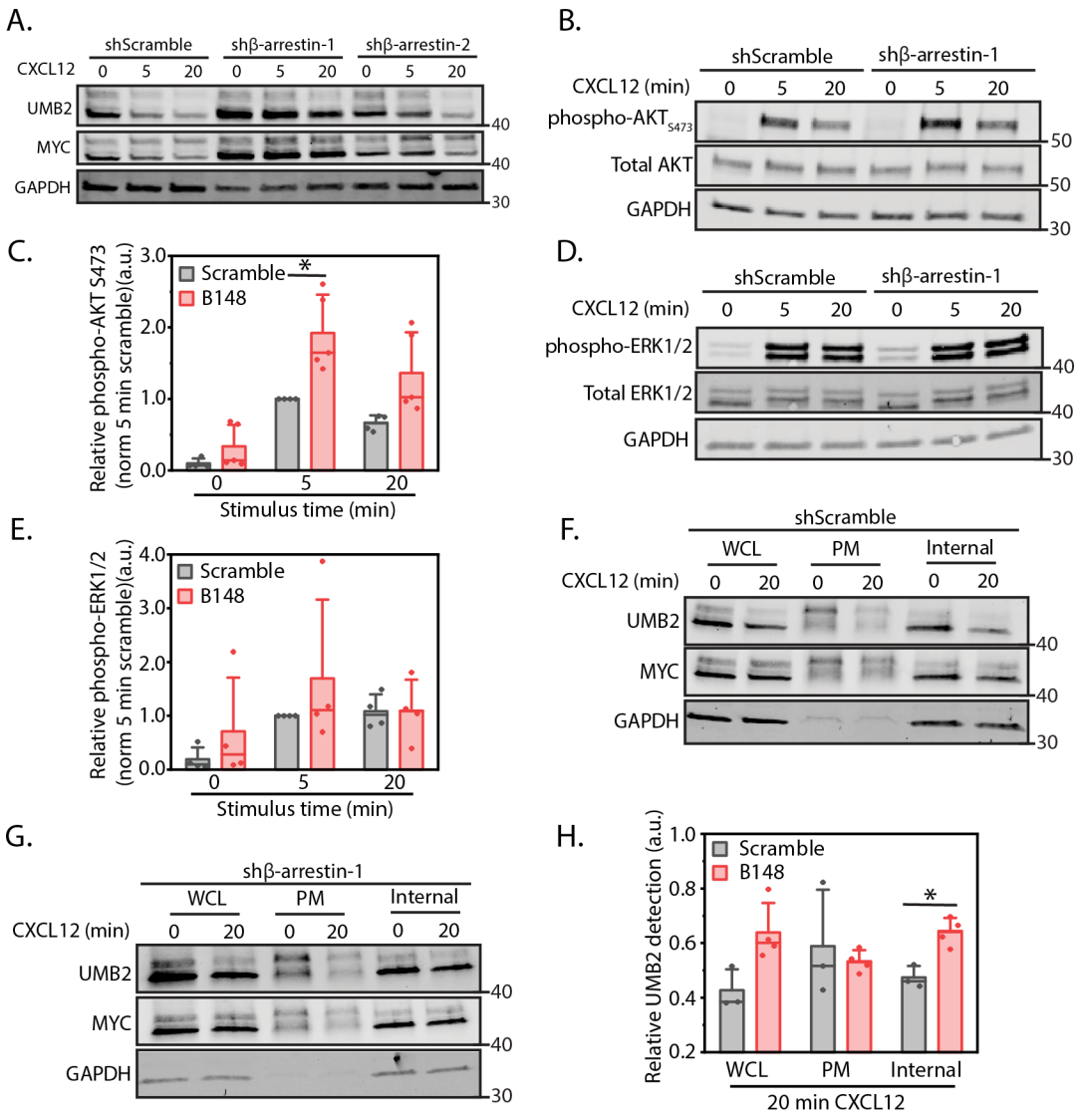


Figure 3-8 β-arrestin-1 regulates agonist-induced internal CXCR4 PTM

(A) Representative western blot illustrating the effect of β-arrestin-1 and β-arrestin-2 knockdown on CXCR4 PTM. Relative shRNA knockdown efficiency is shown in Figure 3-1a-c. **(B)** Representative western blot illustrating the effect of β-arrestin-1 knockdown on CXCL12-dependent AKT S473 phosphorylation. **(C)** Western blot quantification of CXCL12-dependent AKT S473 phosphorylation upon β-arrestin-1 knockdown. Data was normalized to phospho-AKT:total AKT and to 5 min normalized control shRNA sample. **(D)** Representative western blot illustrating the effect of β-arrestin-1 knockdown on CXCL12-dependent ERK1/2 phosphorylation. **(E)** Western blot quantification of CXCL12-dependent ERK1/2

phosphorylation upon β-arrestin-1 knockdown. Data was normalized to phospho-ERK1/2:total ERK1/2 and to 5 min normalized control shRNA sample **(F-G)** Representative western blots illustrating total, plasma membrane, and internal pools of CXCR4 PTM upon either scramble or β-arrestin-1 shRNA knockdown. **(H)** Quantification of CXCR4 PTM at plasma membrane and internal locations upon β-arrestin-1 knockdown. CXCR4 PTMs were calculated by dividing UMB2 detection (non-post-translationally modified CXCR4) by MYC intensity (total CXCR4) and normalized to the 0 min time point at each location. For all experiments a minimum of 3 independent replicates were performed. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course. β-arrestin-1 knockdown experiments were conducted using two validated shRNAs (Supplemental. Fig 3). Individual data points from each experiment are plotted; mean, SD, median line. Statistical significance (*) denotes $p < 0.05$.

Since non-plasma membrane CXCR4 overexpression has been associated with metastatic potential[215,219], we wanted to investigate whether the internal pool of CXCR4 activated distinct signaling pathways compared to plasma membrane-localized receptors. GPCR signaling at intracellular compartments has become increasingly apparent and has been shown to activate different signaling cascades compared to plasma membrane-localized counterparts[54,133,135,237]. Recent work has shown that activation of G protein signaling at the Golgi and endosomes regulates PI4P hydrolysis and *PCK1* transcription respectively[133,154]. Therefore, we investigated whether activation of intracellular CXCR4 differentially activates downstream signaling compared to plasma membrane receptors. Rather than using mutant receptors that have preexisting signaling defects (Figure 3-3), we decoupled the effects of CXCR4 localization from these mutations by removing a synthetic plasma membrane localization sequence commonly used to increase CXCR4 plasma membrane trafficking[114]. Consistent with our earlier findings,

AKT and ERK1/2 phosphorylation as well as total CXCR4 PTM were not affected by modulating receptor localization (Figure 3-9).

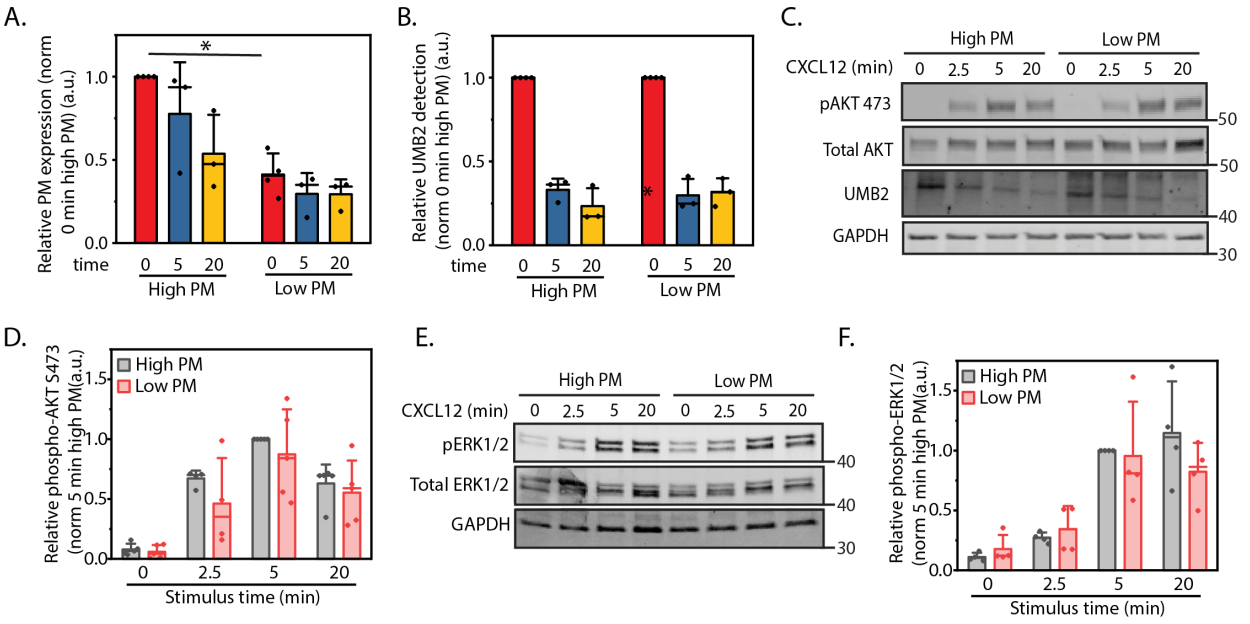


Figure 3-9 Modulation of CXCR4 plasma membrane localization does not affect CXCL12-dependent AKT S473 and ERK1/2 phosphorylation or CXCR4 PTM.

(A) Flow cytometry analysis of CXCR4 plasma membrane expression of high and low plasma membrane (PM) expressing CXCR4 constructs. Plasma membrane expression was normalized to total CXCR4 expression and secondly to the 0 min high plasma membrane CXCR4 sample. **(B)** Flow cytometry analysis of high and low plasma membrane expressing WT CXCR4 constructs. UMB2 detection was normalized to total CXCR4 expression and secondly to the 0 min control samples. **(C)** Representative western blot illustrating CXCL12-induced AKT S473 phosphorylation and UMB2 detection in high and low plasma membrane expressing CXCR4 cells. **(D)** Western blot quantification of agonist-induced AKT S473 phosphorylation. Data was normalized to phospho-AKT S473:total AKT and to 5 min high plasma membrane expression sample. **(E)** Representative western blot illustrating CXCL12-induced ERK1/2 phosphorylation in high and low plasma membrane expressing CXCR4 cells. **(F)** Western blot quantification of CXCL12-induced ERK1/2 phosphorylation. Data was normalized to phospho-ERK/12:total ERK1/2 and to 5 min high plasma membrane expression sample. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for

the stated time course. Individual data points from each experiment are plotted; mean, SD, median line. Statistical significance (*) denotes $p < 0.05$.

Since no overt defect in signaling was observed, we hypothesized that signal location is responsible for differential CXCL12-dependent transcription. To investigate this, we measured early growth response gene 1 (*EGR1)* transcript levels upon CXCL12 stimulus in RPE cells with high and low plasma membrane CXCR4 expression. *EGR1* transcription is downstream of the ERK1/2 pathway and has been shown to be induced by CXCL12[238,239]. As expected, WT RPE cells (not overexpressing CXCR4) were unresponsive to CXCL12 (Figure 3-10A). However, compared to cells with high plasma membrane expression, cells with low plasma membrane CXCR4 expression had significantly increased CXCL12-induced *EGR1* transcript levels (Figure 3-10A). This result is inconsistent with the spare receptor model. Furthermore, in agreement with previous work[133,154,210], this suggests that while cells often use some of the same signaling machinery, the localization of a signaling event can lead to different cellular responses. Since β-arrestin-1 plays a role in activating internal CXCR4, we investigated whether inhibition of β-arrestin-1 decreased CXCL12-induced *EGR1* transcription. Indeed, β-arrestin-1 knockdown reduced agonist-induced *EGR1* transcript levels (Figure 3-10B), providing additional evidence that intracellular pools of CXCR4 are physiologically relevant and that their function is dependent on β-arrestin-1.

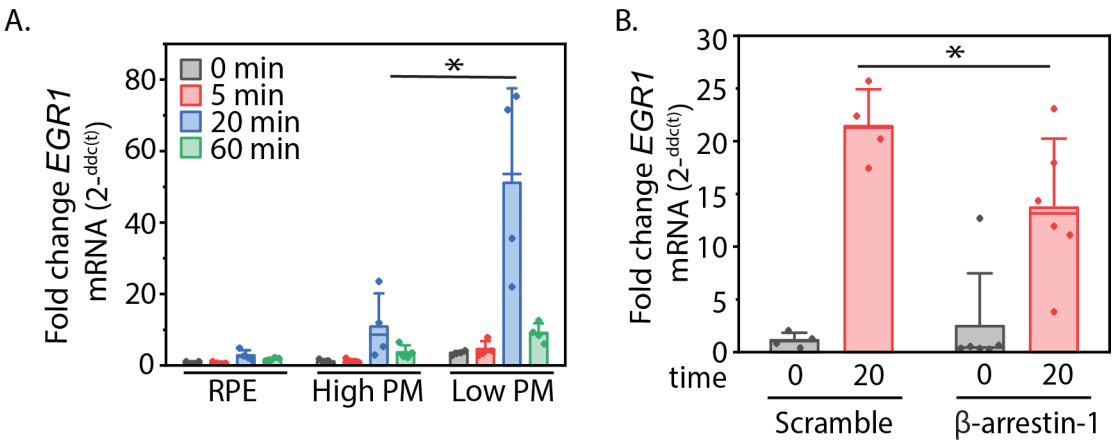


Figure 3-10 Intracellular pools of CXCR4 are primarily responsible for *EGR1* transcription.

(A) qPCR analysis of *EGR1* transcription in WT RPE or RPE cells overexpressing high or low plasma membrane localized CXCR4. *EGR1* transcript levels were calculated using the ΔΔCT method normalized to GAPDH and 0 min high plasma membrane CXCR4. **(B)** β-arrestin-1 knockdown reduces CXCL12-dependent *EGR1* transcript levels in RPE cells overexpressing CXCR4. *EGR1* transcript levels were calculated using the ΔΔCT method. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 for the stated time course unless noted. β-arrestin-1 knockdown experiments were conducted using two validated shRNAs (Figure 3-2). Individual data points from each experiment are plotted; mean, SD, median line. Statistical significance (*) denotes $p < 0.05$.

3.4 Discussion

This work has revealed a new element of GPCR signaling whereby plasma membrane and internal pools of CXCR4 communicate to regulate cell signaling. These observations are distinct from previous work investigating intracellular GPCR signaling, where receptor internalization or OCT3 channel-permeable ligands were required[131,135,143,153,154,160]. CXCR4 PTM is dependent on $G_{\beta\gamma}$ activation and β-arrestin-1 plays a specific role in regulating intracellular CXCR4 PTM. This work expands upon the β-arrestin signaling at a distance concept and supports a model where β-arrestins are not only able signal at a distance at the plasma

membrane but also regulate communication between plasma membrane and internal GPCR populations to influence agonist-dependent transcriptional programs. A model for communication between plasma membrane and intracellular pools of CXCR4 and its ramification on signaling is summarized in Figure 3-11. There are several potential mechanisms for how this communication may occur that warrant additional research.

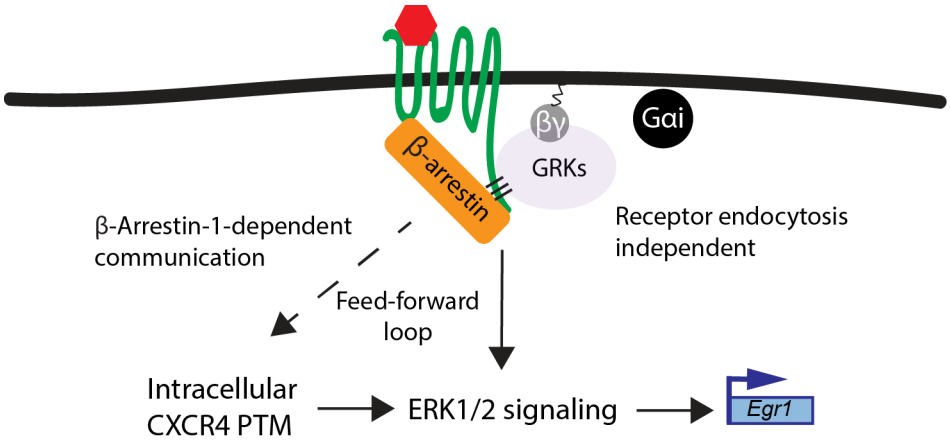


Figure 3-11 Proposed model for intracellular CXCR4 PTM

Schematic summarizing a potential model for communication between plasma membrane and internal GPCR pools.

