AN EXAMINATION OF HOW RAB GTPASES AND MOLECULAR CHAPERONES INFLUENCE PLASMA MEMBRANE EXPRESSION OF CHEMOKINE RECEPTOR DIMERS

by

Kelsie Gillies

Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
November 2013

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Signal termination processes of GPCRs are well established, unlike processes that regulate the assembly and intracellular trafficking of these signaling complexes. Bimolecular fluorescence complementation was used to study GPCR dimer formation in two projects. Firstly, the importance of Rab GTPases on the cell surface expression and signaling of two chemokine receptors expressed on prostate cancer cells was examined. Rab GTPases necessary for CXCR4 and CCR2 cell surface expression and signaling were different from those necessary for the CXCR4/CCR2 heterodimer. Therefore, this project emphasizes the importance of studying heterodimers as unique entities from their constituent receptors. Secondly, interactions between molecular chaperones and two co-receptors necessary for HIV infection – CCR5, a chemokine GPCR, and the main HIV receptor, CD4, a glycoprotein – were investigated. Further emphasizing the unique characteristics of GPCR dimers, this project found that molecular chaperones interact differently with CCR5 homodimers, when compared to CCR5/CD4 heterodimers.

LIST OF ABBREVIATIONS USED

AC	Adenylyl Cyclase
AMP	Adenosine Monophosphate
$A_{2A}R$	A2A Adenosine Receptor
ATCC	American Type Tissue Culture Collection
ATP	Adenosine-5'-Triphosphate
AT1R	Angiotensin II Type IA Receptor
α2-AR	Alpha2-Adrenergic Receptor
β2-AR	Beta2-Adrenergic Receptor
BiFC	Bimolecular Fluorescence Complementation
BiP	Binding Immunoglobulin Protein
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	3',5'-Cyclic Adenosine Monophosphate
CCL31	Chemokine C-C Motif Ligand 31
CCR2	C-C Motif Chemokine Receptor Type 2
CCR5	C-C Motif Chemokine Receptor Type 5
CD4	Cluster of Differentiation 4
cDNA	Complementary Deoxyribonucleic Acid
CO_2	Carbon Dioxide
Co-IP	Co-Immunoprecipitation
COP	Coat Protein
C-Terminus	Carboxy-Terminus
CXCL12	C-X-C Motif Ligand 12
CXCR4	C-X-C Motif Chemokine Receptor Type 4
DAG	Diacyl Glycerol
DMEM	Dulbecco's Modified Eagle's Medium
DN	Dominant Negative
D_1R	Dopamine 1 Receptor
D_2R	Dopamine 2 Receptor
DRiP78	Dopamine Receptor Interacting Protein, 78 Kilodaltons
DTT	Dithiothreitol
E/DRY	Glutamic Acid/Aspartic Acid-Arginine-Tyrosine
EDTA	Ethylenediaminetetraacetic Acid
ER	Endoplasmic Reticulum
ERAD	Endoplasmic Reticulum-Associated Degradation
ERGIC	Endoplasmic Reticulum-Golgi Intermediate Complex
ERp	Endoplasmic Reticulum Protein
FAK	Focal Adhesion Kinase
FBS	Fetal Bovine Serum
GABA	Gamma-Aminobutyric Acid
$GABA_BR1$	Gamma-Aminobutyric Acid B Receptor 1
$GABA_BR2$	Gamma-Aminobutyric Acid B Receptor 2
GDI	Guanosine Diphosphate-Dissociation Inhibitors
GDP	Guanosine Diphosphate

GFP	Green Fluorescent Protein
6'GNTI	6'-Guanidinonaltrindole
GPCR	G-Protein-Coupled Receptor
G-Protein	Guanosine Nucleotide-Binding Protein
gp120	Envelope Glycoprotein GP120
gp41	Envelope Glycoprotein GP41
GRK	G-Protein-Coupled Receptor Kinase
GRP94	Glucose-Related Protein, 94 Kildaltons
GTP	Guanosine Triphosphate
HAART	Highly Active Antiretroviral Therapy
HCl	Hydrogen Chloride
HEK293A	Human Embryonic Kidney 293 Cells
HIV	Human Immunodeficiency Virus
HRP	Horse Radish Peroxidase
HSC	Hematopoietic Stem Cells
Hsp	Heat Shock Protein
IP_3	Inositol 1,4,5-Triphosphate
MCP-1	Monocyte Chemoattractant Protein-1
$MgCl_2$	Magnesium Chloride
MIP	Macrophage Inflammatory Protein
MRAP	Melanocortin-2 Receptor Protein
NHERF1	Sodium-Hydrogen Antiporter 3 Regulator 1
N-Terminus	Amino-Terminus
OPR	Opioid Receptor
PBS	Phosphate Buffered Saline
PC3	PC3 Human Prostatic Small Cell Carcinoma Cell Line
PDI	Protein Disulfide Isomerase
PEI	Polyethylenimine
p-FAK	Phospho-Focal Adhesion Kinase
PLC	Phospholipase C
PS	Penicillin-Streptomycin
Rab	Rat Sarcoma-Related Protein in Brain
RAN	Rat Sarcoma-Related Nuclear Protein
RANTES	Regulated on Activation, Normal T Cell Expressed and Secreted
Ras	Rat Sarcoma
Rep	Rab Escort Protein
RIPA	Radioimmune Precipitation Assay Buffer
RLuc	Renilla Luciferase
RNA	Ribonucleic Acid
SDF-1α	Stromal Derived Factor-1alpha
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
shRNA	Short Hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
SNSR4	Sensory Neuron-Specific Receptor-4
SSTR1	Somatostatin Receptor 1
SSTR2	Somatostatin Receptor 2

$SSTR_{2A}$	Somatostatin Receptor 2A
TBS	Tris(hydroxymethyl)aminomethane Buffered Saline
TBST	Tween-20 Tris(hydroxymethyl)aminomethane Buffered Saline
TGN	Trans-Golgi Network
Tris	Tris(hydroxymethyl)aminomethane
UPR	Unfolded Protein Response
V1aR	Vasopressin 1a Receptor
V2R	Vasopressin 2 Receptor
V1	Venus 1 (N-terminal Fragment of Venus Protein, Amino Acids 1 to 157)
V2	Venus 2 (C-terminal Fragment of Venus Protein, Amino Acids 158 to 238)
WT	Wild Type
YFP	Yellow Fluorescent Protein

ACKNOWLEDGEMENTS

I would like to thank Dr. Denis Dupré and all members of the Dupré lab – Brent Young, Jaime Wertman and Nigel Chapman – for their guidance and enthusiasm while I was completing this work. I would like to thank Luisa Vaughn and the members of my advisory committee – Dr. Eileen Denovan-Wright, Dr. Kerry Goralski and Dr. Kishore Pasumarthi – for their support while I was preparing this thesis. A heartfelt thanks is in order for my family and their patience with me as I find my place in life. I would also like to thank Julia Tufts and Katie MacLennan for being amazing sources of confidence and friendship. Last, but certainly not least, I would like to thank Adam Fancy for all of his help, encouraging words, and his unwavering faith in me.

CHAPTER 1 INTRODUCTION

1.1 G-Protein-Coupled Receptors (GPCRs)

G-protein coupled receptors (GPCRs), also referred to as seven-transmembrane domain receptors, are a superfamily of integral membrane receptors [1]. Since the cloning of bovine rhodopsin in 1983 [2], followed by that of the β-adrenergic receptor in 1986 [3], a staggering number of GPCRs have been identified. These identified structures span a variety of eukaryotic genomes and prokaryotic genomes [4]. As a result, GPCRs constitute the largest family of cellular signaling proteins that are capable of generating responses from extracellular signaling molecules [5] such as ions, biogenic amines, peptides, hormones, and lipids [4]. Not surprisingly, GPCRs are heavily involved in almost all physiological processes. This physiological relevance is not only due to their large number or ability to respond to diverse extracellular stimuli, but also due to their regulatory roles relating to signal transduction cascades, coupled with ubiquitous expression in almost all organs of the body [6].

The importance of GPCRs is underlined by the clinical relevance of agonists and antagonists capable of modifying GPCR signal transduction cascades to treat a variety of disease states. Pharmacological interventions targeting GPCRs have been shown as effective treatments for respiratory, cardiovascular, central nervous system, urogenital, and metabolic disorders [7]. Furthermore, according to with most recent estimates, approximately 30 to 40% of today's prescription drugs target GPCRs [8-10].

A phylogenetic investigation concluded that there are at least 800 functional GPCRs encoded by the human genome [11]. Several different GPCR classification

systems have been proposed, with the most popular classification system being based on structural similarities among mature receptors [12,13].

In regards to shared features, all GPCRs consist of a seven-transmembrane α-helical core [14]. This core begins as an extracellular amino-terminus (N-terminus) and alternates between intracellular and extracellular loops as seven transmembrane segments before terminating as an intracellular carboxy terminus (C-terminus) [14]. It is the structure of these loops, along with the structure of each terminus, which can differ among GPCRs.

Structural similarities among mature receptors have been used to classify GPCRs into Classes A through F [12,13]. Classes A, B and C are found in all vertebrates [11], with at least 80% of all GPCRs belonging to Class A, the rhodopsin-like GPCRs [15,16]. Other than rhodopsin, this family comprises neuropeptide, neurotransmitter, odorant, chemokine, and glycoprotein hormone receptors [15]. Class A GPCRs also have transmembrane domains that are often referred to as "tilted" or "kinked" because of amino acids like proline that act to distort the helical domains [15]. Class B GPCRs respond to hormones like secretin, glucagon, and parathyroid hormone [15]. Class B GPCRs also have a long N-terminus tail in which multiple cysteines form numerous disulfide bridges [15,16]. Like Class B GPCRs, Class C GPCRs have a long amino terminus [15], but are also characterized by a long C-terminus [15,17,18]. GPCRs of this family are obligatory dimers and include the metabotropic glutatmate, gamma-aminobutyric acid B ($GABA_B$) and Ca^{2+}-sensing receptors [15].

1.2 GPCR Activation and Signal Transduction Cascades

1.2.1 G-protein Dependent Signaling

Ligand binding to a GPCR induces a conformational change in the receptor that initiates activation of the α subunit of a receptor-associated heterotrimeric guanosine nucleotide-binding protein (G-protein) [19]. This activation is accomplished by guanosine nucleotide exchange (GDP to GTP) and results in dissociation of the α monomer from the βγ complex [19]. Signaling is accomplished by means of the Gα subunit or the Gβγ complex [19]. Four families of Gα subunits have been defined based on sequence homology. These families include G_s (G_s and G_{olf}), G_i (G_{i1-3}, G_t, G_g, G_o, G_z), G_q (Gq, G_{11}, G_{14}, $G_{15/16}$) and G_{12} (G_{12} and G_{13}) [20].

As the subject of one of the first cell signaling pathways to be described [20], the G_s pathway has been shown to activate adenylyl cyclase (AC) – the enzyme that catalyzes the conversion of ATP to 3',5'-cyclic AMP (cAMP), a second messenger that can influence the activities of transcription factors, enzymes, and ion channels [20,21]. G_i proteins inhibit AC, whereas the G_q family has been shown to activate phospholipase C (PLC) [20]. PLC cleaves phosphatidylinositol 4,5-bisphosphate into two constituent compounds – diacyl glycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). IP_3 becomes distributed throughout the cytosol, but DAG remains membrane-bound [22]. IP_3 then binds to its receptors, such as calcium channels in the smooth endoplasminc reticulum (ER). Binding of IP_3 to these receptors causes an increase of cytosolic calcium ions, which can act in concert with DAG to activate protein kinase C (PKC) [20]. By phosphorylating a myriad of proteins, PKC can then initiate numerous cellular signaling cascades. Similarly, the Gβγ complex can also initiate this series of molecular events

associated with PLC activation [20]. Additionally, the G_{12} family has been shown to stimulate PKC, phospholipase D, c-Src Brunton's tyrosine kinase and the Gap1M Ras GTPase activating

protein [20].

1.2.2 G-protein Independent Signaling

GPCR kinases (GRKs) phosphorylate cytosolic domains of GPCRs so that these sites can then bind β-arrestins, proteins that prevent further signaling through the GPCR and initialize internalization of the GPCR [23]. Internalized GPCRs can be recycled by recycling endosomes for later expression at the plasma membrane or directed to lysosomes for degradation [23]. However, GRKs are also involved in G-protein independent signaling cascades. These cascades rely on signaling events associated with β-arrestins and mass spectroscopy studies have elucidated numerous proteins that assemble with β-arrestins for downstream signaling to occur [24].

1.3 Dimerization of GPCRs

It was originally thought that GPCRs acted as monomeric receptors, but the belief that GPCRs can act as heterodimers or even higher-oligomers became popular due to a fundamental study by Maggio et al. (1993) [25]. In this study, chimeric α2-adrenergic/M3 muscarinic receptors were created such that the C-terminus receptor portions were exchanged between the two receptors [25]. It was found that, when expressed alone, each chimera was unable to bind either a muscarinic or adrenergic ligand and no signaling activity was detected in the presence of either ligand [25]. However, coexpression of both chimeras resulted in binding of both ligands and resultant signaling [25].