Many new questions regarding the molecular mechanism and physiological relevance of plasma membrane and intracellular CXCR4 activation remain unanswered. A limitation of our surface biotinylation approach to study intracellular CXCR4 PTMs is that it is unable parse out which subpopulation(s) of CXCR4 are being post-translationally modified. While agonist-induced internal CXCR4 PTM appear to partially occur at the Golgi (Figure 3-4A), CXCR4 may also be post-translationally modified at other intracellular compartments. Understanding which intracellular CXCR4 populations are post-translationally modified is important for understanding which receptor pool is responsible for CXCL12-induced *EGR1* transcription. Furthermore,

neither β-arrestin or $G_{\beta\gamma}$ are believed to actively post-translationally modify proteins. However, $G_{\beta\gamma}$ is a potent kinase activator therefore identification of the kinase or other protein machinery responsible for internal CXCR4 PTMs is necessary. Interestingly, $G_{\beta\gamma}$ has been shown to traffick to intracellular compartments including the Golgi after agonist activation, independent of receptor endocytosis[240,241]. Understanding the specific PTMs of plasma membrane and internal CXCR4 populations could also provide important insights pertaining to the function and fate of activated intracellular receptors. It is possible that this mechanism may regulate receptor trafficking to the plasma membrane, effectively providing cells with a short-term memory of prior signaling events.

While many questions remain, the data presented expand the role of $G_{\beta\gamma}$ and β-arrestins in regulating GPCRs and support a new model of GPCR signaling whereby plasma membrane and internal pools of receptors communicate to collectively determine a cellular response. These observations may resolve the paradox that while CXCR4 overexpression is associated with metastatic potential, plasma membrane localization is not[215,216]. Additionally, this work supports a growing amount of evidence supporting that targeting specifically intracellular GPCR populations or the downstream signaling cascades activated by these pools might lead to improved therapeutic strategies for treating cancer, cardiovascular disease, and pain management[154,155,210].

3.5 Acknowledgements

MSD thanks support from the National Science Foundation Graduate Research Fellowship and the University of Michigan Rackham Pre-doctoral Fellowship. The work is supported in part by Pardee Foundation and a gift from Kendall and Susan Warren. MSD would also like to thank Greg Thurber for allowing us to use his LiCor CLX imager for preliminary

work in this study. MSD would like to thank Manoj Puthenveedu for discussion and Wylie Stroberg for data interpretation feedback.

Chapter 4 Development of a Spatially Resolved Proteomics Approach to Study the CXCR4 Interactome During Endocytic Trafficking

Work in this chapter is unpublished. I would like to extend a sincerest thank you to Venky Basrur, Datta Mellacheruvu, and Alexey Nesvizhskii for help with mass spectrometry and data analysis. Without their help, this work would not have been possible.

4.1 Abstract

C-X-C chemokine receptor 4 (CXCR4) is a GPCR and cognate clathrin-coated pit cargo. It is an important receptor for a variety of biological processes including immune cell homeostasis, embryonic development, and cell migration. CXCR4 signaling is activated by agonist binding (CXCL12) and depending on C-terminal tail post-translational modifications (PTM), regulates cell proliferation and growth, chromatin remodeling, or cell migration. CXCR4 expression is deregulated in 23 cancers and has been shown to increase cancer cell metastasis towards CXCL12 expressing cells. Additionally, CXCR4 is a HIV co-receptor. While CXCR4 endocytic trafficking is well established, the molecular players involved in regulating this process remain poorly understood. In this work we adopted APEX2 proteomics to study how the CXCR4 interactome changes during endocytic trafficking. Unfortunately, we were unable to confirm the top protein-protein interactions predicted by our initial proteomics experiments. Improved experimental design and additional experiments are necessary to advance this research.

4.2 Introduction

There is compelling evidence that a more complex model of CXCR4 regulation at the cell membrane exists and it is unlikely that all of the molecular players involved in this process are known. Unfortunately, traditional experimental methodologies are not well suited to address this question. Fluorophore properties limit the number of proteins that can be simultaneously visualized and it is currently not possible to track protein PTMs or discover new protein-protein interactions using live cell imaging without *a priori* knowledge. Furthermore, it is not feasible to isolate endocytic pits or plasma membrane microdomains for proteomic analysis. Consequently, to truly interrogate this process, a technique with both the spatial resolution of microscopy and discovery science capabilities of global proteomics is necessary.

While proteomic approaches to study biological complexity are rapidly revolutionizing basic cell biology, traditional mass spectrometry-based proteomics is fundamentally limited by spatial resolution. One approach used by researchers to overcome this limitation is co-immunoprecipitation of protein complexes. However, during this protocol, transient protein-protein interactions are oftentimes lost and not identified. This is particularly true for membrane proteins as the use of harsh detergents to solubilize membrane proteins also disassociates protein complexes. Recently, two different approaches – BioID/TurboID and APEX2 - have been developed to overcome these limitations and add spatial resolution to proteomics experiments [242–244]. Both techniques use enzymes to promiscuously label neighboring proteins with biotin. After protein extraction, biotinylated proteins can be isolated by immunoprecipitation and quantified by mass spectrometry to reveal protein-protein interactions. These techniques remove the necessity of maintaining protein-complex integrity during protein extraction allowing researchers to use harsh protein extraction protocols to solubilize membrane proteins. By

creating a fusion protein between a protein of interest and the biotin-labeling enzyme, researchers can study the local interactome of their protein of interest.

BioID and APEX2 work by different mechanisms. BioID is an *E. coli* biotin ligase that has been mutated to promiscuously biotinylate neighboring proteins at lysine residues via a Biotin-AMP intermediate[244]. Originally, BioID experiments were limited to longer time scales as effective labeling required 12-18 hours, however, recent advancements have shortened labeling time to within a couple minutes[242,244]. APEX2 is a modified soybean ascorbate peroxidase that utilizes hydrogen peroxide to convert biotin-phenol into biotin-phenol radicals [243,245,246]. These short-lived radicals are highly reactive and covalently label endogenous proteins at electron rich tyrosine residues promiscuously in cells (Figure 4-1), which can then be isolated and identified by mass spectrometry. One advantage of the APEX2 methodology is that labeling happens within 30 seconds and is dependent on hydrogen peroxide, which gives researchers sub-minute resolution. This is particularly useful when studying how the GPCR interactome changes during receptor signaling and has been used to study δ opioid and β2 adrenergic receptor interacting partners during endocytic trafficking[247,248].

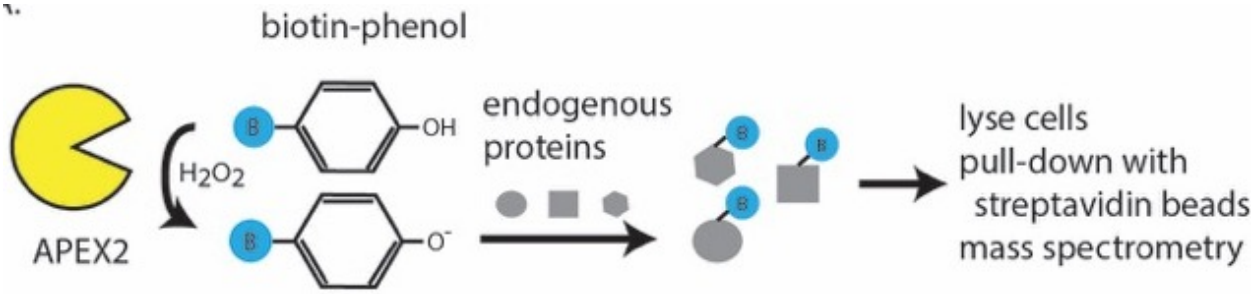


Figure 4-1 APEX2 Proteomic labeling

Upon hydrogen peroxide addition, APEX2 converts biotin-phenol into biotin-phenol radicals. Biotin-phenol radicals are highly reactive and covalently bind to endogenous proteins at electron rich tyrosine residues (20 nm labeling radius). Afterwards, biotinylated proteins can be isolated by immunoprecipitation and identified and quantified by mass spectrometry.

We recently adopted the APEX2 approach to study how the CXCR4 interactome changes during endocytic trafficking. While we were able to confirm proper localization and trafficking of the CXCR4-APEX2 fusion protein, we were unable to confirm the biological relevance of the top protein targets from our proteomics experiments. To make definite conclusion from this work, further experimental optimization and additional mass spectrometry experiments are necessary.

4.3 Materials and Methods

Cell culture

Retinal pigment epithelial (RPE) cells were obtained from Dr. Sandra Schmid at UT Southwestern and all stable cell lines were derived directly from this line. RPE cell lines were cultured in DMEM/F12 media (Corning) supplemented with 10% FBS.

Constructs and stable cell line generation.

FLAG-CXCR4-APEX2, APEX-NES (cytosol control), Lyn11-EGFP-APEX2 (plasma membrane control), and EGFP-APEX2-Rab5 (endosomal control) constructs were generated and cloned into the pLVX lentiviral transfer plasmid by PCR and restriction enzyme cloning. Individual stable RPE cell lines expressing each construct were generated by lentiviral transduction. Lentiviruses were generated in our lab by co-transfecting HEK293T cells (ATCC) with the transfer plasmid, psPAX2, and pMD2.G lentiviral envelope and packaging plasmids. Cell supernatant containing mature lentiviral particles was collected 4 days after transfection. RPE cells were transduced in DMEM/F12 media supplemented with FBS and 10 μg/mL polybrene and stable cell lines were generated through puromycin selection (3 μg/mL).

Biotinylation experiments

RPE cells stably expressing an APEX2 construct were incubated with biotin-phenol (500 μM) for 30 min. Afterwards, hydrogen peroxide (at a final concentration of 1 mM) was added to initiate APEX2-mediated biotinylation for 30 seconds at room temperature. The biotinylation was quench by washing the cells with quencher solution (10 nM sodium azide, 5 nM Trolox dissolved in DPBS) 3 times as previously described[246]. If cells were going to be used for protein or mass spectrometry analysis, samples were lysed (RIPA buffer supplemented with protease inhibitor) and either stored for western blotting or immediately used for immunoprecipitation (described below). Please see Immunofluorescence section for information on downstream immunofluorescence methods.

Cell signaling and time course experiments

Prior to all time course experiments, cells were serum-starved for 4 hrs. Serum starvation creates a starting point for ligand-induced CXCR4 internalization as well as increased plasma membrane localization. In order to track how the CXCR4 proteome changed endocytic trafficking, cells were stimulated with CXCL12 (50 nM) for the following time course: 0, 5, 10, 30, 60, and 180 minutes. While data was collected at later time points, we decided to focus our analysis on the 0 and 30 min time points. Biotinylation experiments using cytosol-localized APEX2 (APEX2-NES), plasma membrane-localized APEX2 (Lyn11-EGFP-APEX2), and endosome-localized APEX2 (EGFP-APEX2-Rab5) were included as spatial references/experimental controls. Additionally, a streptavidin bead control was used to control

for off-target protein-bead associations. Besides accounting for off-target biotinylation, these controls provided us with expected proteomic hits (spatial references) during CXCR4 endocytic trafficking.

Western Blotting

Prior to western blotting, samples were incubated with Laminelli buffer supplemented with β-mercaptoethanol (loading buffer). Samples were run on SDS-PAGE 4-20% BioRad gels (15 well/15 µl or 10 well/50 µl gels). For all signaling and biotinylation experiments, 12.5 - 40 µl of lysate was loaded. SDS-PAGE gels were run at constant 140 V for approximately 60 min. Afterwards, proteins were transferred to PVDF or nitrocellulose membranes using the iBlot transfer systems (mixed range proteins 7 min setting) or Thermo Fisher Fast Blotter (mixed range transfer). Resulting membranes were incubated in blocking solution (1% BSA in TBST or 5% nonfat milk in TBST) rocking for 1 hr at room temperature. Afterwards, blots were incubated with their respective antibodies (Table 4-1) overnight at 4°C. Prior to secondary labeling, blots were wash 3x for 5 min per wash with TBST. Blots were then incubated with the corresponding secondary antibody (Table 4-1) for 1 hr at room temperature. Blots were then washed with TBST as described above. Western blots were imaged using a LiCor Odessey SA System.

Immunofluorescence Assays

Cells were seeded in 6-well plates on glass coverslips 24 hr prior to each experiment and serum-starved for 4 hr as described above. Cells were stimulated with CXCL12 as specified in each figure legend and immediately washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. Cells were permeabilized for 10 min with 0.2% Triton-X100 diluted in

PBS and subsequently blocked with 2.5% BSA diluted in PBS (blocking solution) for 1 hr. Cells were incubated with primary antibody diluted in blocking solution and incubated overnight at 4 ^{o}C (Table 4-1). Slides were wash 3x for 5 min each with PBS and incubated with secondary antibodies diluted in blocking solution for 1 hr at room temperature (Table 4-1). Cells were washed with PBS 3x, 5 min per wash and incubated with DAPI (Table 4-1) diluted in PBS for 10 min at room temperature. Afterwards, cells were washed with PBS and mounted onto glass slides using Fluoromount G. Slides were imaged by spinning disk confocal microscopy as specified in the figure legends. Different experimental samples were imaged using the same imaging settings each day.

Flow cytometry experiments

Cells were plated onto 10 cm dishes 24 hrs prior to each experiment, washed and serum-starved as described above. Afterwards, cells were non-enzymatically disassociated from each plate using 50 μM EDTA in Ca^{2+}-free PBS. Cells were pelleted by centrifugation (500 *g* for 10 min) and resuspended in serum-free media on ice. Cells were transferred into 1.5 ml eppendorf tubes and treated with either a vehicle or ligand and transferred to a heat block at 37 ^{o}C for the time course described in figure legends. Immediately afterwards, cells were transferred back on ice, pelleted (centrifuged 3,000 *g* for 3 min), and resuspended in 4% paraformaldehyde for 10 min on ice. Cells were pelleted and incubated with conjugated antibody (Table 4-1) diluted in 2.5% BSA for 1.5 hrs on ice. Afterwards, cells were washed 1x with PBS and 10,000 cells were analyzed by the Guava EasyCyte Flow Cytometer and its accompanied software. Internalization was quantified as previously described [197,225].

Immunoprecipitation (IP)

For APEX2 biotinylation mass spectrometry experiments, biotinylated plasma membrane proteins were isolated from whole cell lysate using high capacity streptavidin agarose beads. Approximately 70 µl of bead slurry was added to 900 µl whole cell lysate and incubated overnight (~ 18 hr) rotating at 4 °C. Afterwards, samples were pelleted (centrifuged for 3 min at 2,000 *g*) and non-biotinylated/bead bound proteins removed. Beads were washed 5 times with RIPA buffer containing protease inhibitors. Ammonium bicarbonate was used for the final wash and afterwards all remaining buffer was remove as previously described [249]. To ensure high capture efficiency, prior to mass spectrometry total protein and biotinylated protein levels were evaluated by SDS-PAGE and western blotting. Upon biotinylation IP confirmation, captured biotinylated proteins were submitted for mass spectrometry analysis at the UM Medical School Proteomics Resource Facility (PRF).

Mass spectrometry

A minimum of 3 independent biological replicates was submitted for mass spectrometry analysis for each experiment. Biotinylated protein peptide fragments were released from beads via trypsin digestion. Resulting peptides were dried to completeness in a lyophilizer and reconstituted in 0.1% formic acid and cleaned up with C18 tips before being redried [250]. Dried peptides were reconstituted in formic acid/acetonitrile solution before being analyzed by mass spectrometry. Peptides were separated using reverse phase nano-capillary column (PepMap C18, 2m, 75 m x 25 cm) at a flow rate of 300 nl/min and directly sprayed onto the Orbitrap Fusion mass spectrometer using an EasySpray source. The mass spectrometer was set to collect a survey scan (resolution 240,000@200 m/z) followed by data-dependent, higher-energy collisional

dissociation spectra on the 20 most abundant ions using the instrument settings optimized at the Facility. For help with analyzing the proteomic data we were assisted by Alexey Nesvizhskii's lab. Acquired tandem mass spectra were searched against a UniProt protein sequence database using the Proteome Discovery software (ThermoFisher). This resulted in a summary matrix of protein identifications and the corresponding quantification values across all samples and time points. The quantification was performed using spectral counts [251]. We took several steps to remove contaminants and solely identify high yield peptide hits. Firstly, only high confidence hits as determined by the Proteome Discovery software were considered. Secondly, common contaminants proteins such as keratins, myosins, and heterogenous ribonucleoproteins were discarded. We also removed samples that did not have greater than or equal to 10 spectral counts in at least one of the CXCR4-APEX experimental conditions. Once establishing our list of high confidence hits, we imported the data into REPRINT, an online tool developed by the Nesvizhskii lab for analyzing proteomic data (unpublished). As part of REPRINT, we used the statistical scoring tool SAINT [252–254] and prior knowledge of the most common background proteins assembled in the Contaminant Repository for Affinity Purification (CRAPome; *www.crapome.org*) to determine high confidence protein-protein interactions [255].

Table 4-1 Reagents

Reagent	**Supplier**	**Dilution**	**Experiment**
ms-FLAG-647	Genscript	1:1000	WB, IF, FC
Gt-anti-rb Dylight 800	Invitrogen	1:10000	WB
Gt-anti-ms Dylight 680	Invitrogen	1:10000	WB
Human CXCR4 R-PE	Invitrogen	1:500	FC

ms-GAPDH	Santa Cruz	1:1000	WB
rb-Phospho-pERK1/2	CST	1:2000	WB
rb-Phospho-AKTS473	CST	1:2000	WB
rb-UMB2	ABCAM	1:2000	WB
STREP-568	Thermo Fisher	1:1000	IF
STREP-700	Thermo Fisher	1:2000	WB
CXCL12	R&D Systems	50 nM	NA
Streptavidin agarose	Thermo Fisher	NA	NA
AMD3100	Sigma	10 nM	NA
AG-1296	Santa Cruz	10 uM	NA
AG-556	Santa Cruz	10 uM	NA

4.4 Results

APEX2 is functional when attached to the C-terminus of CXCR4 and spatial references

Before proceeding to the proteomics experiments we first needed to confirm that generation of the APEX2 fusion proteins did not impact APEX2 biotinylation activity. Additionally, since we were interested in studying how the CXCR4 interactome changed during endocytic trafficking with sub-minute temporal resolution, we needed to confirm that protein biotinylation was dependent on both biotin-phenol, and H_2O_2. To test whether APEX2 was necessary for protein biotinylation, we expressed both WT CXCR4 and CXCR4-APEX2 constructs in RPE cells and tested whether biotin-phenol and H_2O_2 addition induced protein biotinylation. Indeed, while

expression of CXCR4 alone was unable to induced protein biotinylation, CXCR4-APEX2 robustly biotinylated proteins upon biotin-phenol and H_2O_2 addition (Figure 4-2A).

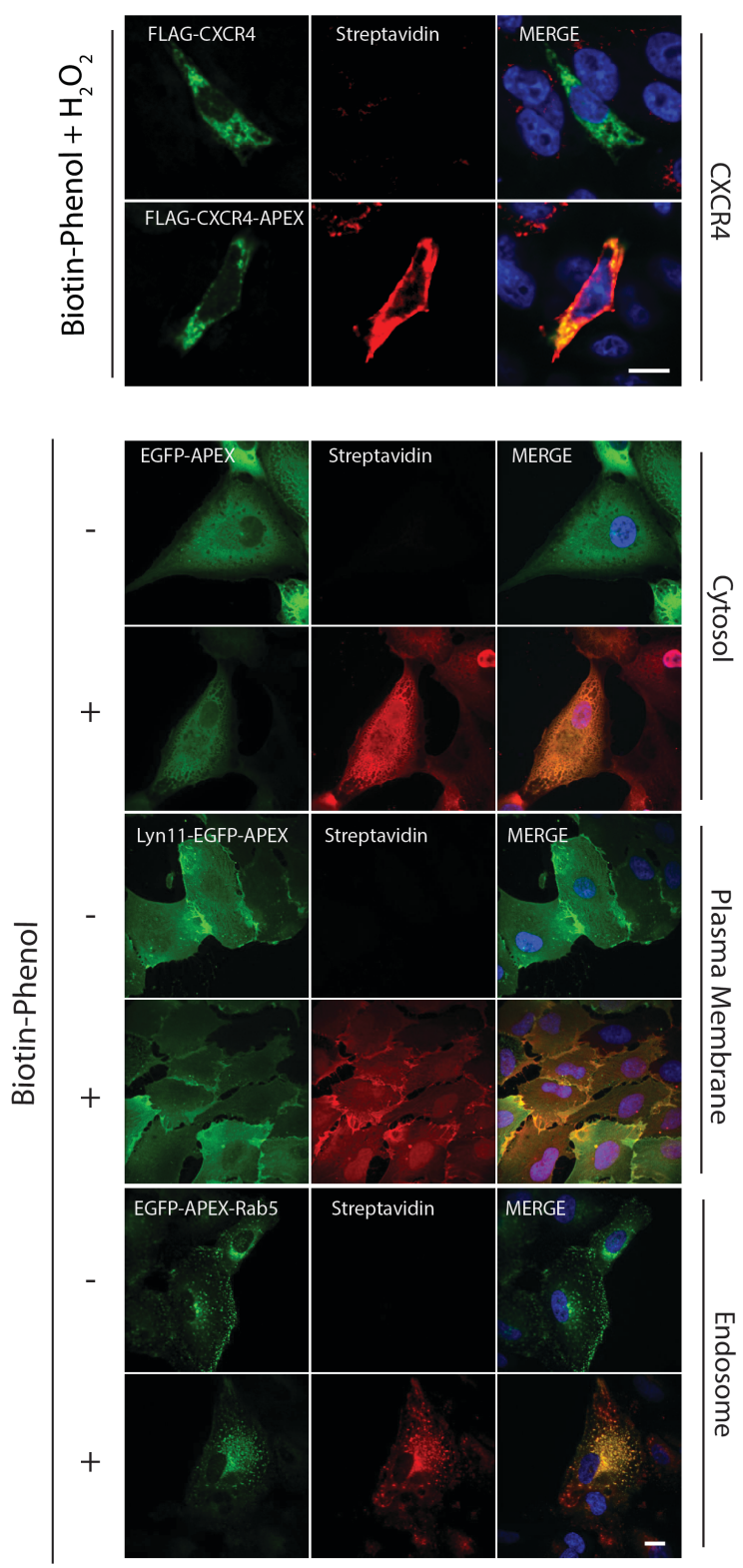


Figure 4-2 APEX2 fusion proteins retain biotinylation activity

(A) CXCR4-APEX2 fusion protein biotinylation activity is dependent on APEX2 expression, biotin-phenol and H_2O_2 addition. This experiment was conduct in CHO cells transiently overexpressing FLAG-CXCR4 or FLAG-CXCR4-APEX2 constructs. (B) Confirmation of APEX2 fusion protein control construct, expression, localization, and biotinyation activity in RPE cells stably overexpressing each construct. Where noted all samples were incubated with biotin-phenol (100 μM) for 30 min prior to H_2O_2 addition (1 mM) for 1 min. DAPI was used to label nuclei, FLAG antibody to label each construct, and fluorescently conjugated

streptavidin to label biotinylated proteins. All samples were imaged using a 60x objective on a spinning disk confocal microscope. Scale bar is 10 μm.

Next we evaluated whether APEX2 biotinylation activity was dependent on biotin-phenol. To do so we performed the APEX2 biotinylation experiment +/- pre-incubation with biotin-phenol. As expected, protein biotinylation was restricted to samples that were treated with both biotin-phenol and H_2O_2 (Figure 4-2B). This was confirmed for each construct used in the study (Figure 4-2B).

As CXCR4 traffics through the cell, it's local proteome changes significantly and therefore the local background (non-specific proteome) also changes. Therefore, it was important to consider this in our proteomics experiments. To do so, we used cytosol-, plasma membrane- and endosome-localized APEX2 controls. These controls allowed us to probe the local proteome at different intracellular locations and identify which CXCR4-APEX proteomic targets were *bona fide* protein-protein interactions instead of non-specific resident bystanders present at the same intracellular compartments. Therefore it was necessary to confirm that each APEX2 control construct localized properly. As expected, the cytosol-APEX was diffuse within the cytosol, plasma membrane-APEX (by Lyn11) was enriched at the plasma membrane, and endosomal APEX (by Rab5b) control primarily localized at intracellular vesicles/EEA1-positive endosomes (Figure 4-2B and data not shown).

CXCR4-APEX2 retains internalization, signaling and post-translational modification ability

After confirming that APEX2 fusion proteins retained APEX2 biotinylation activity, we needed to confirm that the CXCR4-APEX2 fusion protein did not alter CXCR4 function.

Confirmation of biological functions of control constructs was not necessary as only proper intracellular localization and APEX2 biotinlyation activity were necessary for the proteomics experiments. There were 3 different aspects of CXCR4 function that we tested: receptor post-translational modification (PTM), signaling, and internalization. Since the C-terminal tail of GPCRs are important for regulating GPCR activity, we were concerned that the addition of a bulky protein to the receptor C-terminus might interfere with agonist-induced CXCR4 signaling, PTM and internalization. While the negative control (BMP receptor ALK2-APEX) was unresponsive to CXCL12 addition, cells overexpressing CXCR4-APEX2 robustly induced AKT and ERK1/2 phosphorylation (Figure 4-3A). This experiment confirmed that WT RPE cells are unresponsive to CXCL12 and that the CXCR4-APEX2 construct was able induce CXCL12 dependent signaling (Figure 4-3A). Since PTMs regulate CXCR4 protein-protein interactions [61,256], we needed to confirm that CXCR4-APEX2 was able to be post-translationally modified. To test this, we used the UMB2 antibody to measure agonist-induced CXCR4 PTM. As described in Chapters 2 and 3, we have previously established this antibody as a robust tool to detect CXCR4 PTM. Indeed, upon CXCL12 stimulus, both WT CXCR4 and CXCR4-APEX2 proteins were rapidly post-translationally modified (Figure 4-3B). The observed band shift in CXCR4 detection was expected as the CXCR4-APEX2 fusion protein has a higher molecular weight. This also provided a good control for UMB2 antibody specificity (Figure 4-3B). AKT phosphorylation was included as a signaling control (Figure 4-3B). Interestingly, during this process we also discovered that, unlike APEX2, addition of EGFP to the C-terminus of CXCR4 drastically decreased agonist-induced CXCR4 PTM (Figure 4-3C). This highlights the necessity for confirming function of fusion proteins prior to conducting experiments. Interestingly, while not to the extent of WT CXCR4, CXCR4-EGFP was able to induce AKT phosphorylation,

however this was not quantified (Figure 4-3C). Lastly, we needed to confirm that the CXCR4-APEX2 fusion protein internalized in response to CXCL12. As expected, CXCR4-APEX2 rapidly internalized upon agonist addition (Figure 4-3D). CXCR4del19-APEX2 was used as a control as this mutant has attenuated internalization (Figure 4-3D) [82].

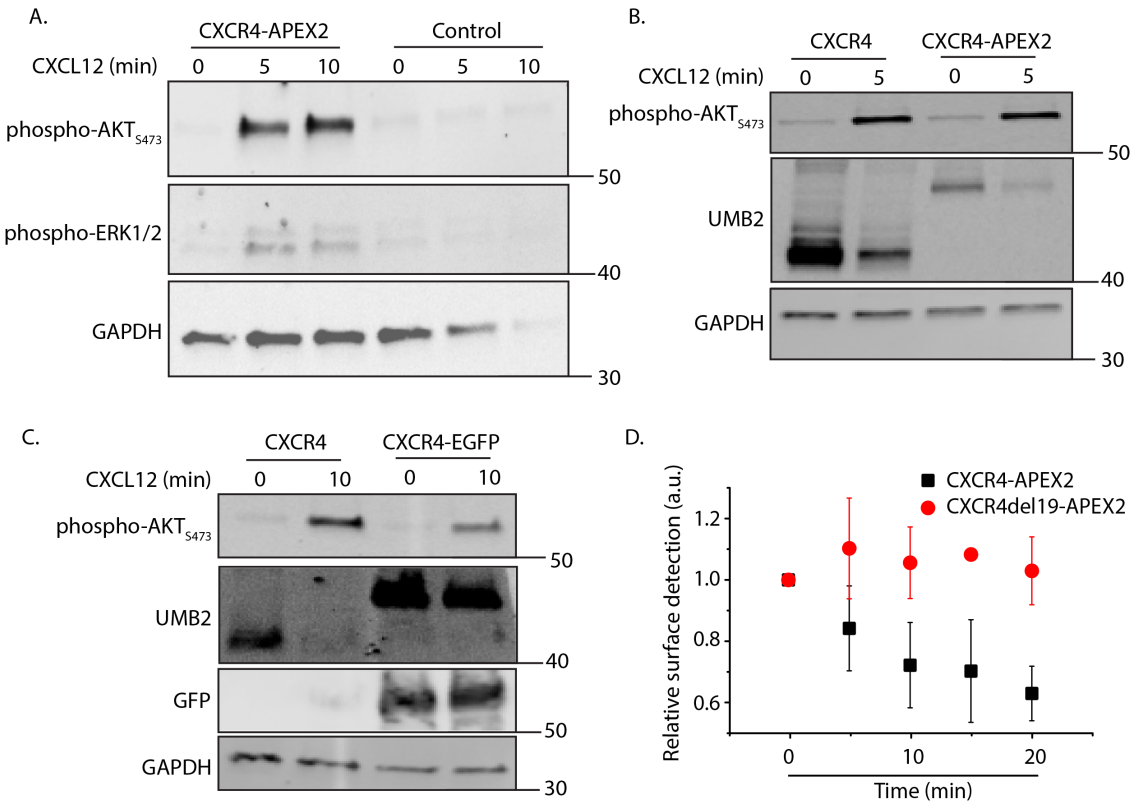


Figure 4-3 CXCR4-APEX does not alter normal CXCR4 function

(A) Representative western blot illustrating that RPE cells overexpressing CXCR4-APEX2 activates AKT S473 and ERK1/2 phosphorylation upon CXCL12 addition. Control RPE cells not expressing CXCR4 were unresponsive to CXCL12. (B) Representative western blot illustrating that the CXCR4-APEX2 fusion protein exhibits similar agonist-induced UMB2 detection compared to WT CXCR4. AKT phosphorylation is shown as a control. (C) Representative western blot illustrating that the addition of EGFP to the C-terminus of CXCR4 alters agonist-induced CXCR4 UMB2 detection. EGFP is shown to illustrate total CXCR4 for the CXCR4-EGFP fusion construct. AKT phosphorylation is shown as a control. (D) Flow

cytometry analysis illustrating that CXCR4-APEX2 internalizes upon CXCL12 stimulation. RPE cells overexpressing CXCR4-APEX2 or CXCR4del19-APEX were stimulated with CXCL12 (50 nM) for the described time course. CXCR4del19 is a mutant receptor that does not internalize upon agonist addition. Data was normalized to plasma membrane receptor detection at 0 min and is plotted mean +/- SEM. With the exception of the 15 min time point for the CXCR4del19-APEX mutant (single replicate), 3 independent biological replicates were conducted. Unless noted all experiments were conducted in RPE cells overexpressing each stated construct. CXCL12 stimulus was 50 nM unless stated.