Perhaps one of the most well known lines of evidence for the dimerization of GPCRs comes from the $GABA_B$ receptor, a Class C GPCR. It was found that when the $GABA_B$ receptor 1 ($GABA_BR1$) gene was expressed alone, the resultant receptor was not functional because it was retained within the cell, unable to be expressed at the plasma membrane [26]. Upon isolation of the $GABA_B$ receptor 2 ($GABA_BR2$) gene, it was found that this resultant protein did not bind $GABA_B$ ligands. When the genes were coexpressed, a functional $GABA_B$ receptor was trafficked to the plasma membrane and had the functional properties predicted for the $GABA_B$ receptor [26,27]. It has been discovered that an arginine-serine-arginine-arginine C-terminus ER retention motif on the $GABA_BR1$ receptor is masked upon interaction with $GABA_BR2$ [26]. Dimerization of GPCRs, along with functional consequences of dimer formation, has since proven to be a popular area of further research.

Evidence supporting GPCR dimerization has been collected through analysis of co-immunoprecipitation (co-IP) and western blotting experiments [28,29], as well as yeast two-hybrid screens [27,30]. GPCR dimerization has also been examined in living cells by the use of fusion constructs and resonance energy transfer. These experiments utilize GPCRs fused with fluorescent or bioluminescent proteins and are completed using fluorescence resonance energy transfer (FRET) and bioluminescence resonance energy transfer (BRET), respectively [31].

Although dimerization studies have been criticized for using overexpression systems in *in vitro* settings, numerous *in vivo* studies have confirmed what has been documented using cell lines. For example, a transgenic mouse model was used to examine the murine lutenizing hormone receptor (LHR) to further probe the

physiological relevance of GPCR dimers or higher-order oligomers [32]. Using a LHR knockout mouse, it was shown that the coexpression of a binding-deficient and signaling-deficient LHR could restore normal LHR function by means of intermolecular functional complementation [32]. Not only did this study demonstrate that GPCRs function as more than just monomeric units, but it was also shown that the binding of a ligand to one member of the signaling complex has the potential to alter the conformation of another member in the same signaling complex [32].

It is interesting to note that as the knowledge of GPCR signaling complexes evolves, there is a possibility that these receptors act as higher-order oligomers. This is especially important because current research methods are generally unable to distinguish experimental outcomes that are the result of dimers or higher-order oligomers [33].

1.4 Novel Pharmacological Properties Resulting from Heterodimerization

It has become increasingly apparent that signal transduction cascades and ligand-binding interactions must be studied in the context of GPCR oligomerization. This is because, as evident in Figure 1.1, the pharmacological properties of each constituent receptor may be altered once they are associated with a larger receptor complex [34].

Expression patterns of individual GPCRs are especially interesting since it was found that dimerization could influence the plasma membrane expression of some GPCRs. For example, $GABA_B$ dimers [35], along with those of oxytocin [36], vasopressin [36], CXCR4 [37] and CCR5 [38] form in the ER soon after each constituent receptor is synthesized. Since plasma membrane expression of GPCRs is essential for proper receptor function, studying the means by which these receptors reach the plasma

membrane and how dimerization may affect trafficking pathways could lead to the identification of novel therapeutic targets.

Heterodimerization can also affect ligand binding to either receptor of the complex. This occurs by means of allosteric modulation, resulting in either transinhibition or transactivation. Transinhibition has been documented in several studies, as exemplified by a study of the μ-opioid receptor (μOPR) and A2A adenosine receptor ($A_{2A}R$) [39]. In this study, it was found that heterodimerization resulted in heightened signaling through the μOPR upon stimulation with morphine, a μOPR agonist [39]. However, simultaneous addition of both morphine and norepinephrine, an $A_{2A}R$ agonist, caused decreased μOPR signaling [39]. Similarly, signaling elicited by norepinephrine stimulation of $A_{2A}R$ in the presence of morphine was also hampered [39]. Alternatively, transactivation has been observed in a study of the $GABA_B$ receptors [40]. Whereas $GABA_BR1$ is capable of binding GABA, it is not able to activate Gαi-mediated signaling [40]. However, $GABA_BR2$ is not capable of GABA-binding, but is able to initiate Gαi-mediated signaling [40]. Therefore, upon ligand binding to $GABA_BR1$, $GABA_BR2$ adopts an active conformation [40].

Novel binding sites may also arise as a result of heterodimerization, as observed by studies of the OPRs. While the κOPR can heterodimerize with the δOPR, it does not form a heterodimer with the μOPR [41]. The κOPR/ δOPR heterodimer has been found to bind partially selective agonists of either the κOPR or δOPR with enhanced affinity [42]. Furthermore, simultaneous addition of an agonist for each receptor resulted in increased potency and simultaneous addition of an antagonist for each receptor resulted in

enhanced antagonism [42]. Interestingly, the κOPR/δOPR heterodimer has been established as the target for 6'-guanidinonaltrindole (6'GNTI), a κOPR agonist [43]. 6'GNTI is selective for this heterodimer as it exhibits low affinity in binding to the κOPR homodimer and does not even bind to the δOPR homodimer [43].

Heterodimerization introduces the concept of G-protein selectivity [44,45]. While each receptor of the heterodimer is associated with a particular G-protein, the resultant heterodimer can normally associate with a G-protein known to couple to each receptor itself. The heterodimer can also couple to a G-protein that has not been previously linked to either receptor in the complex. For instance, μOPR and δOPR both couple to $G_{\alpha i}$, but the resultant μOPR/δOPR heterodimer couples to $G_{\alpha z}$ in a ligand-independent fashion [45]. However, ligand binding to a heterodimer can also influence G-protein coupling, as is observed when the δOPR heterodimerizes with the sensory neuron-specific receptor-4 (SNSR4). When the δOPR/SNSR4 heterodimer was stimulated by the ligand for each receptor, G-protein coupling to each receptor was not affected [44]. Stimulation of SNSR4 resulted in activation of $G_{\alpha q}$ and stimulation of δOPR resulted in $G_{\alpha i/o}$ activation [44]. When agonists of each receptor were co-administered or the heterodimer was stimulated by a mixed agonist, $G_{\alpha q}$-mediated signaling was evident [44].

Internalization and desensitization may also be altered in response to heterodimerization. It has been shown that activation of one partner in the receptor complex is capable of inducing internalization of both receptors. This phenomenon has been documented with somatostatin receptor 1 (SSTR1)/somatostatin receptor 2 (SSTR2)[46], δOPR/β1-adrenergic receptor (β1-AR) [47], $A_{2A}R$ / dopamine 2 receptor (D_2R) [48], somatostatin receptor 2A ($SSTR_{2A}$)/μOPR [49] and α_{2A}-adrenergic receptor

(α_{2A}-AR)/β1-AR dimers [50]. Differences in recycling kinetics between the partners of a heterodimer are also apparent. For example, the vasopressin 1a receptor (V1aR) is recycled to the plasma membrane more rapidly than the vasopressin 2 receptor (V2R) as a result of β-arrestin rapidly dissociating from the V1aR, but not the V2R, in endosomes [51]. When present as a V1aR/V2R heterodimer, this complex exhibits recycles similarly to the V2R [51].

1.5 Trafficking of GPCRs

Whereas GPCR signal termination processes (desensitization and endocytosis) are well understood, processes responsible for the assembly of GPCR signaling complexes and their subsequent trafficking within a cell are less understood. The degree of cellular response to a particular stimulus depends on the plasma membrane expression of a given GPCR, which in turn depends on the balance between endocytic and exocytic events. Therefore, studying the assembly and subsequent anterograde trafficking of GPCRs to the plasma membrane is an essential area of research that has garnered relatively little attention until recent years.

1.5.1 Role of the Endoplasmic Reticulum (ER)

Following transcription, ribosomes are responsible for the translation of proteins [52]. The small ribosomal subunit is responsible for encoding mRNA sequences, whereas the large ribosomal subunit forms primary amino acid chains by the linking of individual amino acids [53]. Ribosomes are classified based on their cellular distribution. As a result, two different ribosomal classifications exist – free or membrane bound [54]. Free ribosomes are situated throughout the cytosol and are associated with the synthesis of proteins destined for intracellular use, such as cytosolic or nuclear proteins [52].

Membrane bound ribosomes are found lining a portion of the ER [54]. The presence of ribosomes along this portion of the ER is responsible for it being aptly titled the rough ER. More specifically, ribosomes associate with Sec61 translocation sites, pores of the ER that control transport of polypeptides across the membrane of the ER [52]. Upon interaction of a nascent polypeptide with Sec61, the newly synthesized protein is released into the lumen of the ER [52]. Proteins synthesized at the rough ER are destined to be membrane bound or secreted [52].

In the rough ER, newly synthesized GPCRs must be properly folded and assembled prior to being transported to the plasma membrane. For example, it has been proposed that the $GABA_BR1$, inwardly rectifying potassium cannel Kir 3 and G-protein βγ subunits assemble as a complex in the ER [55]. The β2-AR receptor [56], along with the $GABA_B$ [26] and CCR5 [57] receptors have been shown to assemble as oligomers in the ER. Not only do signaling complexes form in the ER, but their assembly is now believed to be independent of recruitment initiated by ligand binding [58-60].

Transport from the ER requires the recruitment of GPCRs into ER-derived COPII-coated vesicles [61]. The export of GPCRs from the ER is determined by export motifs of the C-termini of these transmembrane receptors. These motifs guide the interaction of GPCRs with Sar1 and the Sec23/24 complex of COPII-coated vesicles [61]. Interestingly, ER export has been shown to be a rate-limiting step in regards to transport of the δ-OPR receptor to the plasma membrane [62]. As a result, ER export can affect both the maturation and function of receptors.

Upon leaving the ER, newly synthesized proteins progress through an extensive endomembrane system. After the ER, proteins are directed to the ER-Golgi intermediate

complex (ERGIC) [63], Golgi apparatus and then the *trans*-Golgi network (TGN) [64]. Ultimately, the Golgi cisternae are responsible for numerous post-translational modifications that can influence both the structure and function of proteins directed towards the plasma membrane.

Once a protein reaches the *trans*-Golgi, it is either directed to further compartments of the endomembrane network for additional modifications or the plasma membrane [65]. When expressed at the plasma membrane, GPCRs have access to the extracellular space to allow ligand stimulation and subsequent internalization or desensitization. Accordingly, both anterograde and retrograde trafficking pathways influence GPCR expression at the cell surface and GPCR signal transduction cascades.

The anterograde pathway, as reviewed in Figure 1.2 and described above, assumes that nascent polypeptides are properly folded. If these proteins are not properly folded, they are retained within the ER and initiate the unfolded protein response (UPR). The UPR results in slowed translation of further proteins, with the exception of molecular chaperones that can aid in proper protein folding [66]. The UPR can also lead to degradation of misfolded proteins through the ER-associated degradation (ERAD) pathway [66]. ERAD occurs by means of recognition of the misfolded protein by molecular chaperones, translocation of the misfolded protein from the ER to the cytosol, ubiquitination and proteasomal degradation [66]. The quality control system of the ER ensures that proteins are not included in ER-derived transport vesicles until proteins are properly folded.

1.6 Importance of Molecular Chaperones

Proper protein biosynthesis and subsequent folding are highly regulated cellular processes. These processes contribute to quality control mechanisms and protein homeostasis, helping to prevent the misfolding of proteins. Misfolded proteins that are not corrected by ER quality control mechanisms form protein aggregates implicated in the progression of numerous pathological states, including several neurodegenerative diseases [67,68]. Correctly folded conformations are achieved through interactions with scaffold proteins [69], folding enzymes [70] and, what will be a focus of this thesis as part of an exploratory project, molecular chaperones (Figure 1.3) [71,72].

1.6.1 Lectin Chaperones

Lectin chaperones (calnexin and calreticulin) regulate calcium concentration in the ER [73]. Calcium homeostatsis in the ER dictates not only interactions of chaperones with target polypeptides, but also associations between chaperones [74,75]. Calnexin and calreticulin are able to control the ratio of free to bound calcium by binding free calcium ions [76,77]. Consequently, their interactions with polypeptides are calcium-dependent. Although it was originally thought that polypeptides had to be glycosylated prior to interacting with either calnexin or calreticulin, some studies suggest that non-glycosylated proteins may also bind to these chaperones [78-80]. Both calnexin and calreticulin have a single carbohydrate-binding domain and a proline-rich domain that is responsible for calcium binding [81-84]. The proline-rich domain is also known to recruit PDIA3 [71,83].

1.6.2 PDIA3

PDIA3, also known by a number of aliases including ERp57, is a member of the protein disulfide isomerase (PDI) family. This family regulates the formation of disulfide bonds required for proper protein maturation [85]. PDI chaperones act as electron acceptors in oxidation reactions or electron donors in reduction reactions, while also being capable of isomerizing disulfide bonds (essentially rearranging existing disulfide bonds) [85]. Members of this family are comprised of thioredoxin-like domains that help make up the active site motif [85]. The interaction with lectin chaperones positions PDIA3 so that it can carry out its enzymatic activites on either immature or misfolded glycoproteins [71].

1.6.3 Hsp70 and BiP

A member of the heat shock protein 70 (Hsp70) class, BiP is classified as heat shock 70 kDa protein 5 (HSPA5). Like other members of the Hsp70 family, BiP is composed of three domains – an ATPase domain at its N-terminus, a peptide-binding domain, and a C-terminus domain that helps regulate peptide binding [86,87]. When ATP is bound to its N-terminus, this allows for the binding and rapid release of peptides [86,87]. When ADP is bound to its N-terminus, peptides are tightly bound to the chaperone [86,87]. Members of the Hsp70 family are responsible for maintaining housekeeping functions within a cell, but are also required for proper folding and maturation of newly-synthesized proteins [88]. They are also responsible for the refolding of improperly folded or aggregrated proteins, along with controling activities of a wide variety of regulatory proteins [88]. Hsp70 is known to be present predominantly

in the nucleus and cytoplasm, with one isoform present in the mitochondria [89], whereas BiP is present in the ER [90].