CXCR4-APEX2 proteomic hits confirm proper fusion protein endocytic trafficking

Having established that the CXCR4-APEX2 fusion protein retained biotinylation activity and did not alter CXCR4 signaling, PTM, or internalization, we proceeded with preliminary mass spectrometry experiments. To confirm that the proteomics experiments recapitulated CXCR4 trafficking, we conducted Gene Ontology (GO) analysis of the top hits enriched at the 0 and 30 minute time points. We decided to start with these time points as they represented two distinct CXCR4 localizations – plasma membrane and endosomal. Consequently we hypothesized that these time points would be easily identifiable and that the CXCR4 interactome at each time point would be noticeably different. Prior to CXCL12 stimulus, cell membrane proteins were highly enriched compared to cytosolic controls and 30 min post-CXCL12 stimulus samples. Interestingly, both clathrin endocytic machinery and lipid rafts (a hallmark of clathrin independent endocytosis) proteins were significantly enriched in this population (Figure 4-4) [257,258]. We also observed an enrichment of focal adhesion proteins at both time points (Figure 4-4). This was particularly promising, as CXCR4 is important for cell migration and recent evidence has implicated CXCR4 stimulation in focal adhesion kinase and β-integrin signaling [60]. As expected, at 30 min post-stimulus, we found significant enrichment of early endosome

resident proteins (Figure 4-4). Together, these results suggest the feasibility of our proteomic approach to monitor CXCR4 interactome during its trafficking.

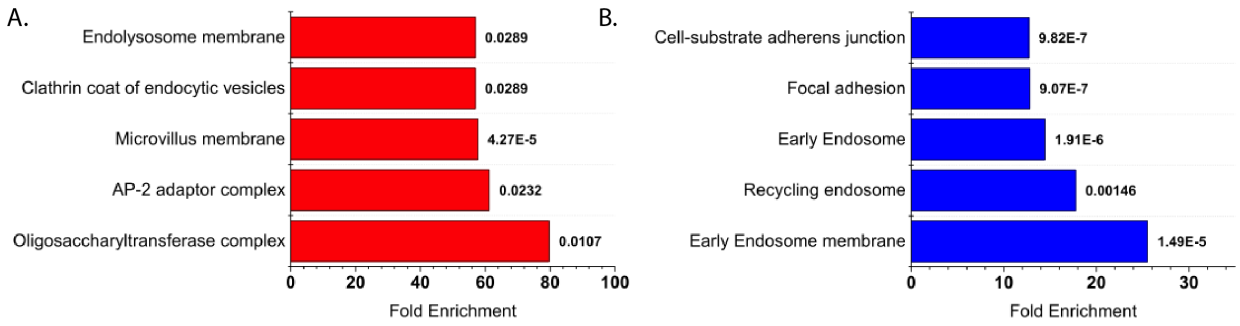


Figure 4-4 CXCR4-APEX2 Gene Ontology (GO) Analysis

(A) Pre-stimulus and (B) 30 min post-CXCL12 stimulus GO analysis. After data processing and cytosolic protein background subtraction, proteins with > 10 spectral counts and > 2 fold enrichment over other conditions were identified and GO determined. For each time point > 50 proteins met the criteria. The top 5 GO fold enrichment categories are listed as well as their corresponding p-values compared to a random protein population (with Bonferroni correction).

PDGFRβ inhibition does not impact CXCR4 signaling, PTM or internalization in RPE cells overexpressing CXCR4.

Platelet derived growth factor receptor beta (PDGFRβ) was one of the top hits at the 0 min time point and intrigued by the potential crosstalk with CXCR4, we investigated whether PDGFRβ was involved in regulating CXCR4 signaling, internalization, or PTM. CXCR4 and PDGFRβ crosstalk has been previously described in glioblastoma cells and researchers found that inhibition of either receptor influenced agonist-induced migration by the other receptor when activated [222]. Therefore, we hypothesized that CXCR4 or PDGFRβ inhibition would impact (increase or decrease) CXCL12 or PDGF-BB stimulus-induced AKT phosphorylation. As expected, blocking CXCR4 or PDGFRβ activity inhibited CXCL12- and PDGF-BB-dependent AKT phosphorylation respectively (Figure 4-5A). However, inhibition of PDGFRβ had no effect

on CXCL12-dependent AKT phosphorylation (Figure 4-5A). Likewise, inhibition of CXCR4 did not affect PDGF-BB-induced AKT phosphorylation (Figure 4-5A). Next we investigated whether PDGF-BB stimulus increased CXCR4 PTM. In contrast to previously literature, PDGF-BB did not induced CXCR4 PTM (Figure 4-5A). Furthermore, inhibition of PDGFRβ did not impact CXCL12-induced CXCR4 PTM (Figure 4-5A). EGF stimulus was used as a control and, as expected, it did not impact either CXCL12- or PDGF-BB-induced signaling or CXCR4 PTM (Figure 4-5A).

Next we explored whether PDGF-BB could induce heterologous CXCR4 internalization. Since, CXCR4 phosphorylation is believed to be a precursor of CXCR4 internalization, we expected that PDGF-BB stimulation would not lead to CXCR4 internalization. Indeed, while CXCL12 resulted in rapid CXCR4 internalization, PDGF-BB did not (Figure 4-5B). Together these results suggest that PDGFRβ and CXCR4 do not have functional crosstalk in RPE cells.

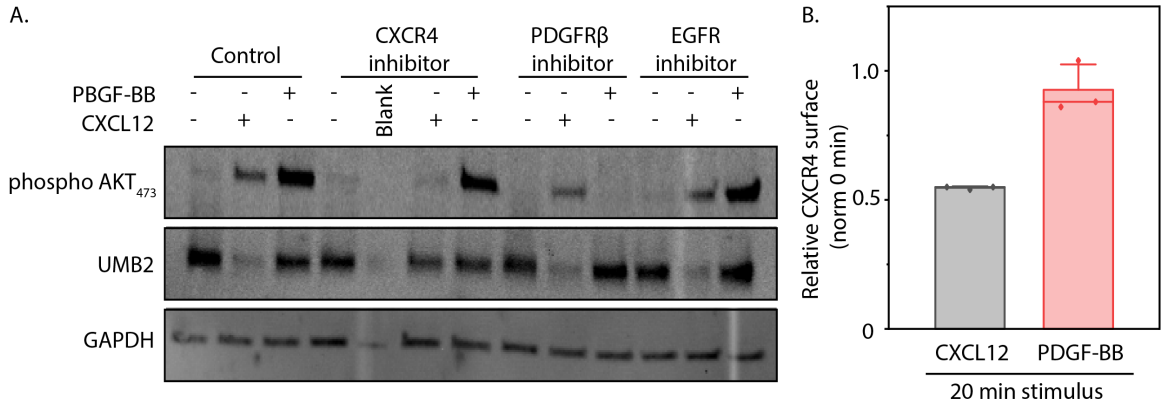


Figure 4-5 PDGFRβ and CXCR4 do not have functional crosstalk in RPE cells overexpressing CXCR4

(A) Representative western blot illustrating the effects of CXCL12 and PDGF-BB stimulation and receptor inhibition on AKT S473 phosphorylation and CXCR4 PTM. (B) Flow cytometry analysis of CXCL12 or PDGF-BB dependent CXCR4 internalization. Data was normalized to plasma membrane receptor detection at 0 min and is plotted mean +/- SD. Three independent biological replicates were conducted. Unless noted all experiments were conducted in RPE cells

overexpressing each stated construct. CXCL12 stimulus was 50 nM unless stated. PDGF-BB stimulus was 10 nM unless stated. Inhibitor concentrations are listed in Table 4-1.

4.5 Discussion

Through this work we adopted an APEX2 proteomics approach to study how the CXCR4 interactome changes during endocytic trafficking. While our work suggests that this technique has great potential to inform future CXCR4 research, interpretation of our current results is limited and to this point we are unable to confirm the biological relevance of the identified CXCR4-PDGFRβ protein-protein interaction.

During this study we encountered several challenges that might be useful to disclose to others who are considering using APEX2 proteomics in their work. Firstly, it is important to confirm that fusion proteins localize and function as expected prior to conducting any proteomics experiments. Early on we discovered that in RPE cells, overexpressed CXCR4 primarily resides at intracellular compartments and has poor plasma membrane expression (Figure-4-6). This was concerning as we were interested in studying how the CXCR4 interactome changes during endocytic trafficking from the plasma membrane to endosomes. Interestingly, while not considered at the time, intracellular localization of CXCR4 has been previously reported in cancer tumors and cell lines [148,163] suggesting that this intracellular pool might be functional. This observation would later inspire the work described in Chapter 3. Regardless, plasma membrane localization was necessary for this study. We were able to increase plasma membrane trafficking by adding an N-terminal plasma membrane localization peptide (hemagglutinin; HA) to CXCR4. Addition of this peptide significantly improved CXCR4 membrane trafficking and allowed us to proceed with our study (Figure 4-6A). This was particularly important for this

study as plasma membrane-localized CXCR4 are exposed to the cytosol and maximizing the difference between experimental conditions is best for assay optimization.

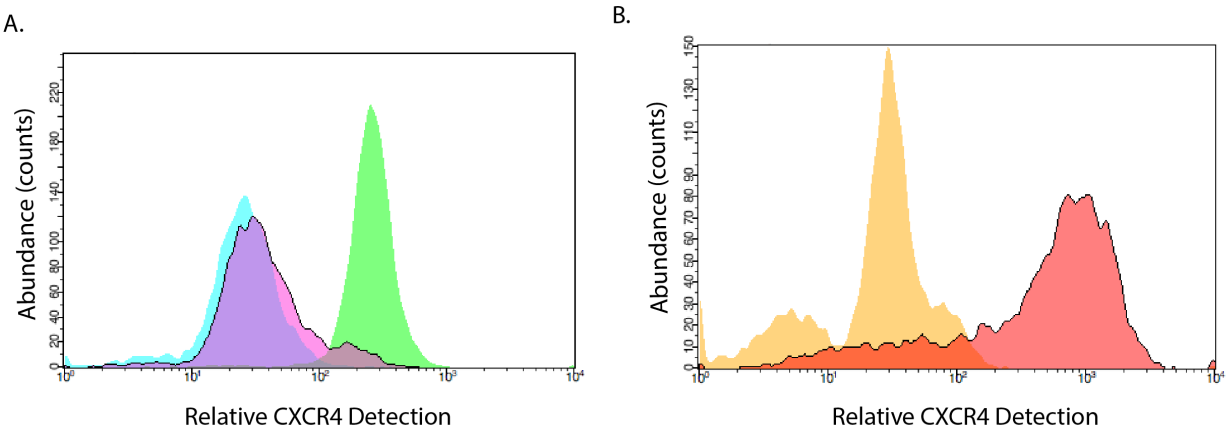


Figure 4-6 Addition of a viral membrane localization peptide to the C-terminus of CXCR4 improves receptor plasma membrane trafficking

(A) Flow cytometry analysis of CXCR4-APEX2 and APEX-NES plasma membrane localization in RPE cells overexpressing either construct. Green is total CXCR4 detection, purple is plasma membrane localized CXCR4-APEX detection and cyan is plasma membrane localized APEX-NES. APEX2-NES is strictly intracellular and as expected has low plasma membrane detection. Therefore, this data suggest that CXCR4-APEX2 has poor plasma membrane localization. (B) Flow cytometry detection of HA-CXCR4-APEX2 plasma membrane localization compared to APEX2-NES. HA-CXCR4-APEX2 is denoted in red while APEX2-NES is denoted in yellow. Compared to (A), the addition of the HA plasma membrane localization peptide drastically improves CXCR4 plasma membrane localization.

Moving forward with mass spectrometry analysis there are several other things that should be considered. Firstly, it was important to ensure that the bait protein (CXCR4-APEX2) spectral counts were equally detected in each experimental condition. This was particularly important, as GPCRs are notoriously difficult to isolate from membrane fractions and are highly post-translationally modified during endocytic trafficking, which can affect peptide detection. A second important feature that unfortunately was overlooked in our experiments was protein

expression and biotinylation efficacy of each bait protein, as well as day-to-day experimental variability. One issue that we encountered was differences in CXCR4-APEX2, plasma membrane, and endosomal spatial reference control spectral counts. While normalization to total spectral counts can account for some of this variation, differences in biotinylation efficiency and bait protein expression were not considered and made comparisons between spatial references and CXCR4-APEX2 difficult particularly when changes in detected protein abundance were minor (i.e. ~ 2 fold difference).

While our initial proteomics results confirmed proper endocytic trafficking and suggested that our approach was working, we were unable to confirm the biological relevance of identified protein-protein interactions. Our investigation of crosstalk between CXCR4 and PDGFRβ was one example where we were unable to confirm the biological relevance of this potential interaction in follow-up experiments. This was particularly disappointing as crosstalk between CXCR4 and PDGFRβ has been previously reported and shown in impact CXCR4-dependent migration[222]. There are several potential explanations for the differences between these seemingly conflicting data. Firstly, it is possible that the CXCR4-PDGFRβ interaction regulates an aspect of CXCR4 biology that we did not test such as CXCR4 degradation, cell migration, receptor recycling, or downstream transcriptional programming. This is possible as we only investigated whether PDGFRβ inhibition altered CXCR4 AKT phosphorylation, PTM, and internalization. In Chapter 3 we show that while CXCL12-dependent AKT and ERK1/2 phosphorylation levels are independent of CXCR4 localization, downstream transcriptional implications are drastically altered by CXCR4 localization. Therefore we cannot rule out that a similar observation may occur with PDGFRβ inhibition as well. Secondly, it is possible that CXCR4 regulates PDGFRβ PTM or internalization, as this was not tested by our experiments.

Thirdly, glioblastoma cells are inherently different than RPE cells and it is possible that CXCR4-PDGFRβ crosstalk may be cell type specific. One reason why GPCRs are advantageous therapeutic targets is that they often have highly restricted expression profiles and therefore, since RPE cells do not express endogenous CXCR4 it is possible that PDGFRβ-CXCR4 crosstalk might require the presence of a cell type specific intermediate that is not expressed in RPE cells. This led us to further investigate the co-expression of CXCR4 and top hits from our proteomics experiment.

We hypothesized that evolutionarily regulators of CXCR4 would likely be co-expressed with CXCR4 or ubiquitously expressed in all cell types. Consequently, proteins that were likely non-specific protein-protein interactors would likely have a negative correlation with CXCR4 expression. To investigate this, we utilized publically available RNAseq data from the Cancer Cell Line Encyclopedia (CCLE) and made pairwise comparisons between CXCR4 and top target proteins identified in our proteomics screen. Interestingly, CXCR4 and PDGFRβ co-expression was slightly negatively correlated (Figure 4-7A-B). Notably, cell lines that had similar expression of CXCR4 and PDGFRβ were brain cancer cell lines (data not shown) suggesting that the CXCR4-PDGFRβ crosstalk might be cell type specific. For comparison, we conducted the same analysis on TOM1 a high-confidence interacting protein identified in a recently published APEX2 proteomics experiment investigating δ opioid receptor interacting proteins [247]. TOM1 is an ubiquitin-linked protein that binds ubiquitin and localizes to endosomes. However prior to the work of Lobingier et al., its link to GPCR trafficking was unknown [247]. They found that TOM1 was involved in sorting GPCRs at endosomes, highlighting the potential of using APEX2 proteomics to study GPCR-protein interactions. Similarly to CXCR4 expression, δ opioid receptor expression is cell line specific (Figure 4-7C). However, in contrast to PDGFRβ, TOM1

is ubiquitously expressed (Figure 4-7D). Interestingly, while TOM1 was not the highest-ranking protein in terms of false discover rate or abundance in their dataset it was selected for follow-up investigation [247]. Together these data suggest that additional information of bait and prey co-expression might be a useful additional tool to parse complex proteomic data. A major limitation of this approach is that it is well established that RNA transcript levels do not necessarily correlate with protein expression [259]. However, if this information is considered qualitatively instead of quantitatively – i.e. if no transcript is detected, then no protein will be present – or used to identify ubiquitously expressed proteins, this approach may have some utility in successfully parsing complex proteomic data.

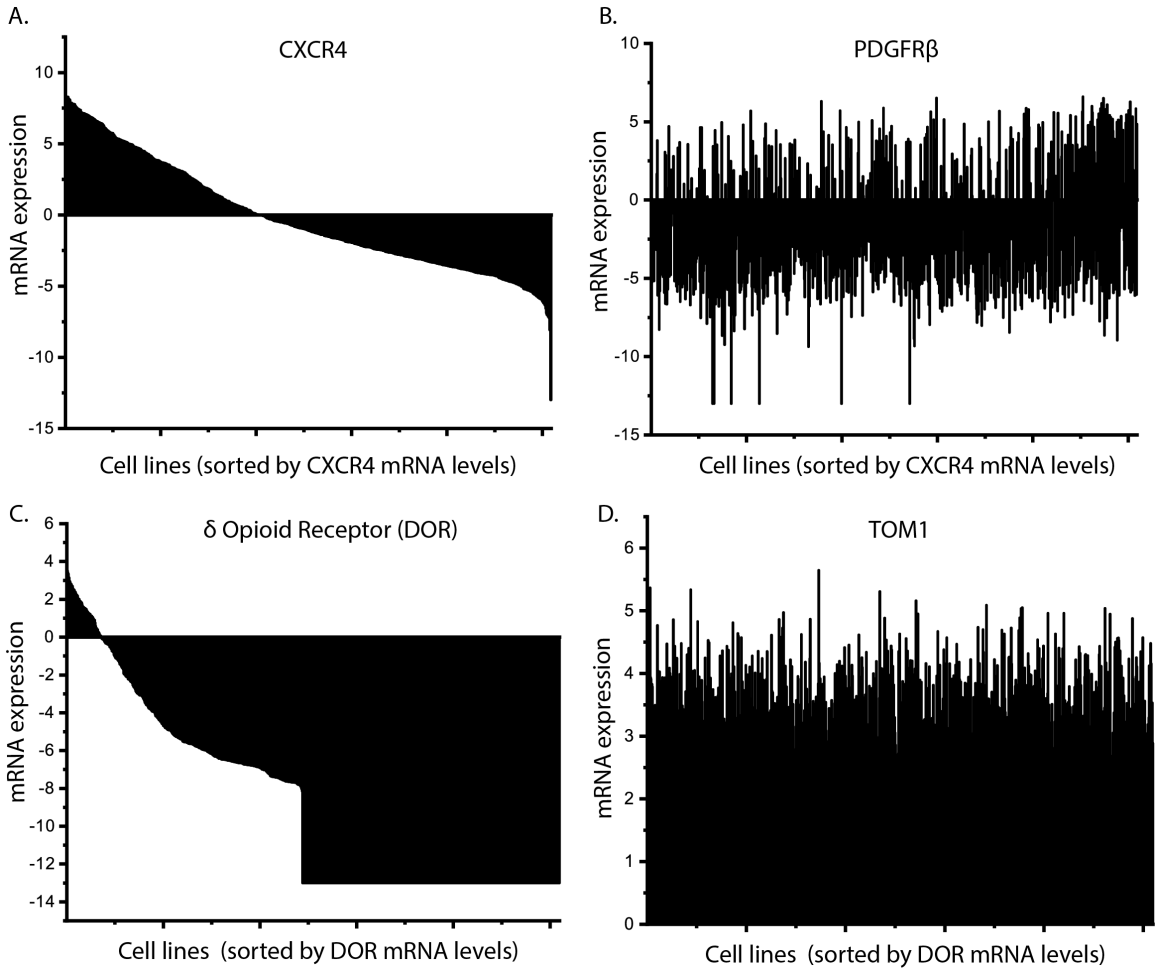


Figure 4-7 Relative mRNA abundance in different cell lines

(A) CXCR4 transcript levels across various cell lines. Data is sorted by CXCR4 abundance. (B) PDGFRβ transcript levels in cancer cell lines. Data is sorted by CXCR4 transcript level. Interestingly, there is a general trend of increased PDGFRβ expression in cell lines with low CXCR4 expression. (C) δ opioid receptor mRNA abundance in different cell lines. Cell lines are sorted by receptor expression level. (D) TOM1 transcript levels in cancer cell lines. Data is sorted by δ opioid receptor transcript level. Unlike PDGFRβ, TOM1 is ubiquitously express in all cell lines tested suggesting that this protein is highly conversed and likely plays an essential cellular function. Data was obtained from the Cancer Cell Line Encyclopedia (https://portals.broadinstitute.org/ccle).

In conclusion, while we established that APEX2 proteomics could be a useful tool to study the CXCR4 interactome and confirmed that the CXCR4-APEX2 fusion protein does not drastically alter CXCR4 signaling, PTM, and internalization, I believe that the current data is not useful for identifying *bona fide* CXCR4 interacting partners. To move forward with this research project, I recommend repeating all proteomics experiments (CXCR4 time course as well as controls and replicates) under the same experimental conditions and with better experimental techniques. Additionally, I recommend using intensity-based analysis and parallel reaction monitoring to obtain higher resolution mass spectrometry peptide quantification.

4.6 Acknowledgements

I would like to extend a sincerest thank you to Venky Basrur, Datta Mellacheruvu, and Alexey Nesvizhskii for help with mass spectrometry and data analysis. Without their help, this work would not have been possible.

Chapter 5 Biometrology in Cell Biology

This chapter is currently in preparation for submission. MSD and SS conceived and wrote the manuscript. MSD conducted all experiments.

5.1 Abstract

As technological and analytical innovations rapidly advance our ability to reveal increasingly complex biological processes, the importance of understanding the assumptions behind biological measurements and sources of uncertainty are essential for data interpretation. This is particularly important in fields such as cell signaling, as due to its importance for both homeostatic and pathogenic biological processes, a quantitative understanding of the basic mechanisms of these transient events is fundamental to drug development. While developed decades ago, western blotting remains an indispensible research tool to probe cell signaling, protein expression, and protein-protein interactions. While improvements in statistical and methodology reporting have improved data quality, understanding the basic experimental assumptions and visual inspection of western blots provides additional information that is useful when evaluating experimental conclusions. Using agonist-induced receptor post-translational modification as an example we highlight the assumptions of western blotting and showcase how clues from raw western blots can hint at experimental variability that is not captured by statistics and methods that influences quantification. The purpose of this article is not to serve as a detailed review of the technical nuances and caveats of western blotting. Instead using an

example we illustrate how experimental assumptions, design, and data normalization can be identified in raw data and influence data interpretation.

5.1 Introduction

The ability to measure and determine the uncertainty of measurements is fundamental to all science. Quantities in cell biology are inherently small, but vary across multiple scales. For instance, intracellular protein concentrations range from pM to nM, length of cellular structures range from nm to μm, forces generated by molecular motors in the pN range, and timescale of signaling transduction events from sub-seconds to hours. Biological systems also interact in complicated ways with their internal and external environment, and are dynamically changing, making specific attributes difficult to measure. As a consequence, biological measurements often result in a distribution of values. Coupled with biological complexity and the observational nature of the biological sciences, much of our current knowledge of molecular mechanisms is largely semi-quantitative. Recent advancements in -omics approaches as well as technical and analytical improvements in microscopy, flow cytometry, and western blotting have ushered in a new era of cell biology in which increasingly high-throughput screening and data collection are rapidly becoming feasible and minute differences between single cells increasingly quantifiable.

A measurement consists of two parts: a value determined by a particular method as well as the uncertainty of the measured value[260,261]. While uncertainty is commonly defined as the experimental variability in a measurement between replicates, in theory it is additive of all uncertainties throughout the experimental process[262]. One often overlooked source of uncertainty as highlighted in the Guide to Expression of Uncertainty in Measurement (GUM), is the incomplete definition of the measurand (thing being measured) and assumptions about what is being measured[262]. Additionally, the possibility of non-representative or incomplete sampling of

what was intended to be measured as well as an incomplete definition of the measurand are common sources of uncertainty defined within the GUM[262]. While these additional sources of uncertainty are not always readily apparent in biological systems, the inherent nature of biological measurements makes understanding these sources of uncertainty critically important when interpreting research results.

While new tools are continually developed to protein abundance, post-translational modification (PTM) and cell signaling – flow cytometry, FRET biosensors, microscopy, etc. [102,263–266] – due to cost and easy implementation, for almost a half-century, western blotting has been and remains an indispensable tool for biologist[267]. In this perspective we take a closer look at several factors that may influence western blot interpretation. The purpose of this perspective is not to review the best practices for western blotting. Several exceptional articles have previously delved into these issues in great deal and readers are strongly encouraged to read if interested[268,269]. Instead we focus on assumptions and aspects of western blots that experimentalists should consider when evaluating data.

5.2 Materials and Methods

Retinal pigment epithelial (RPE) cells overexpressing CXCR4 were grown to 75% confluence a treated with fresh FBS (10%) supplemented media (Gibco PN: 11330-032) 24 hours before the experiment. Cells were serum starved for 4 hours and treated with 12.5 nM CXCL12 (R&D Systems PN: 350-NS-050) for the described time course. Lysates were extracted using RIPA buffer (Pierce PN: 89900) supplemented with protease and phosphatase inhibitors (Thermo Scientific PN: and respectively) and incubated ice for 20 minutes and centrifuged at 16,100g for 45 minutes at 4°C. Loading buffer supplemented with β mercaptoethanol was added to denature lysates and samples were loaded on a 4-20% BioRad gel and transferred onto PVDF

membranes using the iBlot system mixed range transfer (Thermo Scientific). Total protein was quantified using the REVERT Total Protein Stain (LiCor PN: 926-11016). Afterwards, blots were blocked with 5% BSA (Thermo Scientific PN: 37520) in TBST for 1 hour and incubated with primary antibodies ms-GAPDH (1:1000), rb-UMB2 (1:2000) overnight at 4°C. Blots were incubated with secondary antibodies (Gt anti ms-700, gt anti rb-800) for 1 hour in 5% BSA in TBST and imaged using the LiCor Odyssey SA Imaging System. Afterwards, blots were stripped using NewBlot stripping buffer (LiCor PN:928-40032) per manufacture instructions and reprobed with rb-MYC antibody (1:5000) as described above. UMB2 antibody was purchased from ABCAM (PN: Ab124824), MYC from Bethyl (PN: A190-105A) and GAPDH from Santa Cruz Biotechnology (PN: sc-47724). Secondary antibodies were purchased from invitrogen (PN: SA535571 and 35518).

5.3 Results

Experimental assumptions of western blotting and how they can influence results

While statistical analyses and the practice of plotting individual data points improve the accurate portrayal of experimental results, additional information can be gained from looking at raw data. This is particularly true when evaluating western blots or immunofluorescence images. One example from our research that showcases several important factors to consider when evaluating western blots is shown in Figure 5-1. The goal of this experiment was to investigate how CXCR4 (Chemokine receptor 4) mutation (S338/39A and S324/25A) impacts CXCR4 expression and downstream signaling. In addition to probing for total protein and GAPDH (loading controls), CXCR4 was detected using two antibodies: UMB2 and MYC (Figure 5-1). UMB2 is a commercial monoclonal antibody that was initially marketed to detect total CXCR4. However, it has recently been shown to be sensitive to CXCR4 PTMs[225,270]. MYC is a polyclonal

antibody that detects the MYC epitope tag. Experiments were conducted in RPE cells overexpressing individual CXCR4 constructs, which had a C-terminal MYC tag. Wildtype RPE cells have negligible endogenous CXCR4 expression and are unresponsive to CXCL12 (CXCR4 agonist)[225,271].

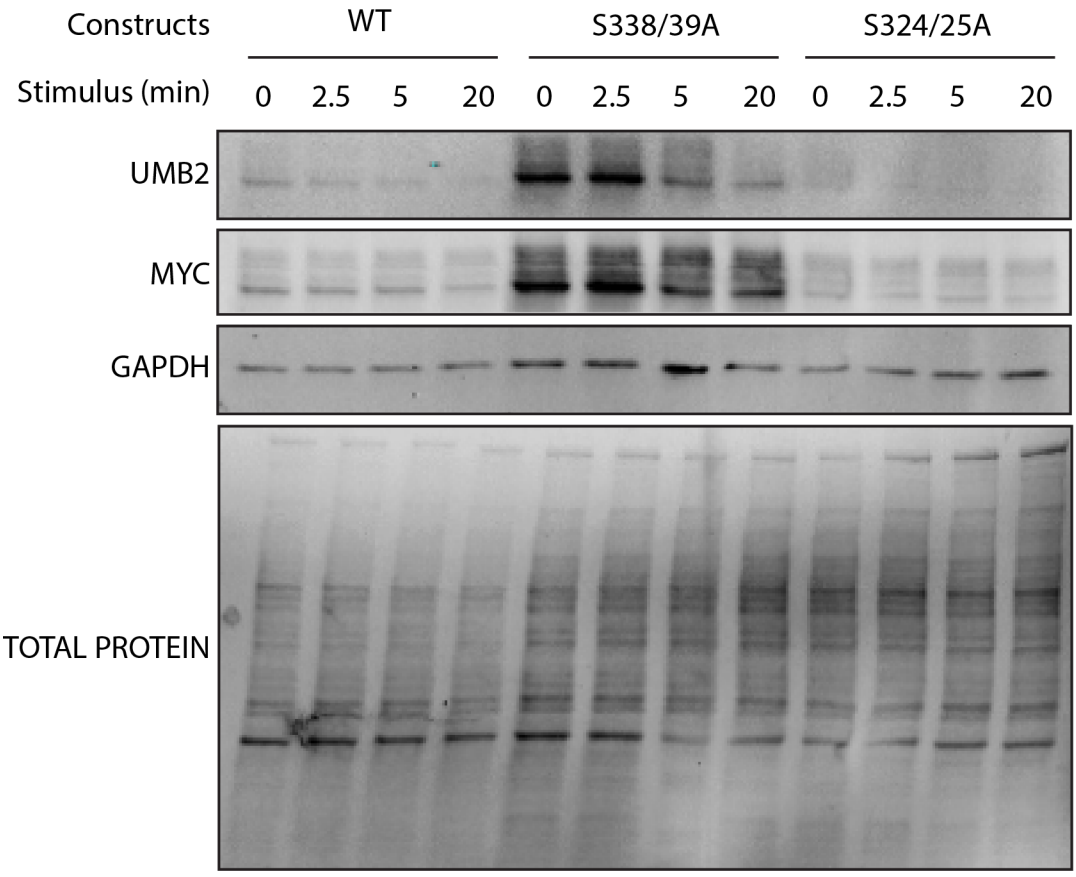


Figure 5-1 Western blotting assumptions are not always true

Representative western blot of non-post-translationally modified (UMB2) and total CXCR4 (MYC) detection for WT and serine mutant receptors (S338/39A and S324/25A). Total Protein and GAPDH staining illustrate protein loading for each replicate.

When initially evaluating a western blot, it is first important to examine the loading control. While loading controls such as GAPDH and Actin are still common practice, total

protein stain should be used whenever possible[269,272–274]. However, when not feasible, loading controls should be confirmed to not fluctuate with experimental treatment[269]. In this case, while loading controls appear similar within each signaling time course, WT and mutant CXCR4 (S338/39A and S324/25A) samples are different. While not ideal, loading protein abundance variability is relatively common in experiments where multiple cell lines or +/- protein knockdown are compared due to unaccounted factors such as differences in cell growth. However, this should be considered when interpreting results as it could suggest that cells were not grown under the same conditions, were seeded differently or have differential growth rates. Protein concentration quantification prior to western blotting can be used to ensure equal loading. In this case, unless sample protein concentrations are listed, information about changes growth conditions or cell density are indistinguishable by western blot. A second assumption is that subsequent antibody detection is within the linear range. However, unless western blot bands are clearly over saturated, is difficult to assess by inspecting individual western blot without additional information. Additional details of this are explained in depth in other reviews[268,269].