Since BiP has been shown to bind directly to hydrophobic residues, it can bind to misfolded proteins that often have exposed hydrophobic regions. More specifically, BiP forms transient bonds with newly synthesized proteins, but forms stable bonds with misfolded proteins, preventing their export from the ER [71]. Not only is BiP responsible for the translocation of newly synthesized proteins across the ER, but BiP also regulates retrograde transport of misassembled proteins back into the ER, where they will be targeted to the proteasome for degradation [91].

1.6.4 GRP94

GRP94, also known as heat shock protein 90 kDa β member 1 (HSP90B1), is present in the ER. GRP94 is composed of three domains, including an N-terminus regulatory domain, substrate-binding domain, and a C-terminus dimerization domain [71]. GRP94 has been shown to bind substrates once they have been released from BiP [92]. Although the exact mechanisms regulating to the control of substrate-binding domain are unclear, it is thought that nucleotide binding to the N-terminus regulates the binding of substrates [71].

1.6.5 DRiP78

DRiP78 (also known as DnaJC14 – DnaJ (Hsp40) homolog, subfamily C, member 14) belongs to the Hsp40 chaperone family [93] and has been shown to influence the anterograde trafficking of a variety of GPCRs, including the dopamine 1 receptor (D_1R) [94], angiotensin II type 1 receptor (AT1R), and M2 muscarinic receptor [95]. In regards to the trafficking of specific receptors, DRiP78 interacts with a conserved

F*XXX*F*XXX*F motif present in many GPCRs [94,95].

1.6.6 Reliance of GPCRs on Molecular Chaperones

Along with studies relating to how DRiP78 is required for proper ER export and subsequent plasma membrane expression of GPCRs, further research has focused on how other molecular chaperones affect the trafficking and expression of GPCRs. For example, NinaA and RAN-binding protein 2 were both shown to participate in the maturation of rhodopsin in both *Drosophila* and vertebrates, respectively [96,97]. Additionally, both D_1R and D_2R expression rely on calnexin [98], while calreticulin has been shown to be involved in the maturation of the bradykinin β-2 receptor [99]. Nevertheless, additional investigation is required to further our understanding of how molecular chaperones interact with GPCRs. These studies may even lead to differences regarding the specificity of molecular chaperones for homo- or heterodimeric forms. For example, it was shown that PDIA3 is required for AT1R homodimerization, as well as β2-AR heterodimerization with AT1R [100]. However, PDIA3 was not involved in β2-AR homodimerization [100].

1.7 Importance of Rab GTPases

Molecular chaperones are not the only cellular components involved in directing newly synthesized proteins through the cell's complex endomembrane system. They share this role with the Rab protein family, part of the Ras superfamily of small GTPases. Although some members of the Rab protein family are ubiquitously expressed and others have tissue-specific expression profiles, all Rabs act to regulate intracellular trafficking pathways involved in vesicle formation from a donor compartment, movement along

cytoskeletal networks, and fusion to an acceptor compartment [101]. Therefore, both molecular chaperones and Rab GTPases are involved in anterograde transport pathways.

There have been at least 70 different members of the Rab protein family identified in humans [102]. Rabs are localized on the cytosolic side of intracellular membranes due to the post-tanslational modification of a C-terminus cysteine motif with hydrophobic geranylgeranyl groups [103]. A Rab escort protein (REP) ensures interaction of a recently synthesized Rab protein with geranylgeranyl transferase [103]. Subsequently, REPs ensure that each hydrophobic, geranylgeranylated Rab protein is directed to the appropriate intracellular membrane through the formation of a lipid anchor between the Rab and membrane [103].

To accomplish their regulatory roles, Rab GTPases cycle between two conformations – the inactive GDP-bound form and the active GTP-bound form [104]. The nucleotide exchange that accounts for the difference between the GDP- and GTP-bound forms is catalyzed by a GDP/GTP exchange factor (GEF) [104]. In contrast, conversion from the GTP- to GDP-bound form is accomplished by means of GTP hydrolysis, carried out by a GTPase-activating protein (GAP) [104].

Once in their GTP-bound form, Rab proteins associate with Rab effectors and direct vesicle traffic and fusion to appropriate organelles [105]. Upon reaching target membranes, GTP hydrolysis of Rab proteins occurs, followed by association with REPs that recycle Rab proteins back to their membrane of origin [105]. After activation by a GEF, Rab proteins are again able to carry out this cycle of direct transport, as reviewed in Figure 1.4 [105].

By studying the trafficking requirements of multiple GPCRs, the roles of specific Rab GTPase isoforms have been established. As shown in Figure 1.5, there are a variety of Rab GTPases that exist in the cell and are responsible for a myriad of discrete trafficking steps. This thesis work examined Rab GTPase isoforms that were previously reported to influence the trafficking of GPCRs to the plasma membrane. For example, Rab1 has been found to direct traffic from the ER to the Golgi [106,107]. Rab2 is responsible for directing vesicle traffic between the ER and *cis*-Golgi [108], whereas Rab6 functions within the Golgi [109]. Rab8 directs anterograde vesicle transport between the *trans*-Golgi and the plasma membrane [110], but Rab11 is known to regulate both endocytic and exocytic trafficking between the plasma membrane and Golgi complex [111]. Multiple studies have shown that the anterograde trafficking of numerous GPCRs can be altered by transfection with dominant negative (DN) forms of Rab GTPases in a varity of cell lines [106,112-114]. DN versions of Rab GTPases may either retain the Rab GTPase in its inactive GDP-bound state or hinder activation of the Rab GTPase by preventing guanosine nucleotide exchange.

By examining the contributions of molecular chaperones and Rab GTPases to the anterograde trafficking of GPCRs, it is evident that both of these protein families are important for proper plasma membrane expression of GPCRs. Therefore, this thesis will study Rab GTPase involvement in the anterograde transport of two chemokine GPCRs (CXCR4 and CCR2) linked to the progression of prostate cancer. Futhermore, roles of molecular chaperones will be explored as they relate to the anterograde transport of receptors, including the CCR5 chemokine receptor, required for HIV entry into host cells. By uncovering trafficking requirements for plasma membrane expression of chemokine

receptors involved in either prostate cancer or HIV progression, a greater understanding of the cellular mechanisms of these disease states can be gained. Through this understanding, it is possible that novel pharmacological targets could someday be identified for the treatment of these diseases that rely on the cell surface expression of chemokine receptors for their pathology.

1.8 Chemokines and Chemokine Receptors

Cytokines encompass a wide range of cell signaling molecules that are secreted by many cells throughout the body [115]. They are essential in determining cellular behavior and are also known as key regulators of intracellular communication [116,117]. Chemokines are low molecular weight secreted cytokines that were originally named for their immunomodulating properties [116-118]. Chemokines have been shown as essential mediators of chemotaxis during development and the trafficking of innate immune cells [116,117]. Immunological responses associated with chemokines include dendritic, B and T cell maturation, along with Type 1 and Type 2 helper T cell responses [117]. However, chemokines have evolved to control more than just immunological responses. They play integral roles in key physiological responses such as the regulation of gene transcription, wound healing, and angiogenesis [117]. Although chemokines and their receptors exert numerous protective and developmental functions, they are also involved in the pathogenesis of many disease states, as will be mentioned in the following sections.

While the system is comprised of considerable redundancy in terms of overlapping functions, the chemokine system is still able to regulate exceptionally selective processes such as the trafficking of particular cell types to targeted tissues [119]. Chemokines are

often divided into four subfamilies – CC, XC, CXC and CX_3C – corresponding to the positions of the first two cysteines in their highly conserved N-terminus tetracysteine motif [118]. In general, CC chemokines bind CC chemokine receptors (CCRs), XC chemokines bine XC chemokine receptors (XCRs), and CXC chemokines bind CXC receptors (CXCRs) [118], all of which belong to the GPCR superfamily.

1.9 Chemokine Receptor CXCR4

CXCR4 is one of the most commonly studied chemokine receptors. It is ubiquitously expressed throughout the body [120] and is activated by its only known endogenous ligand, stromal derived factor-1 (SDF-1, also referred to as CXCL12) [121-123].

SDF-1 was first described in terms of being a potent factor that stimulated the growth of early B-cell progenitors in the bone marrow [124]. Additional research found that SDF-1 acted as a chemoattractant for both lymphocytes and monocytes [125]. Two SDF-1 isoforms have been identified – SDF-1α and SDF-1β [126]. Both isoforms are encoded by one gene, but differential gene splicing results in SDF-1α being composed of 89 amino acids and SDF-1β consisting of 93 amino acids [126]. The SDF-1α isoform is most abundantly expressed, but both isoforms can bind CXCR4 with comparable affinity [127].

Homozygous mutations of either CXCR4 or SDF-1α are embryonic lethal [128]. Consequently, the CXCR4/SDF-1α signaling axis is essential for the development and maintenance of tissues. One of the most important tissues associated with the CXCR4/SDF-1α signaling axis is the bone marrow. Hematopoietic stem cells (HSCs) become localized in the bone marrow and the HSC niche is maintained due to enhanced

SDF-1α expression [125]. The CXCR4/SDF-1α signaling axis is also involved in B cell development [129] and embryogenesis [130]. This signaling axis is also associated with tissue repair and inflammatory processes [131], as well as a variety of pathological states that include liver [132] and coronary artery disease [133].

This signaling axis supports the "seed and soil" hypothesis introduced by Paget in the 1800s [134]. Paget hypothesized that the spread of malignant cells (seeds) to different tissues (soil) does not occur randomly [134]. It was proposed that this process involved directed migration [134]. Today, researchers understand that it is associated with the migration of certain cancer cells to particular tissues, as dictated by interactions of these cells with pro-migratory factors associated with tissues [134]. Concequently, if cancer cells overexpress CXCR4, migration along a SDF-1α gradient is likely to occur such that these cells may become established in tissues that express SDF-1α [134]. The CXCR4/SDF-1α axis has also been associated with angiogenesis, as well as the proliferation and survival of malignant cells [120].

CXCR4 overexpression has been documented in at least 20 different types of cancers [135]. The interests of this thesis were related to the heightened CXCR4 expression that has been documented in both localized and metastatic prostate cancer [136]. Not only is this heightened expression associated with relatively low survival rates, [137], but it is also likely to be accompanied by more aggressive phenotypes [138]. Signaling through CXCR4/SDF-1α has been reported to have several negative effects in relation to studies investigating this signaling axis in prostate cancer cells. For example, it was found that CXCR4/SDF-1α signaling amplifies perineural invasiveness of prostate

cancer cells [139], while also being able to promote the adhesion of prostate cancer cells to bone marrow endothelial cells [140].

Whereas SDF-1α is highly expressed in tissues that are common destinations for prostate cancer metastases (for example, the bones, kidneys, and liver), it has essentially negligible expression at uncommon sites of prostate cancer metastases (for example, the tongue, lungs, and eyes) [141]. *In vivo* experiments have found that administration of a CXCR4 neutralizing antibody or blocking peptide significantly attenuated intraosseous tumor burden following intratibial tumor cell injections [141]. Bone metastases were also reduced after intracardiac injection of prostate cancer cells [141]. Furthermore, administration of the CXCR4 antagonist, AMD3100, was found to prevent prostate cancer cell invasiveness [139].

1.10 Chemokine Receptor CCR2

The CCR2 chemokine receptor is expressed on many cells of the immune system [142]. It is the only known receptor for monocyte chemotactic protein-1 (MCP-1). Together with its ligand, CCR2 is involved in the directed migration of monocyte-derived macrophages to areas of inflammation, as well as other immune responses [143]. Much like other chemokine receptors, CCR2 has been linked to a variety of disease states, including atherosclerosis [144], and dementia-like pathology [145,146].

CCR2 is implicated in a variety of mechanisms favoring cancer growth, such as the recruitment of myeloid suppressor cells to sites of tumor growth [147] and the promotion of tumor cell extravasation [148]. Furthermore, CCR2 expression on several different types of malignancies, including multiple myeloma [149], breast cancer [150] and prostate cancer [151], has been shown to be involved in disease progression. In

relation to signaling through CCR2, MCP-1 stimulation of this receptor results in the directed migration of prostate cancer cells, as well as increased cell division and invasion [152]. CCR2/MCP-1 signaling has also been associated with angiogenesis and [153] prostate cancer-induced osteoclastogenesis [151]. A murine xenograft model with a prostate cancer cell line was used to examine tumor burden and macrophage infiltration after administration of MCP-1 neutrtralizing antibodies [154]. It was found that both of these parameters were decreased with MCP-1 neutralizing antibodies, indicating a role of CCR2 in the progression of prostate cancer [154].

1.11 Dimerization of Chemokine Receptors CXCR4 and CCR2

As further examples of GPCR dimerization, CXCR4 and CCR2 are able to form both homodimers [155-159] and heterodimers [158,160]. An investigation of the CXCR4/CCR2 heterodimer discovered that AMD3100 inhibited the interaction of MCP-1 with CCR2, whereas the CCR2 antagonist TAK779 inhibited SDF-1α interaction with CXCR4 [160]. This study of the CXCR4/CCR2 heterodimer was conducted *in vitro* and cross-inhibition of each constituent receptor resulted in alterations in functional responses, as measured by chemotaxis assays and calcium flux [160]. Physiological consequences of the CXCR4/CCR2 heterodimer have yet to be determined *in vivo* [160].