For experiments with short timescales in particular (i.e. signaling experiments), a third assumption is that total signaling protein detection remains constant between experimental time points. This is illustrated with the UMB2 antibody detection of CXCR4 (Figure 5-1). The UMB2 antibody was originally thought to detect total CXCR4, however upon agonist-addition, there is a drastic reduction in CXCR4 detection (Figure 5-1 – first 4 lanes). There are several explanations for this observation. Firstly, it is possible that CXCR4 is degraded. However, since this phenomenon happens within minutes, degradation is highly unlikely and inconsistence with knowledge of CXCR4 trafficking[225]. Alternatively, since CXCR4 stimulus induces receptor internalization, it is possible that CXCR4 is more difficult to extract from different intracellular

compartments. However, investigation of CXCR4 detection using a second antibody (MYC) revealed that this is not the case as MYC detection is not lost upon agonist-addition (Figure 5-1 – first 4 lanes) – this has been confirmed in the literature[225]. A third explanation is that upon receptor stimulus there is a change in the ability for the UMB2 antibody to detect CXCR4 which is most likely due to CXCR4 PTM as agonist-induced CXCR4 PTM is well established in the region where the UMB2 antibody recognizes CXCR4[61,114,225,256]. While the UMB2 antibody has become a useful research tool to study CXCR4[225,270], understanding why there is a change in antibody detection can provide useful information regarding quantification accuracy. This phenomenon is not unique to CXCR4 as changes in protein localization, PTM, or structure are often essential for proper protein function. However, understanding experimental design caveats can provide critical information about the underlying biological principles investigated.

Incomplete stripping is another common western blotting issue that can lead to inaccurate data quantification. This is particularly true when evaluating signaling cascade activity using phospho-specific antibodies. It is common that phospho and total signaling protein antibodies are raised in the same species – i.e. both are anti-rb – and therefore removal of the phospho-specific antibody is required to obtain both total and phosopho-specific band quantification. While stripping is relatively effective at removing antibodies, failure to completely remove antibody leads to inaccurate quantification, which can alter interpretation in more complicated experiments if incomplete stripping is not consistent between samples. An example of incomplete stripping is illustrated by MYC antibody staining in Figure 5-1. As we established earlier, UMB2 detection is negatively correlated with CXCR4 PTM. Therefore in the scenario the MYC antibody is the total signaling protein antibody and UMB2 is PTM sensitive. Close inspection of the staining shows that there is a visible decrease in MYC detection at later time

points. This is not biologically relevant as we have previously confirmed that CXCR4 is not degraded or differentially extracted at the 20 min stimulus time point[225]. While most of the UMB2 staining is removed by stripping, some residual staining remains (data not shown). Therefore, earlier time points with higher UMB2 detection have higher background UMB2 detection, which is subsequently detected in the MYC quantification. This breaks the third assumption of western blotting that the total signaling protein is constant during the experiment. In this case, when normalizing UMB2 to MYC detection to quantify CXCR4 PTM, this leads to an underestimate for actual CXCR4 PTM. Only inspection of raw western blots allows this information to become evident to the reader. While in many cases this should not impact overall interpretation, in the case of comparisons between WT and mutant proteins or +/- genetic knockdown, this may change data interpretation as well as statistical significance if incomplete stripping is not consistent between experimental samples.

A more realist experimental situation, where mutant and WT CXCR4 expression and agonist-induced receptor PTM are investigated is illustrated the subsequent western blot lanes (5-12) in Figure 5-1. CXCR4 S338/39A and CXCR4 S324/25A are phospho-null mutant receptors of biological relevant serine residues that regulate CXCR4 internalization and signaling[61]. As is illustrated in Figure 5-1, mutation of just 2 residues in this case can have drastic implications in term of CXCR4 detection. We found that at the 0 min time point S338/39A mutant UMB2 detection was drastically higher compared to WT, while the S324/25A mutant UMB2 detection was negligible (Figure 5-1). Superficially, this could suggest that S338/39A increases CXCR4 expression while the S324/25A mutant is poorly expressed. However, investigation again with the MYC antibody clarifies that the S324/25A mutant is expressed (Figure 5-1 – lanes 9-12). Again, there are several hypotheses that may explain why this occurs. It is possible that

S324/25A completely prevents CXCR4 UMB2 antibody detection due to a change in structure or leads to a different CXCR4 PTM state that decreased UMB2 antibody affinity. In contrast, S338/39A detection with both the UMB2 and MYC antibody was elevated compared to WT CXCR4 suggesting that these mutations may have attenuated degradation and possibly PTM (Figure 5-1). Further comparisons of how agonist-induced PTM is influenced by CXCR4 mutation further highlights the difficulty of solely relying on these data for interpretation as incomplete stripping differentially impacts the MYC detection of each of these receptors and decoupling of receptor mutation from CXCR4 detectability is not possible in this data alone (Figure 5-1). These are important questions that should be considered when evaluating western blots and can only be evaluated by raw data inspection. In this case the interpretation of the presented results are confounding and additional lines of evidence are necessary for interpretation.

Another important aspect to consider when evaluating western blots is antibody-banding pattern. While it is well established that protein PTMs such as ubiquitination or phosphorylation can influence protein migration on SDS-PAGE gels, the phospho-specific antibody should detect a subset of the total antibody detection bands – in this example total antibody refers to the total detection of a single protein such as the total ERK1/2 antibody. In our experiment, this condition was met as the UMB2 detection bands are contained within a subset of the MYC (Figure 5-1). Failure of this assumption can have similar effects to incomplete stripping and inaccurate quantification. Additionally, when evaluating short timescale time course data it is important to consider the experimental approach used to collect data as this can be a source of uncertainty particularly at early time points. Assume that it takes approximately 30 seconds to take cells from the incubator, remove media, and prep samples for protein extraction. In this case, a 1 min

stimulus is in practice closer to 1.5 min – a 50% increase in stimulus time. At later time points, this is less of an issue as 20.5 min vs. 20 min is only a 2.5% increase in stimulus time. Complications arise when collecting data from multiple samples as the order of experiments can lead to unintended but relevant differences in collection time. Therefore, caution should be used when evaluating experiments with short timescales. Among other approaches to mitigate these limitations, conducting all post-experiment processes on ice or using technology (such as microfluidics) to precisely add ligands and lyse cells can be used to improve accuracy[275].

While the caveats described in this section are illustrated through comparison of WT and mutant CXCR4 PTM, the core assumptions are broadly applicably to other western blotting experiments as well as other techniques such as microscopy.

How do western blotting assumptions influence quantification?

In the aforementioned section, we highlighted some of the caveats to consider in western blotting experiments. In this section, we will illustrate how they can influence quantification and interpretation. The first step in quantification is data normalization. While technical and experimental variability between experimental replicates make this step necessary, normalization protocols can drastically influence result interpretation. To illustrate this, a simple hypothetical case is illustrated in Figure 5-2A. In this experiment, total protein loading is assumed constant and we are making a comparison between a control and treatment (i.e. inhibitors, knockdown, mutant protein etc.) group at 0 and 5 min post-stimulus. One approach seen in the literature is to normalize values to the initial pre-treatment sample (Figure 5-2B (I)). An issue with this approach is that typically pre-treatment (no stimulus in the case) samples have significantly lower signal-to noise-ratios compared to later time points in this case when the intensity is much

higher. As illustrated in Figure 5-2B (I) by doing so, minor changes in the base of the normalization can have significant implications on the relative change in the signal. While the reciprocal approach mitigates many of the concerns of amplifying value uncertainty to other samples as maximum values have a higher signal to noise ratio, this approach does not permit comparisons between maximum values between multiple treatments (Figure 5-2B (II)). A third strategy builds on this approach and normalizes samples to the "maximum value time point" of the control experiment. Using this approach, the issue of inter-western blot variability is limited and by normalizing to the maximum or close to maximum value, concerns of amplifying value uncertainty to other time points is greatly reduced (Figure 5-2B (III)). Additionally, by normalizing to the maximum value of the control, comparisons between maximum signaling values of different treatments is possible. However, due to the normalization, the uncertainty of the control maximum value is not considered and the assumption that the treatment does not significantly influence other secondary factors such as signaling protein expression need to be considered. While the practice of multiple normalization should be limited whenever possible, when used it is imperative to describe the approach in explicitly in figure legends and methods.

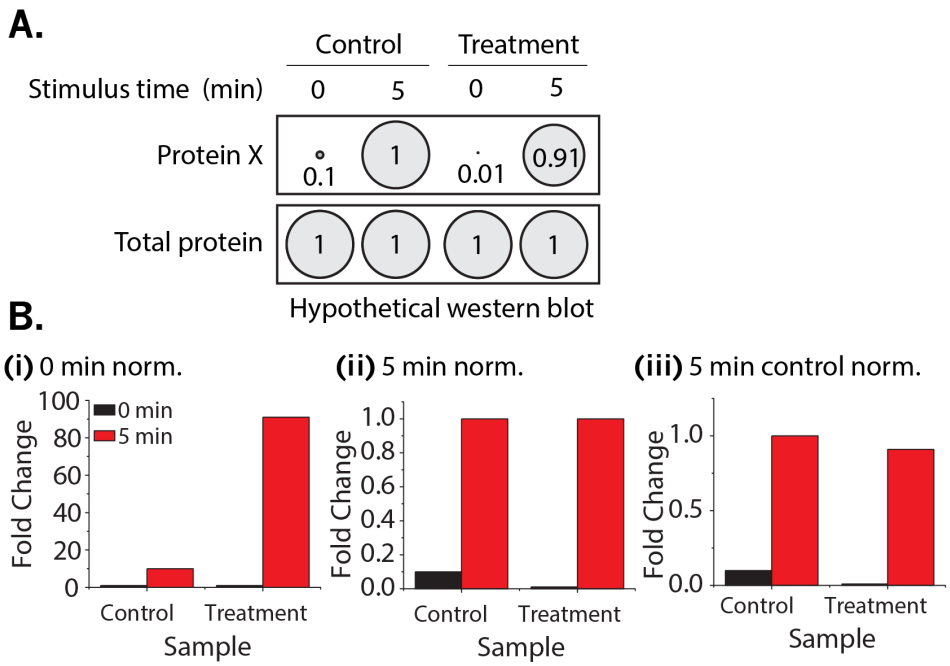


Figure 5-2 Normalization methodology can influence result interpretation

(**A**) Hypothetical western blot results for a control and treatment experiment. Circles diameters are representative of western blot band intensities and are listed. (**B**) Quantification of hypothetical western blot results illustrating that normalizing to samples with low signal to noise ratio can propagate error throughout normalization and influence result interpretation. For this representation, noise was assumed to be constant for each sample. (i) Hypothetical quantification of data when normalized to the 0 min time point of each treatment (i.e. normalizing value: 0.1 and 0.01 for the control and treatment respectively). (ii) Hypothetical quantification of data when normalized to 5 minute time point of each condition (i.e. normalizing value: 1 and 0.91 for the control and treatment respectively). (iii) Hypothetical quantification of data when normalized to the 5 minute time point of the control condition (i.e. normalizing value: one for all samples).

Other considerations for cell signaling experiments

In part due to the recent discoveries of the importance of compartmentalization of receptor signaling, a common approach to study the role of a particular organelle or protein in regulating signaling is to transiently knockdown or knockout a protein of interest and measure

the effect of receptor signaling[127,135,211,225,276]. However, due to the importance of many of these proteins, potential compensatory pathways, and multiple uses of many of the proteins involved in endocytic trafficking, it is important to consider secondary affects of knockdowns/knockouts on receptor signaling and attempt to decouple secondary effects when possible[277]. Recent work has estimated that over 50% of human proteins have multiple intracellular localizations[278]. One classical example from endocytic trafficking is that clathrin-mediated endocytosis occurs at both the plasma membrane and Golgi apparatus[279] (Figure 5-3). Additionally, in unpublished work from our lab, we have shown that an ubiquitination-deficient CXCR4 mutant receptor artificially localizes to intracellular compartments (Chapter 3). Compared to WT, this mutant receptor has reduced signaling and agonist-dependent PTM. However, without additional experiments it was not possible to determine whether these observations were due to the change in receptor localization or mutation. To attempt to decouple these affects, we generated additional constructs that restored mutant receptor plasma membrane localization to confirm that these defects were indeed due to receptor mutation and not localization. Likewise, due to the fundamental importance of signal transduction and receptor regulation, cells have evolved multiple compensatory pathways to control receptor endocytosis. Multiple receptors have been shown to be internalized by both clathrin- and clathrin-independent endocytic mechanisms (Figure 5-3)[118,119] and specific research of epidermal growth factor receptor (EGFR) has revealed that these changes are to due ligand concentration-dependent receptor PTMs[116,119]. Additionally when relating signaling potential back to receptor expression it also is important to consider the spare receptor hypothesis as it has been shown that activation of only a subset of receptors is sufficient for maximal signaling[224]. This is particularly true when evaluating the signaling potential of receptors when treatment (i.e. WT vs. mutant receptor, protein knockdown etc.) affects receptor

trafficking or expression. Lastly, it is important to note that truly rigorous and robust science is ideally supported by multiple methodologies and whenever possible, alternative approaches implemented to test initial conclusions[280,281].

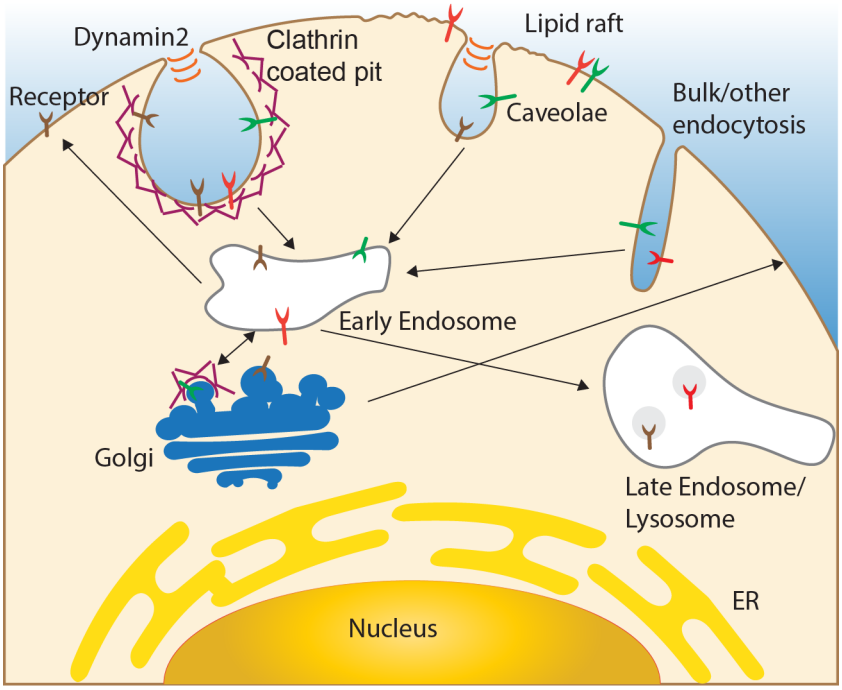


Figure 5-3 Unintended secondary effects of a treatment can influence result interpretation

A schematic representation of endocytic trafficking that highlights some facts to consider when interpreting receptor-signaling experiments. As illustrated by the schematic, receptors can internalize by different and potentially compensatory endocytic mechanisms. Additionally, clathrin-mediated endocytosis occurs at both the plasma membrane and the Golgi, therefore it is important to consider the multiple roles of proteins in cells when conducting isolated knockdown/knockouts experiments as it is possible that the observed effect on cell signaling might be due to a secondary effect of the treatment or genetic perturbation. While this schematic is by no means all encompassing, it highlights some areas that should be considered when designing and interpreting receptor-signaling experiments. Different types of receptors are illustrated in different colors, clathrin triskelia by magenta lattice-like structure and dynamin 2 by the rope-like structure. Internal cellular structures, as well as, the flow of receptors within the cell labeled.