1.12 Chemokine Receptor CCR5

The CCR5 chemokine receptor is expressed on a variety of immune cells [161] and can bind to a myriad of chemokines [162]. Endogenous ligands for this receptor include RANTES (regulated on activation, normal T cell expressed and secreted) [163], CCL3L1 (chemokine C-C motif ligand 3-like 1) [164], macrophage inflammatory protein

(MIP) 1α, and MIP 1β [165]. Although the exact contribution of CCR5 to normal immune function has not been clearly established, it is postulated that CCR5 is involved in inflammatory responses associated with infection [166].

It is interesting to note that a variety of CCR5 genetic variants have been identified in the human population [167]. Some of these genetic variants are associated with mutations that alter the coding sequence of the protein and result in decreased CCR5 expression at the plasma membrane [167]. The CCR5Δ32 mutation has been documented in individuals of North African, West Asian or European heritage [168]. This mutation is associated with a 32 base pair deletion, resulting in accumulation of the truncated receptor in the ER [169]. More specifically, this deletion removes a membrane-proximal basic domain of the receptor's C-terminus that is required for proper transport to the plasma membrane [169]. With the exception of one form of liver disease [170], individuals with the CCR5Δ32 mutation do not exhibit any pathologies that could be attributed to this mutation. These observations are likely due to the fact that the redundancy inherent in the chemokine system can compensate for a lack of CCR5 [171]. Because the CCR5Δ32 mutation is rarely associated with negative health effects, it is possible that therapeutic strategies used to block CCR5 receptor function or plasma membrane expression may also present with limited side effect profiles. Nevertheless, CCR5 has been implicated in a variety of disease states, such as asthma [172], cancer [173], and HIV [174].

1.12.1 CCR5 Involvement in Human Immunodeficiency Virus (HIV) Infection

The human immunodeficiency virus (HIV) envelope houses two different glycoproteins. Envelope glycoprotein GP120 (gp120) is present on the envelope's

exterior and initiates HIV interaction with glycoproteins on host immune cells [175]. Forming a non-covalent bond with gp120, envelope glycoprotein GP41 (gp41) is embedded in the viral envelope. The exterior portion of gp41 is able to interact with gp120 and its interior portion is believed to interact with the matrix underlying the viral envelope [175]. Glycoprotein spikes present on the viral envelope are formed by trimers of gp120-gp41 heterodimers [175].

To gain entry into host cells, gp120 binds to cluster of differentiation 4 (CD4), a glycoprotein present on the surface of immune cells. Binding of gp120 to CD4 initiates conformational changes in each protein that allow the CD4-gp120 complex to interact with CXCR4 or CCR5 [176]. Upon binding to either co-receptor, further conformational changes lead to the insertion of an N-terminus fusion peptide belonging to the gp41 subunit into the membrane of the host cell [176]. The cell and viral membranes are then brought in sufficient proximity for fusion [176]. Therefore, CD4 acts as the primary receptor for HIV infection, but CXCR4 and CCR5 act as co-receptors for viral entry, making these chemokine receptors interesting targets for potential therapies.

It has been estimated that upwards of 90% of HIV infections have originated from HIV strains that employ CCR5 as a co-receptor (R5 HIV strains) [174]. One of the main reasons for this is due to the heightened expression of CCR5 and relatively poor expression of CXCR4 along areas of the genital tract where the virus is likely to come into contact with mucosal surfaces of a potential host [177]. Coupled with the fact that the CCR5Δ32 mutation confers resistance to HIV infection [178], this observation makes CCR5 an especially interesting receptor to study in relation to HIV pathology. Furthermore, CD4 can interact with CCR5 at the plasma membrane in living cells [179].

This interaction initiates in the ER and enhances expression of CCR5 at the cell surface [179]. However, interaction of CD4 with CXCR4 was not found to influence the cellular distribution of CXCR4 [179].

1.13 The Projects

This thesis contains two projects. The first project investigates Rab GTPases required for the anterograde trafficking of two receptors known to be involved in the progression of prostate cancer progression and was published by Gillies et al. (2013) [180]. The second project relates to exploratory work on the involvement of molecular chaperones in the maturation of CD4 and CCR5 receptor complexes.

1.14 Requirements for CXCR4 and CCR2 Anterograde Trafficking in a Prostate Cancer Cell Line

Although desensitization and endocytosis are fairly well understood in terms of how they contribute to the attenuation of GPCR signaling, there is still much to uncover about how GPCR signaling complexes are assembled and trafficked to the plasma membrane. Because cell surface expression of CCR2 and CXCR4 has been suggested to promote prostate cancer progression, it is important to study how processes governing their assembly and trafficking dictate their cell surface expression, including that of their homo- and heterodimers. Two GPCRs – CXCR4 and CCR2 – have been examined in relation to intracellular trafficking events required for transport to the plasma membrane and the effects of receptor trafficking blockade on their signal transduction. It is possible that novel targets capable of preventing CXCR4 or CCR2 cell surface expression could be identified through the characterization of intracellular trafficking requirements. Rab GTPases are capable of controling receptor transport from sites of maturation to the plasma membrane [106,112]. Therefore, this thesis used the PC3 human prostate cancer

cell line to study how different Rab GTPases are involved in the anterograde trafficking of CXCR4 and CCR2 receptors and the CXCR4/CCR2 heterodimer [180].

A large number of signaling cascades are initiated through the activation of these chemokine GPCRs [181], but this thesis measured activation of focal adhesion kinase (FAK) to determine a functional consequence of affecting the plasma mebrane expression of these receptors and dimers with different DN Rab GTPase isoforms. FAK was chosen because it is a a non-receptor tyrosine kinase intimately associated with numerous processes that favor cancer progression, such as cell survival and migration [182].

1.14.1 Hypotheses

This study can be divided into two hypotheses:

1) The anterograde trafficking of CXCR4 and CCR2 homo- and heterodimers will each require a different subset of Rab GTPases;
2) DN forms of Rab GTPases will hinder cell surface expression of CXCR4 and CCR2 homo- and heterodimers, resulting in functional consequences as measured by FAK activation.

1.15 Exploratory Work Relating to Molecular Chaperones Guiding the Maturation of the CCR5 and CD4 Receptors

Currently, many anti-HIV therapies try to antagonize ligand binding sites of the HIV co-receptors [183]. However, because these receptors are involved in such a wide variety of necessary physiological functions, antagonism of these receptors can lead to debilitating side effects. By identifying proteins that may act to restrict the expression of HIV co-receptors at the plasma membrane, novel therapeutic targets may be discovered that avoid problems associated with ligand therapies. The class of proteins that this

project focused on is that of the molecular chaperones. Because the early steps associated with the assembly of signaling complexes are relatively not well characterized, this represents an exciting area of research. If it is possible to block the assembly of the CCR5/CD4 heterodimer, without interfering with the maturation of each individual receptor, only signal transduction cascades initiated by the heterodimer and not each individual receptor would be affected, leaving individual receptors to function normally.

Retaining CCR5 within the cell and preventing its expression at the plasma membrane has been proposed as a way in which heterozygous individuals for the CCR5 mutation (those with the CCR5/ CCR5Δ32 genotype) are more protected from HIV infection, when compared to individuals who do not harbor the CCR5Δ32 mutation [184]. This is because the mutated form of the receptor heterodimerizes with WT CCR5 during early stages of receptor maturation and retains WT CCR5 in the ER [184]. By identifying chaperones required for specific receptor-receptor interactions, novel therapeutic targets could be proposed that prevent only particular assembly and trafficking pathways. The ability of the sodium-hydrogen antiporter 3 regulator 1 (NHERF1) scaffold protein to regulate CCR5 homodimer internalization, but not that of CXCR4/CXCR4 or CXCR4/CCR5 has been documented [185]. Therefore, distinct cellular proteins may interact differentially with receptors depending on the state of receptor dimerization. Since individuals with the CCR5Δ32 mutation (homo- or heterozygotes) rarely have health problems associated with diminished CCR5 plasma membrane expression [170], regulating this receptor's expression at the plasma membrane is a valid avenue to study ways in which the progression of HIV pathology can be circumvented.

The benefits of studying interactions of molecular chaperones with receptor dimers are two-fold. Firstly, signal transduction cascades initiated by the heterodimer can be better understood, with the possibility of targeting only the signaling of the heterodimer with pharmacological interventions. Secondly, plasma membrane expression of CCR5 homo- and heterodimers can be studied with hopes of determining specific interaction partners that may serve as novel therapeutic targets.

1.15.1 Hypothesis

CD4 and CCR5 have been shown to assemble early in maturation, but little is known about the molecular chaperones that promote their assembly. Therefore, this study had the following hypothesis:

1) Molecular chaperones will differentially affect the maturation and plasma membrane expression of the CCR5/CD4 heterodimer, when compared to their effects on each constituent receptor.

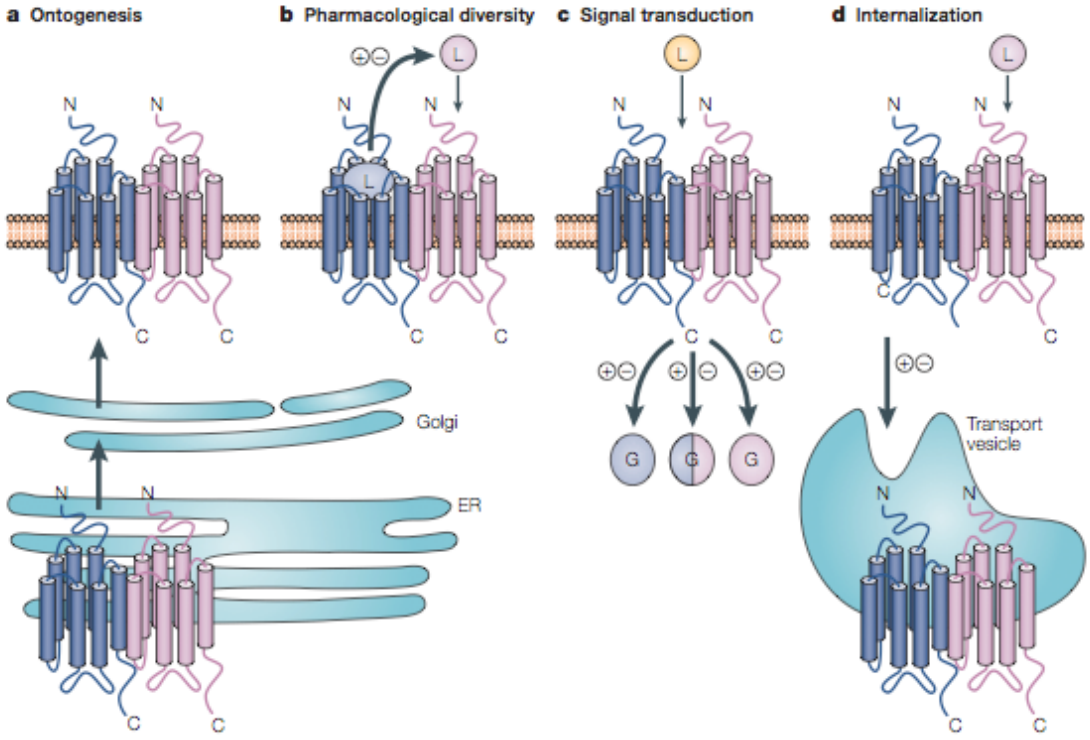


Figure 1.1 Pharmacological consequences of GPCR heterodimer formation. Heterodimerization can either positively or negatively influence (A) maturation and development, (B) binding of a ligand, (C) signal transduction cascades and (D) internalization of GPCRs present as heterodimers. G: G protein; L: ligand (Ellis et al. 2004; Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Drug Discovery] (Ellis et al. 3:577-626), copyright (2004)).

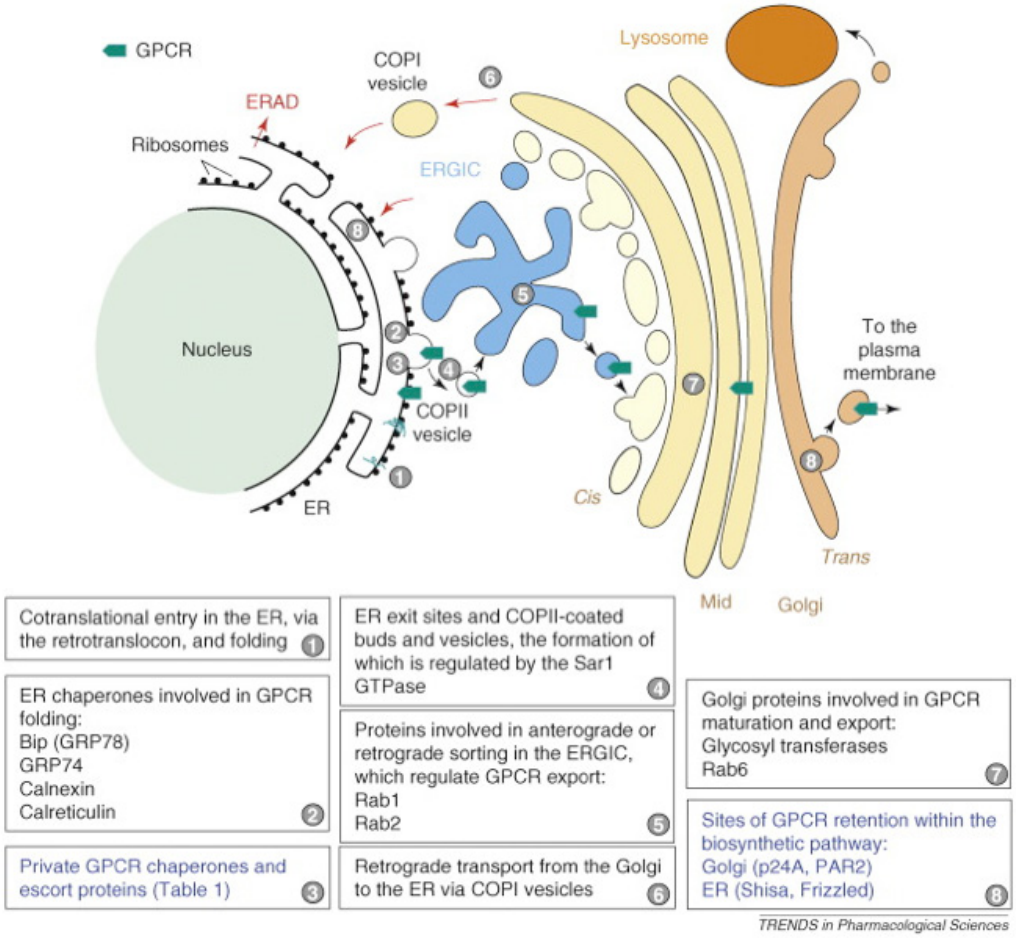


Figure 1.2 Anterograde trafficking pathway. A variety of sequential steps lead to the expression of GPCRs at the plasma membrane. Steps that are unique to GPCRs are written in blue, while the numbered boxes represent transport events associated with proteins in general. COPI: coat protein I; COPII: coat protein II; ER: endoplasmic reticulum; ERAD: ER-associated degradation pathway; ERIG: ER-Golgi intermediate compartment (Achour et al. 2008; Reprinted by permission from Macmillan Publishers Ltd: [Trends in Pharmacological Sciences] (Achour et al. 29:528-535), copyright (2008)).

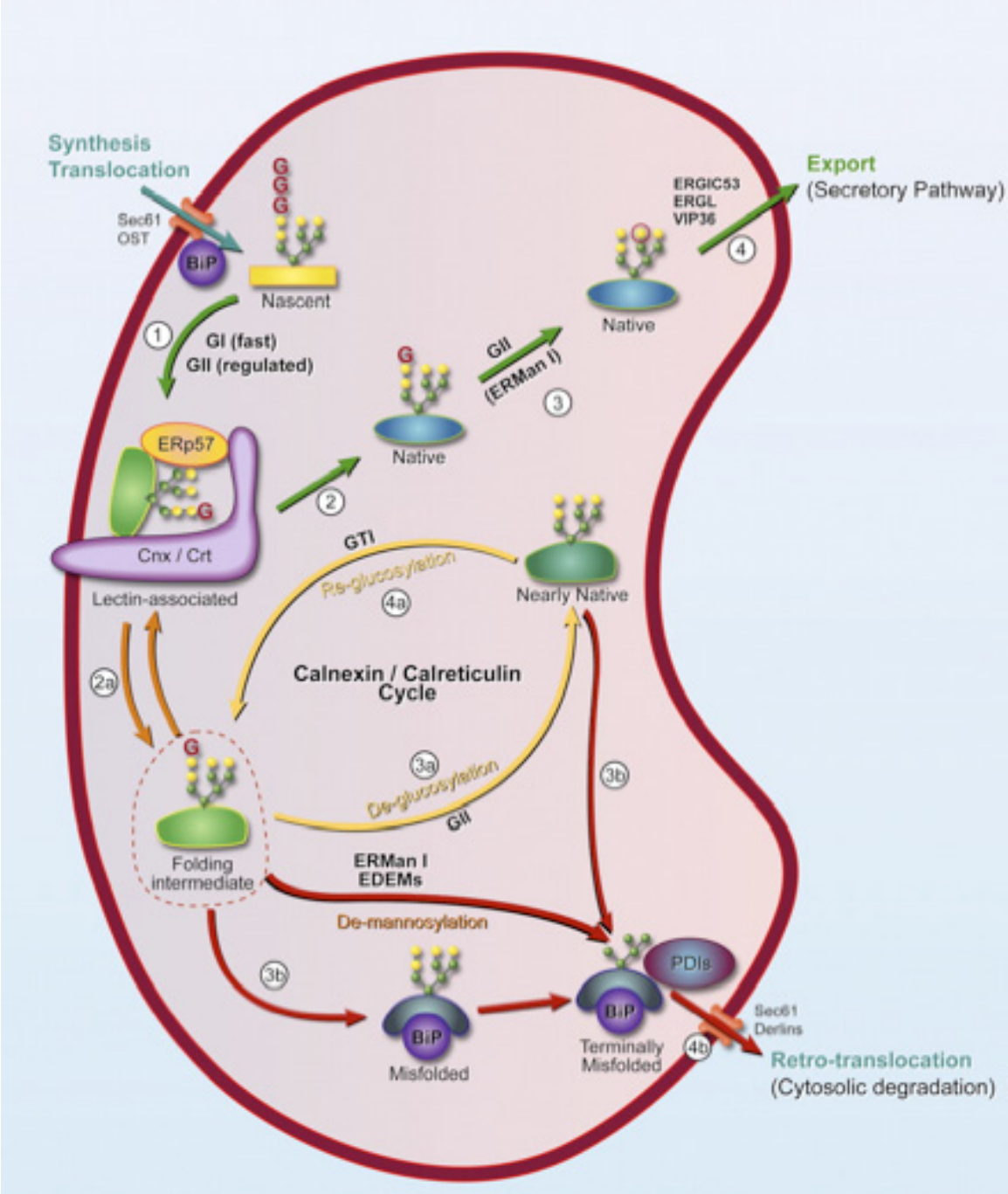


Figure 1.3 Overview of chaperones involved in the maturation of GPCRs. A system of molecular chaperones exists in the cell to guide the folding and maturation of newly synthesized polypeptides (Hebert et al. (2007) [71], permission not required).

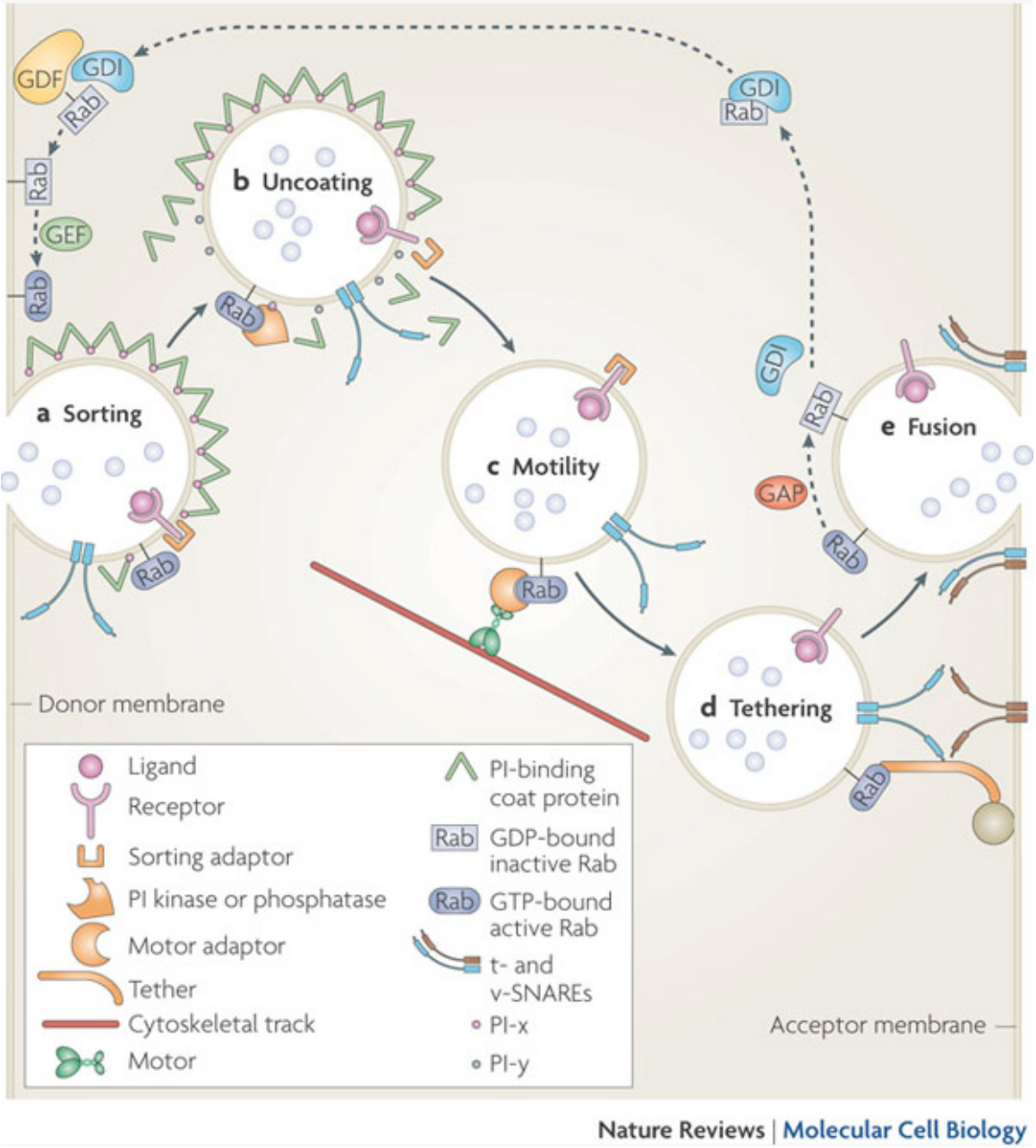


Figure 1.4 Membrane trafficking, as controlled by Rab GTPases and associated effectors. A number of discrete steps are involved with the membrane-targeting action of Rab GTPases and their effectors. As Rab GTPases cycle between active GTP-bound conformations and inactive GDP-bound conformations, proteins contained within transport vesicles are carried along the extensive cytoskeletal network of a cell to reach target membranes. More specifically, (A) GTP-bound Rab GTPases initiate sorting of proteins into a vesicle, (B) transport vesicles may be modified, (C) the vesicles are transported along the cytoskeletal network, (D) vesicles become tethered to target membranes, and (E) fuse with target membranes (Stenmark et al. 2009; Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Stenmark et al. 10:513-525), copyright (2009)).

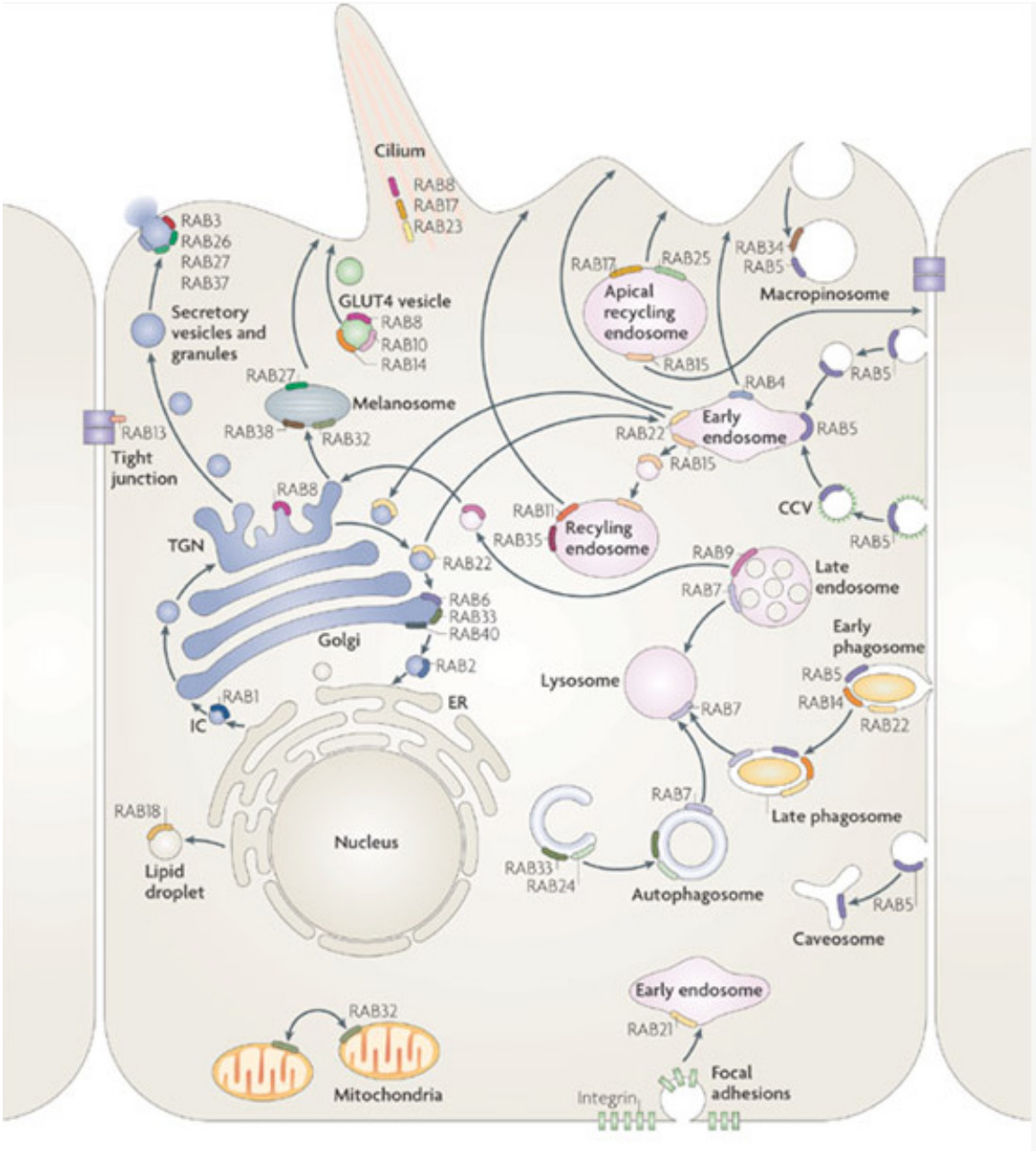


Figure 1.5 Rab GTPases in eukaryotic cells. Many Rab GTPases are present in the cell and regulate numerous trafficking steps. In this thesis, Rab GTPase isoforms that have been shown to influence the plasma membrane localization of GPCRs were studied. These included Rab1, Rab2, Rab6, Rab8 and Rab11. (Stenmark et al. 2009; Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Molecular Cell Biology] (Stenmark et al. 10:513-525), copyright (2009)).