5.5 Discussion

Innovation, creativity, and pushing the limits of technology and research methodologies are fundamental to scientific innovation. Unfortunately, a byproduct of this process is the increased likelihood of honest yet irreplicable results. The failure to replicate could result from heterogenous factors that include a lack of standardized reporting and sharing of experimental protocols, poor experimental design, statistical problems, or biases in hypothesis testing. In this work, we illustrate another important factor that could be playing an important role in the failure to replicate experimental results: the fundamental assumptions made to carry out biological measurements. We believe implementation of core metrology principles within receptor signaling has the potential not only reduce replicability issues but more importantly facilitate discoveries that approach absolute scientific truth. Throughout this article, we have revisited the fundamental assumptions of western blotting and highlighted important features to consider when evaluating western blot data that contribute to data uncertainty.

As the biological sciences become more quantitative, the replicability of measurements between different research groups is the minimum gold standard of science. The culture of replicability requires members of the scientific community to become responsible for the gold standards of reporting measurement protocols and assumptions. These gold standards do not inhibit novelty and significance, but carefully ensure the validity of research findings and whether those finding support the conclusions presented in the research. While western blotting was highlighted in this work, many of the core concepts are applicable to other methodologies used to measure cell signaling and cell biology processes in general.

5.6 Acknowledgements

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Chapter 6 Conclusions and Future Directions

6.1 Overview

Through the work described in this thesis, I have investigated the role of endocytic trafficking and CXCR4 localization in regulating CXCR4 post-translational modification (PTM) and signaling as well as established several new tools to probe CXCR4 biology.

6.2 Chapter 2 summary and future directions

In Chapter 2, I investigated the effects of clathrin heavy chain knockdown in regulating CXCL12-dependent ERK1/2 phosphorylation and CXCR4 PTM. We found that clathrin heavy chain knockdown reduced CXCR4 internalization and increased ERK1/2 phosphorylation. Interestingly, increased ERK1/2 activation was dependent on lipid rafts, as sequestration of plasma membrane cholesterol abrogated this increase. Additionally, while not statistically significant, clathrin knockdown was correlated with increased CXCR4 colocalization with caveolin-1 suggesting potential endocytic pathway compensation. During this work we also discovered that the monoclonal CXCR4 antibody UMB2 is sensitive to CXCR4 PTM and, using this antibody, revealed that clathrin knockdown increased steady-state CXCR4 PTM. Together these results support a model where shifting the localization of CXCR4 within clathrin and clathrin-independent endocytic pits changes receptor steady state PTM and signaling.

While there is growing evidence that receptor plasma membrane microdomain localization regulates signaling, many questions remain unanswered. One exciting question is how receptors move to different plasma membrane microdomains. For EGFR, it has been

suggested that ligand concentration-dependent receptor ubiquitination determines clathrin and clathrin-independent receptor internalization as well as receptor fate and signaling[115,116,119]. One mechanism by which this could occur is that upon sensing ligand concentration, receptors actively cluster in different membrane domains. This could be driven by ligand concentration, as ligands (such as CXCL12) have been shown to oligomerize at high concentrations and induce receptor clustering[84,87]. Alternatively, endocytic components (from clathrin- or clathrin-independent endocytosis) could spontaneously assemble at activated receptors. It is possible that receptor PTM and subsequent protein-protein interactions mediate this process. Recently, receptor tail phospho-barcodes have been shown to favor different β-arrestin conformations that preferentially interact with clathrin or other signaling proteins such as Src. Unfortunately, our inability to use live cell imaging to identify receptor PTMs makes direct investigation of these hypotheses difficult. Harsh detergents to selectively isolate CXCR4 at different plasma membrane microdomains could be used to isolate receptors and the UMB2 antibody to subsequently investigate PTM state. However, this approach is restricted to biochemical analyses and is incompatible with microscopy. Alternatively, receptors could be selectively tagged upon entering specific membrane microdomains. This could be accomplished using a modified BirA biotinylation stategy[169,198,282]. Receptors could be cloned with a BirA acceptor peptide (as previously described)[198,282] and the BirA enzyme localized to different plasma membrane compartments. Therefore, when the receptor moves to a BirA containing plasma membrane microdomain, it would be irreversibly biotinylated at the acceptor peptide. By isolating biotinylated receptors or co-labeling with streptavidin and UMB2 we could identify whether CXCR4 PTM is plasma membrane compartment-specific. Additionally, this could be used to relate receptor trafficking back to initial plasma membrane microdomain receptor localization.

Using this readout, we could explore the molecular mechanism involved in regulating this process. Alternatively, it could be interesting if we could generate a CXCR4 nanobody based on the UMB2 antibody. In theory this tool would allow us to track CXCR4 PTM in real time.

6.3 Chapter 3 summary and future directions

In Chapter 3, I investigated the role of receptor intracellular localization as a regulator of CXCR4 signaling and PTM. This project arose serendipitously as we were originally interested in studying how CXCR4 PTM impacted CXCR4 signaling and endocytosis. However, I noticed that one of the ubiquitin-deficient mutant receptors (K3R) did not traffic properly to the plasma membrane and was retained at intracellular compartments such as the Golgi. An observation that CXCR4 PTM was unchanged by high plasma membrane and low plasma membrane expression inspired us to directly explore whether intracellular CXCR4 is post-translationally modified. Using a modified immunoprecipitation approach to selectively isolate plasma membrane and intracellular receptors, we found that both plasma membrane and intracellular CXCR4 are post-translationally modified in response to agonist. In contrast to previous observations in the literature[131,154], intracellular CXCR4 activation was independent of endocytic trafficking and membrane permeable ligand. We found that intracellular CXCR4 PTM was dependent on Gβγ and β-arrestin-1 and initial results suggest that activation of intracellular CXCR4 is important for CXCL12-dependent *Egr1* transcription. This work revealed a new mechanism for intracellular GPCR activation and adds to the growing evidence that GPCR localization regulates signaling outcomes[131,133,143,154]. There are several important unanswered questions regarding intracellular CXCR4 activation including the location and specific PTM(s) of intracellular CXCR4, the molecular mechanism for intracellular CXCR4 PTM, and the downstream consequences of intracellular CXCR4 activation.

6.3.1 CXCR4 intracellular localization and PTM

Identification of the intracellular localization(s) and PTM state of activated CXCR4 is necessary. While our preliminary data suggest that a subset of CXCR4 is retained in the Golgi, it also stably resides in other secretory pathway intracellular vesicles that we did not stain for (Figure 3-2). Furthermore, inspection of agonist-induced CXCR4 PTM using the UMB2 antibody suggests that CXCR4 PTM may occur at multiple intracellular compartments (Figure 3-3A). Therefore, characterizing CXCR4's precise intracellular localizations and PTM state at each compartment could provide additional clues regarding the biological function or molecular mechanism of intracellular CXCR4 PTM. Comprehensive microscopy experiments investigating CXCR4 co-localization at different intracellular compartments or subcellular fractionation could be used to quantify the relative CXCR4 abundance and PTM at different intracellular compartments pre- and post-CXCL12 stimulation. While these experiments would provide information about CXCR4 localization and CXCR4 PTM, the precise PTMs and potential compartment-dependent differences are indistinguishable as the UMB2 antibody is not PTM specific – site or type.

The C-terminus of CXCR4 is littered with many sites of phosphorylation, ubiquitination, and methylation[61,114,205,226,227]. Each is thought to serve a distinct regulatory purpose[84,256]. Therefore, it would be interesting to investigate whether there are any differences in site specific or type (i.e. phosphorylation, ubiquitination…etc.) of CXCR4 PTM at different localizations. Since the UMB2 antibody cannot resolve specific PTMs, it would be interesting to use mass spectrometry or PTM-specific antibodies to probe CXCR4 PTMs at different intracellular compartments. We previously attempted to use mass spectrometry to identify the PTMs

responsible for the CXCL12-dependent loss of CXCR4 UMB2 detection. Our initial efforts were unsuccessful for two reasons – (1) difficulty in isolating post-translationally modified CXCR4 peptides and (2) PTM complexity/poor protease digestion cleavage sites to resolve different PTMs. To overcome these issues, we could enrich for post-translationally modified CXCR4 peptides and use mutant receptors to systematically reduce the PTM complexity of the C-terminal tail. Alternatively, CXCR4 site-specific phospho-antibodies could be used[61,256]. However, since phosphatase treatment failed to rescue CXCR4 UMB2 detection, we believe that a combination of multiple PTMs (i.e. phosphorylation and non-phosphorylation) are responsible for changes in UMB2 detection and would not be captured by using phospho-specific antibodies alone. Additionally, I have not had reproducible success using CXCR4 phospho-antibodies. Regardless, the ability to identify the specific PTMs at plasma membrane and intracellular CXCR4 would represent a significant advancement in our understanding of the biological relevance of different intracellular receptor populations.

6.3.2 How are intracellular GPCRs activated?

While we discovered that intracellular CXCR4 PTM is dependent on Gβγ activity and β-arrestin-1, it remains unclear how intracellular CXCR4 are post-translationally modified. Since Gβγ activation is necessary for CXCR4 PTM and Gβγ is functional at multiple intracellular compartments[153,154,210,234], it is possible that localized Gβγ activation is responsible for intracellular CXCR4 PTM. To investigate this, we could inhibit Gβγ at different cellular compartments and measure CXCR4 PTM. By exploiting the strong interaction between activated Gβγ and the C-terminus of GRK2, researchers have developed a suite of localized Gβγ protein inhibitors – known as GRK2 C-terminus (GRK2ct)[153,154,210,234]. These inhibitors work by recruiting activated Gβγ to nonfunctional GRK2ct, preventing activation of endogenous GRKs.

By adding peptide localization motifs to localize GRK2ct to different cellular compartments, researchers have used this approach to study Gβγ-dependent processes at the plasma membrane, Golgi, and endosomes[153,154,210,234]. A similar approach could be implemented to investigate whether localized Gβγ activation is necessary for intracellular CXCR4 PTM. One caveat of this approach is that it fails to decouple whether Gβγ was activated at the plasma membrane and trafficked to an intracellular compartment or is directly activated at an intracellular compartment.

Interestingly, it is known that Gβγ rapidly translocates from the plasma membrane to different intracellular compartments including the Golgi upon GPCR activation[240,241]. Gβγ translocation occurs at a variety of timescales (from on the order of seconds to many minutes), is reversible, and independent of receptor internalization[283]. This phenomenon is ubiquitous and occurs with all Gβγ complexes and endogenously in multiple cell types[283]. However, the precise biological function and mechanism by which this Gβγ translocation occurs remains unclear. One proposed mechanism for Gβγ translocation is that agonist-induced Gβγ depalmitoylation decreases plasma membrane localization and leads to the rapid diffusion to intracellular compartments[284]. Using parathyroid hormone receptor as a model system, the Vilardaga group recently found that Gβγ exchanges between activated β2-adrenergic receptors and PTHR and this promoted sustained endosomal PTHR cAMP signaling[143]. Interestingly, while it was initially thought that G proteins and β-arrestin GPCR binding was mutually exclusive, recently researchers have found that Gβγ, β-arrestin, and GPCRs can form mega signaling complexes[143,144]. Building on this model, it is possible that β-arrestins plays a role in mediating Gβγ translocation and would be interesting to investigate agonist-dependent Gβγ and β-arrestin-1 trafficking as well as Gβγ translocation in β-arrestin-1 knockdown cells. Since it is well established that activated Gβγ recruits GRKs to phosphorylate GPCRs, this could be a potential

mechanism for intracellular activation. Alternatively, β-arrestin-1 itself may dissociate from activated plasma membrane CXCR4 and traffic to intracellular CXCR4 to facilitate receptor PTM. Interestingly, there is also experimental precedence for GPCR-independent β-arrestin trafficking; β-arrestin 2 has been shown to traffic independently of its activated GPCR to nearby clathrin-coated structures on the plasma membrane to induce ERK1/2 signaling[211]. Several potential hypotheses are illustrated in Figure 6-1.

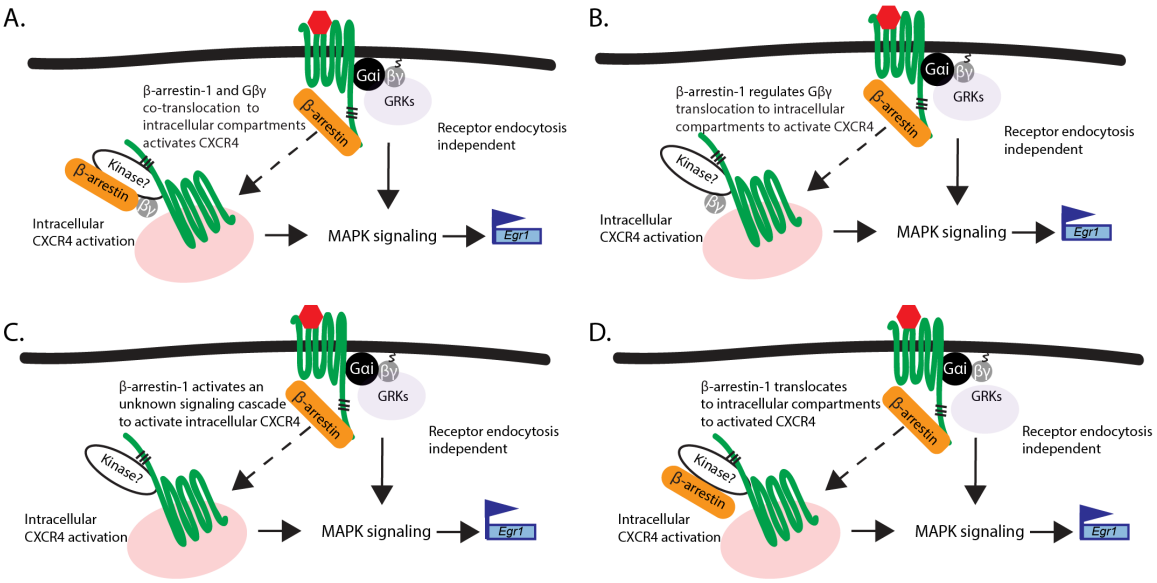

Figure 6-1 Potential mechanism for intracellular CXCR4 post-translational modification.

(A) Gβγ and β-arrestin-1 co-translocate to intracellular compartments to initiate intracellular CXCR4 PTM. **(B)** β-arrestin-1 regulates Gβγ intracellular translocation to initiate intracellular CXCR4 PTM. **(C)** β-arrestin-1 activates an unknown signaling cascade that leads to intracellular CXCR4 PTM. **(D)** β-arrestin-1 translocates to intracellular compartments to activate CXCR4.

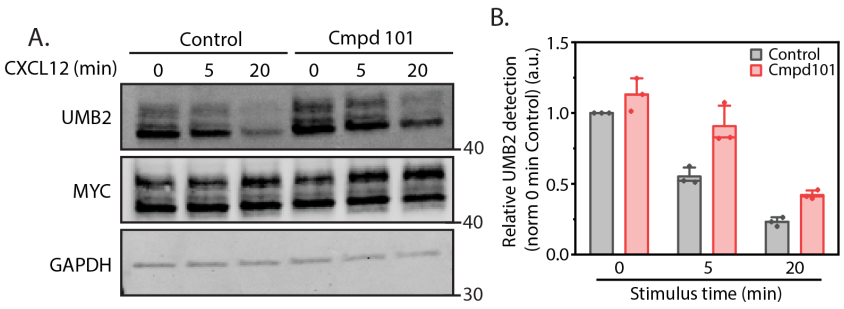

Figure 6-2 GRK2/3 inhibition reduces agonist-induced CXCR4 PTM

(A) Representative western blot showing agonist induced CXCR4 PTM upon GRK2/3 inhibition. **B**) Western blot quantification of CXCR4 PTMs upon GRK2/3 inhibition. CXCR4 PTMs were calculated by dividing UMB2 detection (non-PTM CXCR4) by MYC intensity (total CXCR4) and normalized to the 0 min control sample. All experiments were conducted in RPE cells overexpressing WT CXCR4 and stimulated with 12.5 nM CXCL12 unless specified. GRK2/3 activity was inhibited by Cmp101 (10 μM). Cells were pretreated for 30 min prior to and throughout the experiment. (Individual data points from each experiment are plotted; mean +/- SD, median line. n=3 for all experiments.