CHAPTER 2 MATERIALS AND METHODS

2.1 Reagents

The PC3 human prostatic small cell carcinoma cell line (CRL-1435) and HEK293A human embryonic kidney cell line (CRL-1573) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Fetal bovine serum (FBS), penicillin-streptomycin (PS) and Lipofectamine 2000 were purchased from Invitrogen (Etobicoke, ON, Canada). Dulbecco's Modified Eagle's Medium (DMEM) High Glucose, monoclonal anti-FLAG antibody, Protein A-Sepharose, ready to use Bradford Reagent and all chemicals were from Sigma-Aldrich (Oakville, ON, Canada). Bovine serum albumin (BSA) was from BioBasic Incorporated (Markham, ON, Canada). Laemmli sample buffer was from BioRad (Mississauga, ON, Canada) and BioTrace^{TM} nitrocellulose transfer membrane was from Pall Corporation (Saint Laurent, QC, Canada). CL-XPosure film was from Fisher Scientific (Whitby, ON, Canada). Supersignal West Femto Maximum Sensitivity Substrate, EZ-link Sulfo-NHS-LC-Biotin and streptavidin agarose resin were from Thermo Scientific Pierce Protein Research Products (Rockford, IL, USA). SDF-1α, MCP-1 and monoclonal β-actin were from Abcam (Toronto, ON, Canada). Polyclonal anti-GFP, monoclonal anti-c-myc, monoclonal anti-CCR2, polyclonal anti-fusin (C-20; anti-CXCR4), monoclonal anti-pFAK and horseradish peroxidase (HRP)-conjugated secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal anti-FAK was from Biovision (Milpitas, CA, USA). Polyclonal anti-Hsp70, polyclonal anti-calreticulin, polyclonal anti-calnexin, polyclonal anti-PDIA3, polyclonal anti-BiP and polyclonal anti-GRP94, coelenterazine H and coelenterazine 400a were from Cedarlane Labs (Hornby,

ON, Canada). Polyclonal anti-DRiP78 was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Calreticulin and PDIA3 small interfering RNAs (siRNAs) were from Applied BioSystems (Carlsbad, California, USA). Hsp70 siRNA and DRiP78 small hairpin RNA (shRNA), as well as control siRNA-A were from Santa Cruz Biotechnology (Santa, Cruz, CA, USA). Polyethylenimine (PEI) was from Polysciences Incorporated (Warrington, PA, USA). 96-well microplates (white Optiplates) were from Perkin-Elmer (Waltham, MA, USA). Cell culture plastics were from VWR International (Mississauga, ON, Canada).

2.2 Cell Culture

Both PC3 and HEK293A cell lines were maintained in DMEM, supplemented with 10% heat inactivated FBS and 2% PS in a 5% CO_2 atmosphere at 37°C. Cells were passed with 10 cm cell culture plates when they were 80-90% confluent. Experiments were completed with cells that were of passage 35 or less. Cell lysis experiments were carried out in 6-well plates and co-IP experiments were conducted using 10 cm dishes when cell layers were 70-80% confluent.

2.3 Constructs

CXCR4 and CCR2 receptors were obtained from the Missouri University of Science and Technology cDNA Resource Center and transferred into a pcDNA3.1 vector containing the N-terminus (Venus1) or C-terminus (Venus2) portion of the Venus yellow fluorescent protein as previously detailed [185]. CCR5 and CD4 receptors were purchased from the Missouri University of Science and Technology cDNA Resource Center. Each receptor construct was transferred into a pcDNA3.1 vector containing the N-terminal (Venus1) or C-terminal (Venus2) portion of the Venus yellow fluorescent

protein as previously described [185]. Chaperone constructs (BiP WT, BiP T37G and calnexin, each of which is HA-tagged, along with the FLAG-tagged GRP94) were from Dr. William Green (University of Chicago). The $pGFP_2$-N_2-CD4 construct was from Dr. Jana Stankova (Université de Sherbrooke). Gγβ1-Rluc, Gγ2 and DRiP78 FLAG-tagged contructs were from Dr. Terence Hébert (McGill University). Wild type (WT) and DN Rab GTPase constructs were generated as was previously described [113]. Rab GTPase isoforms used were MYC-tagged Rab1 WT or Rab1 S25N, Rab2 WT or Rab2 S20N, Rab6 WT or Rab6 T27N, Rab11 WT or Rab11 S25N and FLAG-tagged Rab8 WT or Rab8 T22N. Each WT and DN Rab GTPase was expressed in PC3 cells such that expression levels could be confirmed by western blot analysis of cell lysates.

2.4 Transfections

PC3 cells were plated in 6-well plates or 10 cm dishes and transfected with cDNA and Lipofectamine 2000 according to manufacturer's instructions. Experiments were completed 48 h post-transfection, after 24 h of serum deprivation.

HEK293A cells were plated in 6-well plates or 10 cm dishes and transfected with cDNA, siRNA or shRNA using PEI. cDNA or inhibitory RNA and PEI were mixed in a ratio of 1:3 (DNA:PEI), along with 100 μl of serum-free DMEM for each well or 500 μl of serum-free DMEM for each 10 cm dish. Cells were cultured for 48 h post-transfection, at which time cell lysis or co-immunoprecipitation experiments were carried out.

2.5 Bimolecular Fluorescence Complementation (BiFC)

Bimolecular fluorescence complementation (BiFC) takes advantage of the formation of a fluorescent molecular complex and allows protein-protein interactions to be visualized in living cells [31]. This thesis used the Venus variant of yellow fluorescent protein and either an N-terminus (Venus1 (V1), the first 157 amino acids of Venus) or C-terminus (Venus2 (V2), amino acids 158 to 238 of Venus) fragment was coupled to a particular receptor [180]. As evident in Figure 2.1A, each separate Venus fragment does not emit fluorescence and is unable to bind to an anti-GFP antibody. When the receptors coupled to each Venus fragment dimerize, V1 and V2 form a functional Venus. This functional Venus is able to fluorescence and bind to anti-GFP antibody (Figure 2.1B).

For BiFC experiments, cells were co-transfected with either GFP-tagged or Venus1- and Venus2-tagged receptors (total cDNA transfected into each well was kept constant by the addition of a pcDNA vector as needed). 48 h post-transfection, cells were harvested with 100 μl phosphate buffered saline (PBS) and distributed into 96-well microplates. Fluorescence was measured using a Perkin Elmer Wallac EnVision 2104 Multilabel plate reader.

Fluorescence of cells transfected with empty pcDNA was subtracted from each sample's fluorescence reading. All fluorescence values were ultimately corrected for cell counts and normalized to controls transfected with both pcDNA and the receptor. Cell counts were completed by diluting 10 μl of cell suspension, then loading 10 μl of this diluted suspension onto a hemocytometer. Three quadrants were counted and the average number of cells per quadrant was calculated to determine the number of cells per ml (10^4

multiplied by the average cells/quadrant, corrected by the dilution factor).

2.5.1 Fluorescence Microscopy for Visualization of BiFC

To visualize BiFC signals, an Olympus IX81 microscope with a Photometrics coolSNAP HQ2 camera and excite series 120Q light source were used. YFP (Venus1/2) was excited at 488 nm and images were obtained at a fluorescence emission of 525 nm.

2.6 Biotin-Streptavidin Cell Surface Assay

Cells were transfected and harvested after 48 h. As illustrated in Figure 2.2, cells were washed with PBS before being incubated with 0.9 mM EZ-link Sulfo-NHS-LC-Biotin for 30 min. Samples were washed with 100 mM glycine-PBS and lysed with radioimmune precipitation assay buffer (RIPA) buffer (50 mM Tris-HCl, ph 7.5, 10 mM $MgCl_2$, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, and complete EDTA-free protease inhibitors). Supernatants were incubated with streptavidin agarose resin overnight. Samples were then washed three times with RIPA buffer prior to incubation with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer with 2.8 M DTT for 1 h at room temperature in order to elute bound proteins. Immunoblots were probed with appropriate primary antibodies, as well as corresponding horseradish-peroxidase-conjugated secondary antibodies.

2.7 Cell Lysis and Co-Immunoprecipitation (Co-IP)

Cells were harvested 48 h post-transfection, washed with PBS, and lysed with RIPA. Samples were pre-cleared with protein A-Sepharose with BSA and nutated for 30 minutes at 4°C. Centrifugation at 13000 RPM for 15 minutes at 4°C was completed to clarify the lysates. Samples were then incubated with a corresponding primary antibody for 30 minutes at 4°C. The protein-antibody complex was then precipitated with protein-

A sepharose beads that were left overnight on a nutator at 4°C to precipitate proteins of interest. Following overnight incubation, samples were washed with RIPA and proteins were eluted with 710 mM β-mercaptoethanol-Laemmli sample buffer. For cell lysis analysis, 30 μl of the supernatant was added to 30 μl of Laemmli sample buffer containing 1.5 μl of β-mercaptoethanol. Samples were subjected to SDS-PAGE and western blots were completed with applicable antibodies. The co-IP protocol is outlined in Figure 2.3.

2.8 Bradford Assay

BSA was used to produce protein standard curves with concentrations ranging from 0.01 to 10 μg/ml. Protein samples (1 μl of each) were loaded into wells of a 96-well plate with 119 μl of water and 120 μl of Bradford sample buffer. Samples were incubated at room temperature for 5 min before absorbance was read on a Perkin Elmer Wallac EnVision 2104 Multilabel plate reader at 595 nm.

2.9 Western Analysis

Cells were lysed and lysates were clarified as described in Section 2.7. Lysates were nutated with 710 mM β-mercaptoethanol-Laemmli sample buffer at room temperature before being placed in a dry bath at 65˚C for 5 min. Samples were processed with SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk powder in Tris buffered saline (TBS). Immunoblots were probed overnight with primary antibodies at 4°C in 5% TBS milk. The following day, membranes were washed with 0.1% Tween-20 TBS (TBST) and incubated with HRP-conjugated secondary antibodies for 1 h in 5% TBS milk. After membranes were washed again in

0.1% TBST, immunoblots were visualized with Supersignal West Femto Maximum Sensitivity Substrate. ImageJ 4.3 software (NIH) was used to determine immunoblot band densities.

2.10 Bioluminescence Resonance Energy Transfer (BRET)

HEK293A cells were co-transfected with the indicated cDNAs to achieve a 1:1 (donor:acceptor) ratio between Renilla luciferase (RLuc)-fusion (donor) proteins and GFP- or V1/V 2-fusion (acceptor) proteins. 48 h post-transfection, cells were harvested with 100 μl PBS containing 0.1% glucose and 90 μl of each sample was distributed into 96-well microplates. Signals were read using a FLUOstar Omega BMG Labtech plate reader with 460 nm (RLuc) and 520 nm (GFP or Venus1/Venus2) band pass filters. Coelenterazine H was used as a substrate for BRET1 and coelenterazine 400a as a substrate for BRET2, both at a final concentration of 5 μM. The BRET ratio was quantified by dividing emission values at 520 nm by those at 460 nm. A schematic representation of BRET1 and BRET2 is shown in Figure 2.4.

2.11 Statistical Analysis

All statistical comparisons were done using the unpaired two-tailed student's t test. Measurements are presented as mean ± SEM. Levels of significance and subsequent p values are 0.05 (*), 0.01 (**) and 0.001 (***).

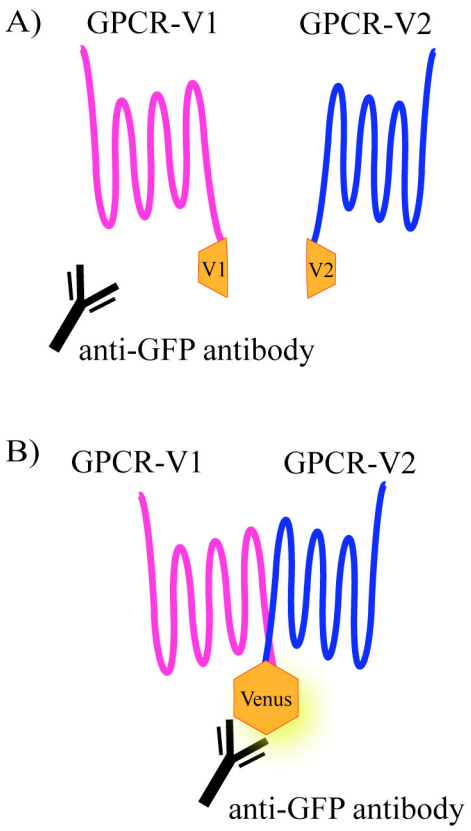


Figure 2.1 Schematic of the basis of BiFC. Each GPCR is tagged with an N-terminus or C-terminus fragment of the YFP variant Venus, referred to as V1 or V2 respectively. (A) V1 and V2 are unable to fluoresce individually and also do not bind an anti-GFP antibody. (B) When in close enough proximity to one another due to dimerization of their fused receptors, the Venus fragments covalently reconstitute to from a functional fluorescent protein that is able to bind an anti-GFP antibody.

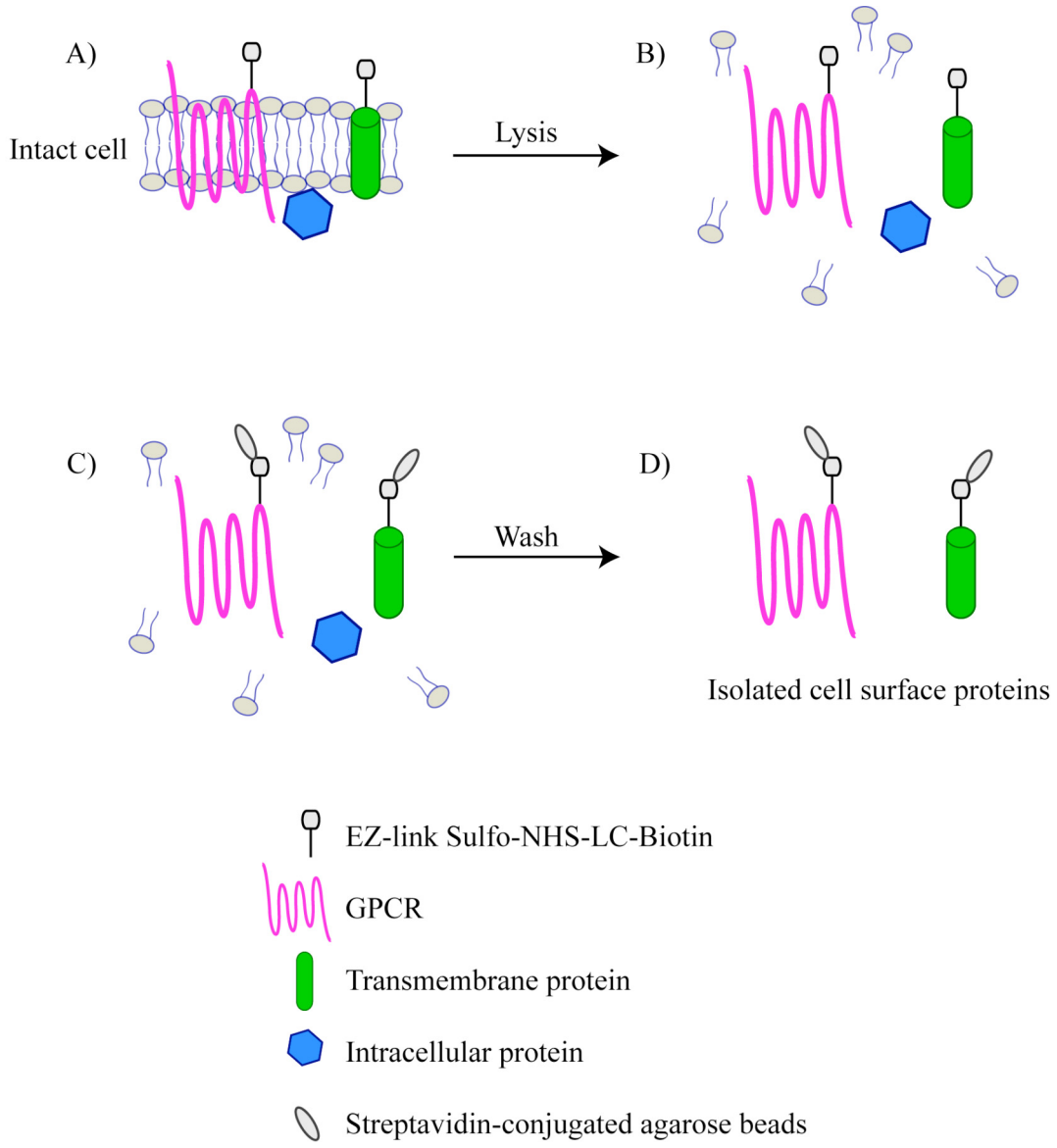


Figure 2.2 Schematic of the biotin-streptavidin cell surface assay. (A) Cells are treated with EZ-link Sulfo-NHS-LC-Biotin that reacts with primary amines of cell surface proteins. Because EZ-link Sulfo-NHS-LC-Biotin is not membrane permeable, it does not interact with intracellular proteins. (B) After biotin-labeling, cells are lysed and (C) mixed with streptavidin-conjugated agarose beads that bind biotinylated proteins. (D) Agarose beads are then washed to remove unbiotinylated proteins and the resulting biotin-labeled cell surface proteins are studied by western blot analysis.

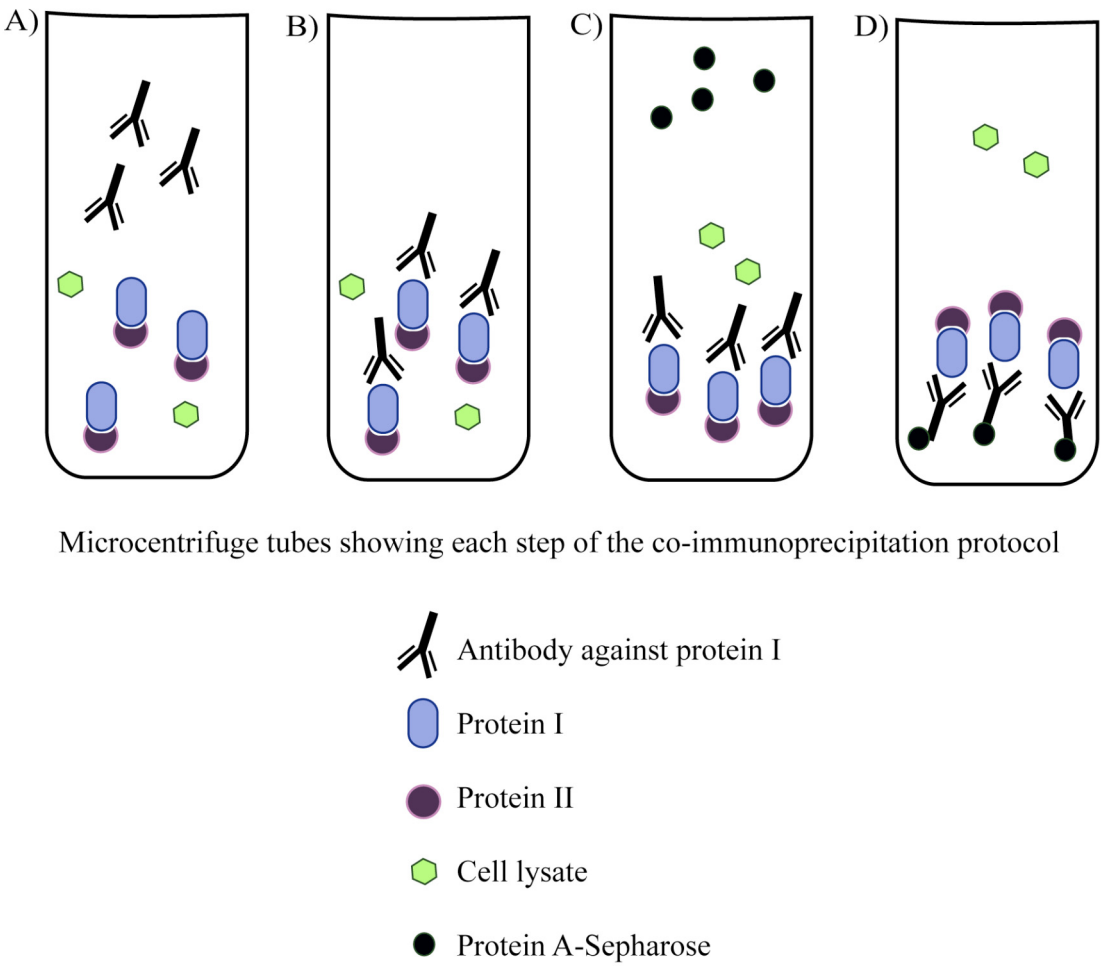


Figure 2.3 Schematic of the co-IP protocol. (A) An antibody against one protein of interest (protein I) is added to the cell lystate. (B) The antibody then binds to protein I, which is complexed with a second protein (protein II). (C) Protein A-Sepharose is added and renders the antibody-protein I/II complex insoluble. (D) This complex is then precipitated through centrifugation and washed. The precipitate is analyzed by means of western blot, immunoblotting against protein II.

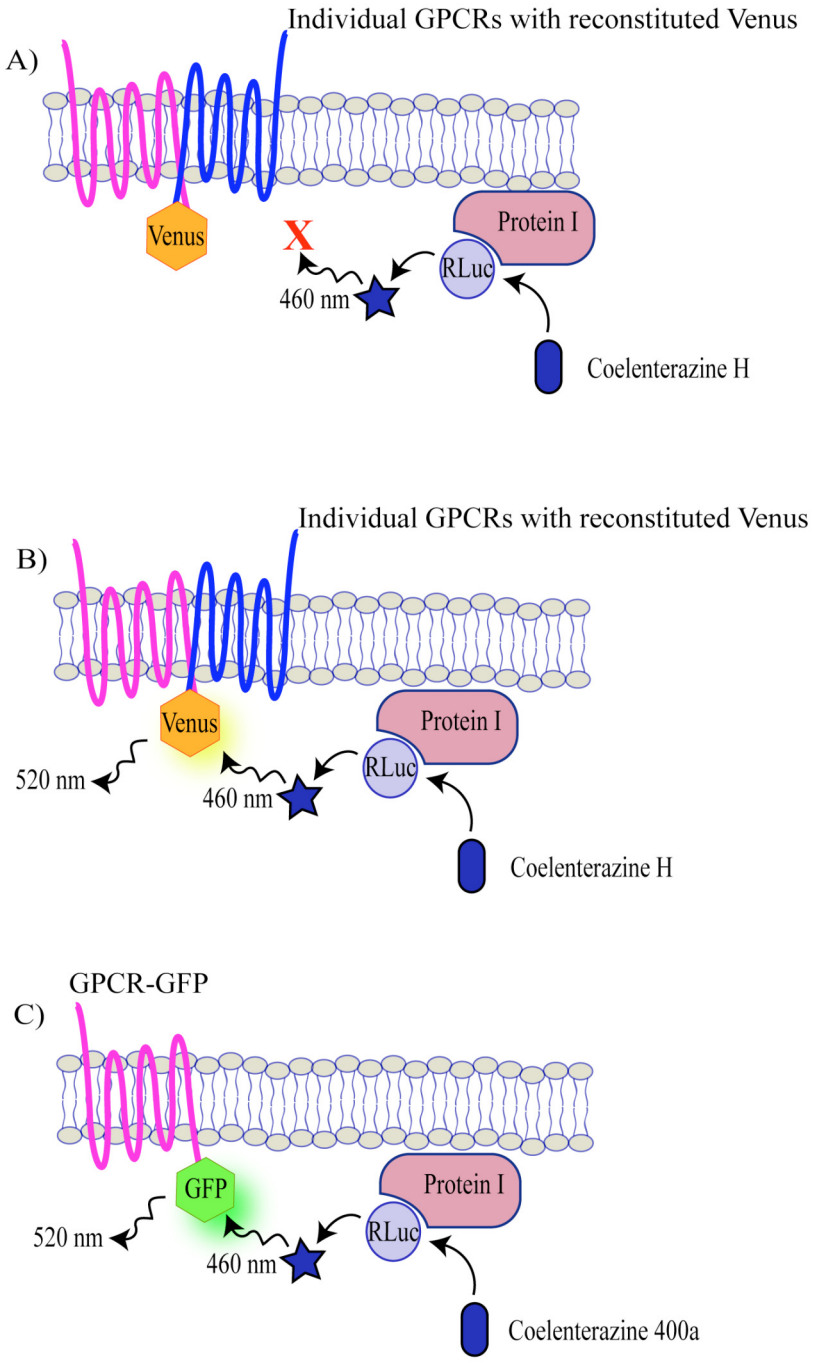


Figure 2.4 Schematic of the BRET protocol. GPCRs are tagged with (A, B) Venus fragments for BRET1 or (C) GFP for BRET2. G-proteins are tagged with RLuc. GFP or the reconstituted Venus fragments act as energy acceptors, whereas RLuc catalyzes oxidation of the substrate (coelenterazine H (BRET1) or coelenterazine 400a (BRET2)) to act as an energy donor. When in close enough proximity, energy emitted from RLuc oxidation of the substrate (460 nm) can be transferred to V1/V2 or GFP. Subsequent emission from V1/V2 or GFP can be read at 520 nm with a plate reader, except when BRET does not occur, as is shown in (A).