It is important to note that neither β-arrestin-1 nor Gβγ are known to actively post-translationally modify GPCRs, therefore it is likely that an unidentified kinase, ubiquitin ligase or other PTM-capable protein is directly involved in intracellular CXCR4 PTM. Particularly, if localized Gβγ activity is necessary for intracellular CXCR4 PTM, GRKs are likely candidate kinases. Intrigued by this hypothesis, we preliminarily investigated the role of GRK2/3 in regulating CXCR4 PTM. As expected, pharmacological inhibition of GRK2/3 increased agonist-induced CXCR4 UMB2 detection (Figure 6-2). Interestingly, while GRK2/3 inhibition led to a reduction in CXCR4 PTM, especially at later time points, CXCR4 UMB2 detection decreased suggesting that some CXCR4 PTMs are GRK2/3 independent. Unfortunately, we did not have time to investigate whether GRK2/3 inhibition specifically inhibited plasma membrane or

intracellular CXCR4 PTM – it would be particularly interesting to see whether GRK2/3 inhibition ubiquitously decreases CXCR4 PTM or is location-specific.

Alternatively, it is possible that β-arrestin-1 and/or Gβγ activate other signaling pathways that lead to intracellular CXCR4 PTM. If this is the case, there are two different approaches that could be used to identify the molecular mechanism. Firstly, I would continue to investigate whether other known downstream signaling proteins were involved in intracellular CXCR4 PTM. To do this, we could systematically inhibit downstream signaling proteins and measure changes in CXCR4 PTM. Preliminary data suggest that neither AKT or ERK1/2 activation are required or CXCR4 PTM (data not shown). However, CXCR4 activation is also known to activate both JAK/STAT signaling as well as calcium signaling[84] which could play a role in modulating intracellular CXCR4 PTM. Alternatively, an unknown signaling mechanism could be responsible for intracellular CXCR4 PTM. To investigate this, we could start by conducting a kinase inhibitor screen to identify kinases that reduce intracellular CXCR4 PTM. Alternatively, APEX2 proteomics can be used to identify CXCR4 interacting partners at intracellular locations. This could be accomplished by removing plasma membrane-localized CXCR4 and enriching for protein interactions at intracellular compartments. Finally, a genome-wide CRISPR screen could be used to determine regulators of CXCR4 PTM. In this case, plasma membrane and intracellular CXCR4 agonist-induced UMB2 detection could be used as the readout.

6.3.3 What is the biological relevance of intracellular CXCR4 activation?

Additional investigation of the downstream implications of intracellular GPCR activation in terms of signaling and receptor fate is another exciting future direction. While we found that *Egr1* transcript levels correlate with intracellular CXCR4 activation, whether intracellular CXCR4 is actually activated should be directly tested. To do so, we could use live cell

microscopy tools to confirm that intracellular CXCR4 activation using mini-G proteins (mG). mG proteins are miniaturized version of the G proteins that are fluorescently tagged that upon receptor stimulus, localize to activated GPCRs[285,286]. While preliminary, using the mGi protein, we saw a CXCL12-dependent increase in mGi-CXCR4 colocalization (Figure 6-3). Identified intracellular puncta suggest that intracellular CXCR4 activation occurs at endosomal compartments and potentially the Golgi (yellow arrows in inset images Figure 6-3). Interestingly, while not directly tested, others have suggested that endosomal CXCR4 signaling is necessary for complete AKT activation[135]. Additional replicate experiments are necessary to confirm these observations and it would also be interesting to explore whether these observations are independent of GPCR endocytosis, Gβγ, or β-arrestin-1. To do so, analogous experiments could be conducted in the presence of inhibitors or shRNA knockdowns for each of these proteins/pathways. In agreement with our previous findings, I expect that mGi and intracellular CXCR4 agonist-induced colocalization is independent of CXCR4 internalization but requires Gβγ and β-arrestin-1. We could further extend these experiments to test whether Gβγ localization regulates this process by using localized GRK2ct inhibitors.

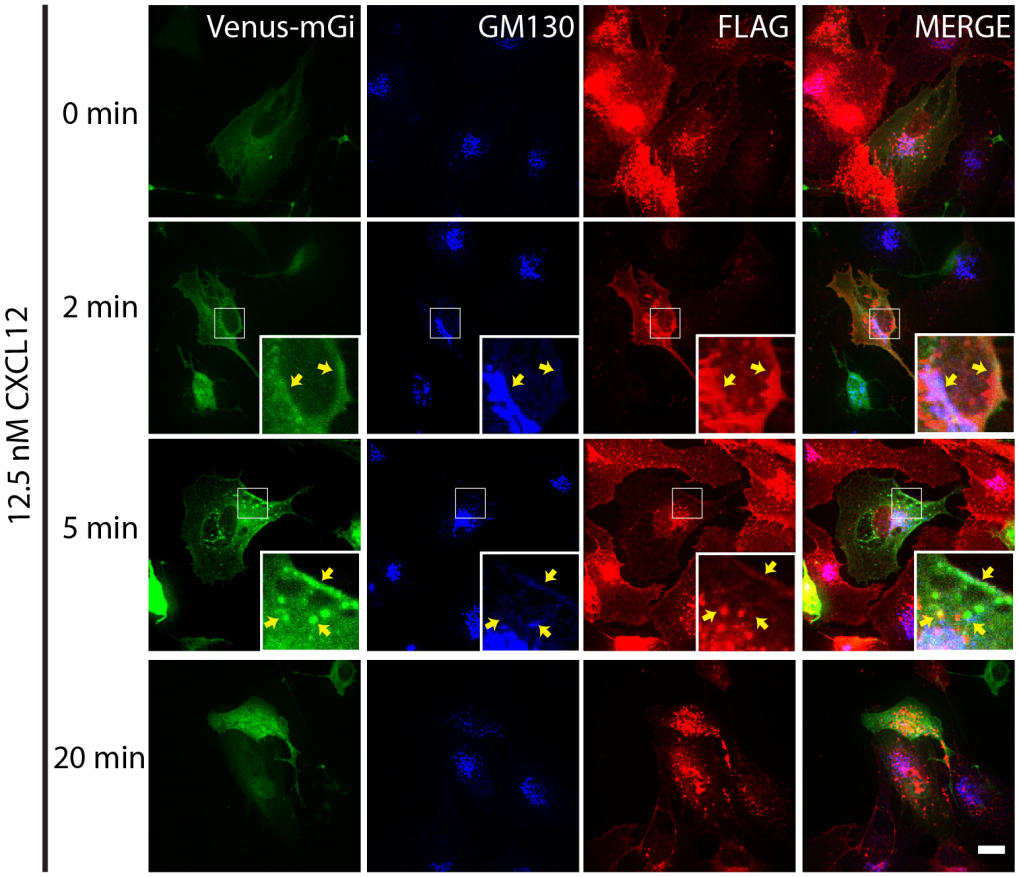

Figure 6-3 mini-G protein CXCR4 agonist-induced colocalization

Representative confocal microscopy images of Venus-mGi and CXCR4 colocalization upon CXCL12 (12.5 nM) stimulus. CXCR4 is labeled using the FLAG antibody whereas the Golgi is denoted by GM130 staining. Yellow arrows show colocalization between mGi and CXCR4. Scale bar 10 μm. Images were captured using 60x magnification on a spinning disk confocal microscope. This data is n = 1.

It would also be interesting to investigate whether there are other transcriptional implications besides *Egr1* transcription of intracellular CXCR4 activation. For example, it is possible that activation of intracellular GPCRs promote cell growth and proliferation whereas activation at the plasma membrane primarily activates transcription of chemotactic factors. Additionally, the temporal sequence of downstream signaling events would be interesting to

explore. RNAseq experiments could be used to determine the broader transcriptional implications of CXCR4 activation and coupled with localized inhibition of CXCR4 to discover transcriptional consequences of localized GPCR signaling. Additionally, it would be interesting to know which transcriptional implications are G protein-dependent vs. independent.

The long-term implications and receptor fate of intracellular CXCR4 PTM are also unknown. Inspired by recent work from the Luker lab, it is possible that intracellular CXCR4 PTM functions as memory for subsequent signaling events. Researchers found that conditioning cells with growth factors prior to CXCL12 increased the number of cells that activate CXCL12-dependent signaling cascades[287]. This finding provides two insights: firstly only a subset of cells actively signal and secondly cells have a short-term memory of prior signal exposure that influences subsequent rounds of signaling. It is possible that CXCR4 PTM could play a role in regulating this process. Additionally, since only a subset of cells signal, it would be interesting to multiplex signaling with CXCR4 PTM and internalization. This could be accomplished by flow cytometry.

We also could investigate how multiple rounds of CXCL12 stimulation affect CXCR4 membrane trafficking, signaling, and PTM. This experiment could also reveal information regarding where post-translationally modified intracellular CXCR4 traffic. To exclude the plasma membrane labeled receptors, we could use the surface biotinylation assay pre-stimulus to label plasma membrane receptors with a biotin-tag and monitor receptor trafficking as well as fate of intracellular vs. plasma membrane CXCR4 post-receptor stimulus. We could also re-stimulate the cells after several hours and investigate subsequent CXCL2-dependent signaling, internalization, and CXCR4 PTM. This could provide insight into whether intracellular CXCR4 PTMs regulate future signaling events or trafficking.

6.3.4 Mathematical modeling to investigate the implications of plasma membrane and intracellular CXCR4 signaling

A modeling approach could also be used to investigate the biological relevance of intracellular GPCR activation. Recently the Schnell lab used a mathematical model to investigate the efficacy of different sensors to detect unfolded protein in the ER[288]. A modified version of this approach could be used to understand the biological and evolutionary rationale of having multiple localization of CXCR4 contribute to controlling a downstream response. Stroberg et al. tested three different mechanisms to sense ER stress: an unfolded-protein sensor, free-chaperone sensor, and a combined sensor. Each sensor was benchmarked for resource utilization and accuracy. While the AND switch coincided with the best of both the unfolded protein and chaperone-mediated sensors, it did not significantly improve the outcomes of either individual mechanism[288]. Interestingly, the combined sensor is what occurs in nature and the authors concluded that it likely serves a different purpose than minimizing the overproduction of chaperones during ER stress[288].

A similar approach could be used to model plasma membrane and intracellular CXCR4-dependent signaling. Instead of having a single compartment, two compartments could be used to model how localized CXCR4 activation leads to downstream transcription in a β-arrestin-1-dependent mechanism. It would be interesting to investigate whether modulating either of these two populations changes a cell's ability to accurately and adeptly respond to CXCL12. As discussed in Chapter 3, plasma membrane and intracellular CXCR4 activation resemble a feed-forward positive feedback loop (Figure 3-11). In nature, cells encounter many different signals and their ability to accurately respond is essential. Feed-forward loops increase sensor robustness and sensitivity and allows for cells to distinguish signaling from noise[289]. It is possible that

modulating the balance of receptors in these two pools could lead to over-activation or increased variability in cellular response. Using the mathematical model we would be able to investigate these different scenarios. Additionally, we have great experimental control of this system and are able to measure total receptor level and PTM state at plasma membrane and intracellular compartments. Furthermore, we have established assays to measure intermediate (ERK1/2 phosphorylation) and final transcriptional output (*Egr1* transcript levels) as well as response variability. These readouts could be used as a proxy for the fitness of each situation. Similarly to Stroberg et al., we could test different mechanisms such as plasma membrane alone vs. the combined signaling mechanism. This could then be expanded to include how the distribution of plasma membrane and intracellular CXCR4 localization may modulate downstream signaling response, resource utilization, and variability. These experiments would provide information about how receptor localization impacts downstream signaling as well as fitness consequences of modulating this balance.

6.4 Chapter 4 summary and future directions

In Chapter 4, we adopted APEX2 proteomics to study how the CXCR4 interactome changes during endocytic trafficking. While we were able to confirm that the CXCR4-APEX2 fusion protein did not drastically affect CXCR4 function, we were unable to confirm the biological relevance of the protein-protein interactions identified by our proteomics experiment. PDGFRβ was one of the top hits identified in our proteomics experiment. Ultimately, we found no functional crosstalk between CXCR4 and PDGFRβ signaling, agonist-induced CXCR4 PTM or CXCR4 internalization in retinal pigment epithelial (RPE) cells. This could be due to several reasons including cell type specificity or the fact that we only tested a subset of potential CXCR4-PDGFRβ crosstalk processes. While we believe that the APEX2 technology is a

promising tool to study the CXCR4 interactome, additional proteomics experiments are necessary to advance this research.

While spatiotemporal proteomics provides information about CXCR4 interacting partners, protein PTMs play an essential role in regulating these interactions as well as the downstream signaling implications[61,290]. Therefore, it would be interesting to investigate how local proteomic architecture correlates with the CXCL12-dependent phospho-proteome. This could be accomplished by running APEX2 proteomics experiments simultaneously with phosphoproteomics. Samples could be divided into two groups one for streptavidin pulldown and the other phosphopeptide enrichment. While CXCR4 phosphoproteomics has been previously investigated[60], this experiment would add important spatial information pertaining to CXCR4 localization and interacting partners during these signaling events. This research could be extended further by inhibiting different signaling cascades or endocytic trafficking pathways to determine how specific proteins not only regulate the CXCR4 interactome but also downstream signaling implications. For example, since caveolae are known to be important for CXCL12-dependent ERK1/2 signaling[102,225], we would expect that inhibition of CME would increase ERK1/2 phosphorylation and potentially shift the balance of downstream signaling events or CXCR4 endocytic trafficking towards CIE-dependent processes.

6.5 Chapter 5 summary and future directions

During the course of my thesis research, I became increasingly interested in how we measure cell biology processes and interpret receptor-signaling data. In Chapter 5, I summarized some useful practices when evaluating western blotting data. Using agonist-induced receptor PTM as an example, I highlighted the assumptions of western blotting and showcased how clues

from raw western blots can hint at experimental variability that is not captured by statistics and methods that influence quantification.

As technological and analytical innovations rapidly advance our ability to reveal increasingly complex biological processes, the importance of understanding the assumptions behind biological measurements and sources of uncertainty are essential for data interpretation. This is particularly important in fields such as cell signaling and synthetic biology. Due to its importance for both homeostatic and pathogenic biological processes, a quantitative understanding of the cell signaling mechanisms is fundamental to drug development. Likewise, the ability to accurately predict and design standard components is necessary for biomedical engineering and synthetic biology innovation.

6.6 Conclusions

Understanding how cells decipher complex extracellular information using limited resources and conserved signaling hubs remains in my opinion one of the biggest outstanding questions in biology. Together with recent work from other groups, the contents of this thesis highlight the important role of receptor localization as a master regulating of cellular decision-making; this not only challenges our traditional understanding of receptor signaling but how we study and interpret cell-signaling events. Additional research is necessary to truly elucidate the mechanisms and importance of intracellular GPCR activation.

The implications of spatiotemporal signaling span both basic and translational science as it is becoming apparent that intracellular GPCR activation leads to distinct signaling outcomes and dysfunction is associated with a growing number of diseases[133,154,155]. An improved understanding of the molecular mechanism of intracellular GPCR activation has the potential to lead to the development of a new class of therapeutics that selectively modulate these pathways.

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