CHAPTER 3 RESULTS

3.1 CXCR4 and CCR2 Interaction in PC3 Cells

Previous studies have indicated that the chemokine GPCRs CXCR4 [136] and CCR2 [151] are expressed at endogenous levels in PC3 cells. Western blotting was carried out to confirm these findings and examine endogenous expression of both receptors in PC3 cells, as shown in Figures 3.1A and 3.1B [180].

Both CXCR4 and CCR2 can form homo- and heterodimers, with ligands showing differential interactions with the CXCR4/CCR2 heterodimer when compared to the individual receptors [158,160]. Co-IP experiments were conducted to show that endogenous CXCR4 could be immunoprecipitated with endogenous CCR2 in PC3 cells (Figure 3.1) [180]. These experiments were carried out by immunprecipitating CXCR4 and immunoblotting against CCR2 (Figure 2.1B) or by immunoprecipitating CCR2 and immunoblotting against CXCR4 (Figure 3.1B) [180]. Each experiment yielded the same result.

By enabling detection of CXCR4/CCR2 heterodimers through visualization with a microscope or detection with an anti-GFP antibody, BiFC facilitated the study of the CXCR4/CCR2 signaling complex [180]. Dimerization of the signaling complex resulted in the formation of a functional Venus that was able to bind to an anti-GFP antibody (Figure 3.2A) [180]. Figure 3.2B indicates the relative fluorescence levels of different chemokine receptor dimer combinations and Figure 3.2C shows fluorescence microscopy of the CXCR4V1/CCR2V2 heterodimer used in this thesis [180].

3.2 Rab GTPase Expression

It was determined how different Rab GTPase constructs could influence the cell surface expression of endogenous CXCR4, CCR2, and the overexpressed CXCR4/CCR2 heterodimer [180]. To do this, PC3 cells were transfected with MYC- or FLAG-tagged WT or DN Rab GTPases constructs for 48 h [180]. Cells were then lysed and processed with SDS-PAGE [180]. Figure 3.3 shows that expression levels for all Rab GTPase constructs were similar when transfected into PC3 cells [180]. Figure 3.3 also indicates that total endogenous CXCR4 and CCR2 levels did not change after transfection with Rab GTPase WT or DN constructs [180].

3.3 Anterograde Trafficking of CXCR4

To facilitate the study of how Rab GTPase DN mutants may alter the anterograde trafficking of endogenous CXCR4 and CCR2, as well as the overexpressed heterodimeric complex, cell surface expression of these receptors was examined after PC3 cells were transfected with WT or DN Rab GTPase isoforms [180]. Cell surface expression of CXCR4 was measured 48 h post transfection by means of biotin-streptavidin cell surface assays and western blot analysis with an anti-CXCR4 antibody [180]. It was found that DN isoforms of Rab1 and Rab11 did not influence CXCR4 plasma membrane expression (Figure 3.4A) [180]. However, Rab2 S20N (38.25 ± 7.71%), Rab6 T27N (66.25 ± 6.10%) and Rab8 T22N (60.75 ± 10.27%) significantly decreased CXCR4 plasma membrane expression when compared to Rab2, Rab6, and Rab8 WT isoforms (Figure 3.4A) [180].

3.4 Anterograde Trafficking of CCR2

Much like the study of the CXCR4 trafficking pathway, biotin-streptavidin cell surface assays and western blot analysis revealed that endogenous CCR2 cell surface depended on expression of particular Rab GTPase isoforms [180]. Whereas Rab1 S25N (37.33 ± 6.74%), Rab6 T27N (35.33 ± 3.76%), and Rab8 T22N (26.00 ± 4.58%) decreased CCR2 cell surface expression when compared to WT isoforms, Rab2 S20N and Rab11 did not influence CCR2 plasma membrane expression (Figure 3.4B) [180].

3.5 Anterograde Trafficking of CXCR4/CCR2

Biotin-streptavidin cell surface assays, followed by western blot analysis, found that Rab1 S25N (30.33 ± 8.69%) and Rab8 T22N (64.18 ± 9.46%) significantly decreased plasma membrane expression of the CXCR4V1/CCR2V2 heterodimer (Figure 3.4C) [180].

3.6 Focal Adhesion Kinase (FAK) Phosphorylation

Because cell migration plays such an integral role in prostate cancer metastasis and FAK is so intimately associated with migratory processes [182], activation of this protein kinase in PC3 cells expressing different Rab GTPase isoforms was investigated [180]. CXCR4 and CCR2 must be expressed at the cell membrane to bind SDF-1α or MCP-1, respectively [180]. Therefore, studying a signaling pathway activated by CXCR4 or CCR2 would further highlight the roles of Rab GTPases in the plasma membrane expression of these receptors [180].

WT and DN Rab GTPases were transfected in PC3 cells that expressed endogenous receptors [180]. After stimulation with SDF-1α at a concentration of 30 ng/ml for 15 min at 37°C and 5% CO_2, levels of phospho-FAK (p-FAK) were quantified

through western blots and compared between non-stimulated and stimulated cells [180]. After stimulation with SDF-1α, cells transfected with Rab2 S20N (p=0.019), Rab6 T27N (p=0.0001) and Rab8 T22N (p=0.0258) exhibited statistically significant decreases in p-FAK activation when compared to their WT counterparts (Figure 3.5A) [180].

When PC3 cells were transfected with WT and DN Rab GTPases, but stimulated with MCP-1 (20 ng/ml for 15 min at 37°C and 5% CO_2), Rab1 S25N (p=0.0158), Rab6 T27N (p=0.0231), and Rab8 T22N (p=0.0035) resulted in statistically significant decreases in p-FAK activation when compared to their WT isoforms (Figure 3.5B) [180].

As was completed with endogenous CXCR4 and CCR2, FAK activation in cells overexpressing the CXCR4V1/CCR2V2 heterodimer was measured after stimulation with SDF-1α or MCP-1 [180]. After stimulation with SDF-1α (Figure 3.5C), there were statistically significant decreases in p-FAK levels in cells transfected with Rab1 S25N (p=0.0037) and Rab8 T22N (p=0.0078), compared to cells transfected with WT isoforms [180]. After stimulation with MCP-1 (Figure 3.5D), significant decreases in FAK phosphorylation were also apparent with Rab1 (p=0.0068) and Rab8 DN GTPases (p=0.0329), compared to their WT isoforms [180].

3.7 Interaction of Molecular Chaperones with CCR5 and CD4 Receptors and Dimers

Because Rab GTPases are not the only proteins involved in the maturation of GPCRs, this portion of the thesis examined how molecular chaperones interact with CCR5 and CD4 receptors. CD4 and CCR5 have been shown to associate in the ER [179]. It is known that this interaction increases CCR5 cell surface expression, but molecular chaperones promoting the assembly of these receptors and their heterodimer are relatively unknown. As a first step in attempting to determine how molecular

chaperones may affect the maturation and plasma membrane expression of CCR5 and its homo- and heterodimers, chaperones shown to be expressed endogenously in HEK293 cells [100,186] were examined for interactions with CCR5-GFP, CD4-GFP, CCR5V1/V2 and CCR5V1/CD4V2. As evident through co-IP experiments presented in Figure 3.6, each chaperone expressed at an endogenous level interacted with overexpressed CCR5 and CD4 receptors. Each endogenously expressed chaperone also interacted with both the overexpressed CCR5 homodimer and CCR5/CD4 heterodimer (Figure 3.6).

3.8 Effects of Molecular Chaperones on Heterodimer Maturation and Dimerization

Before studying the effects of each chaperone on receptor maturation and dimerization, it was important to confirm overexpression of the FLAG- or HA-tagged chaperone constructs (Figure 3.7). It was also important to confirm knockdown of endogenous Hsp70, calreticulin, PDIA3 and DRiP78 (Figure 3.7).

To determine if the molecular chaperones included in this study influenced receptor maturation and dimerization, fluorescence constructs of each receptor or receptor pairing were transfected with each molecular chaperone construct, siRNA or shRNA. If the chaperones affected receptor maturation of CCR5-GFP or CD4-GFP, a significantly different fluorescence reading would occur relative to control. This is because the fluorescence reading would be proportional to the amount of mature receptor in the cell. For instance, if siRNA or shRNA knockdown of a molecular chaperone resulted in a significantly decreased fluorescence reading, the absence of this chaperone adversely affected receptor maturation. Therefore, this molecular chaperone could be deemed essential for proper receptor maturation. Additionally, if either CCR5-GFP or CD4-GFP maturation was affected by alteration of a chaperone's expression levels, we

could attribute subsequent effects on the dimer as a result of this chaperone's effect on the individual receptor. If siRNA or shRNA knockdown of a particular chaperone resulted in decreased fluorescence of CCR5-GFP, this chaperone's effect on CCR5 dimers may be attributed to the fact that it primarily affected CCR5 maturation, as less CCR5 would be available in the cell for dimer formation.

The effects of each chaperone on receptor maturation and dimerization are presented in Figure 3.8. When HEK293A cells were transfected with each construct, siRNA or shRNA, along with CCR5-GFP, DRiP78 WT overexpression resulted in significantly decreased fluorescence ($p=0.0029$), whereas overexpression of WT calnexin resulted in significantly increased fluorescence ($p=0.0179$), when compared with control (Figure 3.8A). When the same experiment was conducted with CD4-GFP, overexpression of BiP WT resulted in significantly increased fluorescence ($p=0.0052$), compared to control (Figure 3.8B). Overexpression of both DRiP78 WT and BiP T37G caused significantly decreased fluorescence ($p=0.0032$ and 0.0034, respectively) compared to control, when the CCR5V1/CCR5V2 homodimer was investigated (Figure 3.8C). Fluorescence relating to the CCR5V1/CD4V2 heterodimer was significantly decreased upon cotransfection with Hsp70 siRNA ($p<0.0001$), calreticulin siRNA ($p=0.0418$) and the GRP WT and BiP T36G constructs ($p=0.0014$ and 0.0039, respectively), compared to control. Overexpression of the BiP WT construct resulted in increased fluorescence ($p=0.0017$) when cotransfected with the CCR5V1/CD4V2 heterodimer and compared to control (Figure 3.8D).

3.9 BRET Experiments to Investigate Effects of Molecular Chaperones on G-Protein Coupling to Receptors

Previous studies have already determined that protein complexes associate with chaperones during their assembly. For example, DRiP78 can promote the assembly of Gβγ subunits of G-proteins [186] and phosducin-like protein, along with the cytosolic chaperonin complex, has also been associated with Gβγ subunit assembly [187]. In order to determine if molecular chaperones influence G-protein coupling to dimers of CCR5, HEK293A cells were transfected similarly as in Section 3.8. However, Gβ1-Rluc and Gγ2 were cotransfected with each chaperone and receptor pairing. CCR5-GFP cotransfected with the vector pRluc-N3 served as the negative control for BRET2 and CCR5V1/CCR5V2 cotransfected with pRluc-N3 served as the negative control for BRET1. No significant differences were found in any of BRET experiments conducted to determine if chaperones influenced G-protein coupling to CCR5, the CCR5V1/CCR5V2 homodimer or the CCR5V1/CD4V2 heterodimer (Figure 3.9).

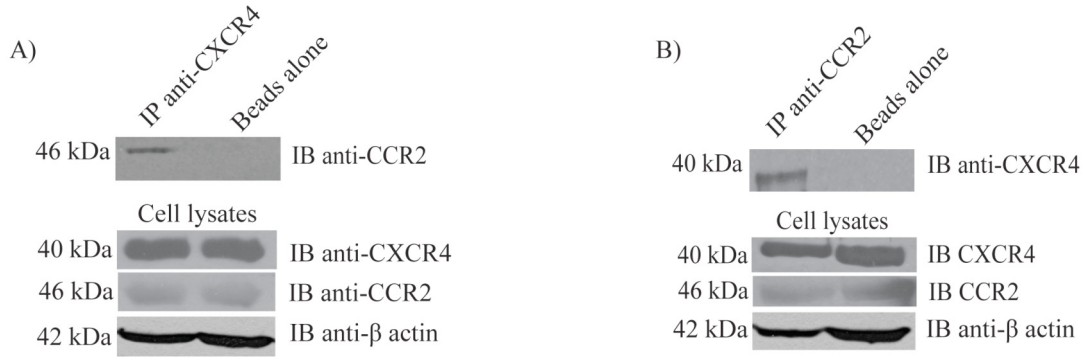


Figure 3.1 Expression and interaction of chemokine receptors in PC3 cells.
(A) Co-immunoprecipitation of the CXCR4/CCR2 complex in PC3 cells. PC3 cells expressing endogenous receptors were lysed and immunoprobed with anti-CXCR4 (1:1000). Samples were processed with SDS-PAGE and immunoblotted with anti-CCR2 (1:1000). Lysates were immunoblotted with anti-CXCR4 (1:000), anti-CCR2 (1:1000) or anti-β actin (1:5000). (B) Same experiment as presented in (A), but probing against CCR2 and blotting against CXCR4. Results are representative of 3 independent experiments (Gillies et al. (2013) [180], Figure and text extract from Cellular Physiology and Biochemistry by S./KARGER AG. Reproduced with permission of S./KARGER AG in the format reuse in a thesis/dissertation via Copyright Clearance Center).

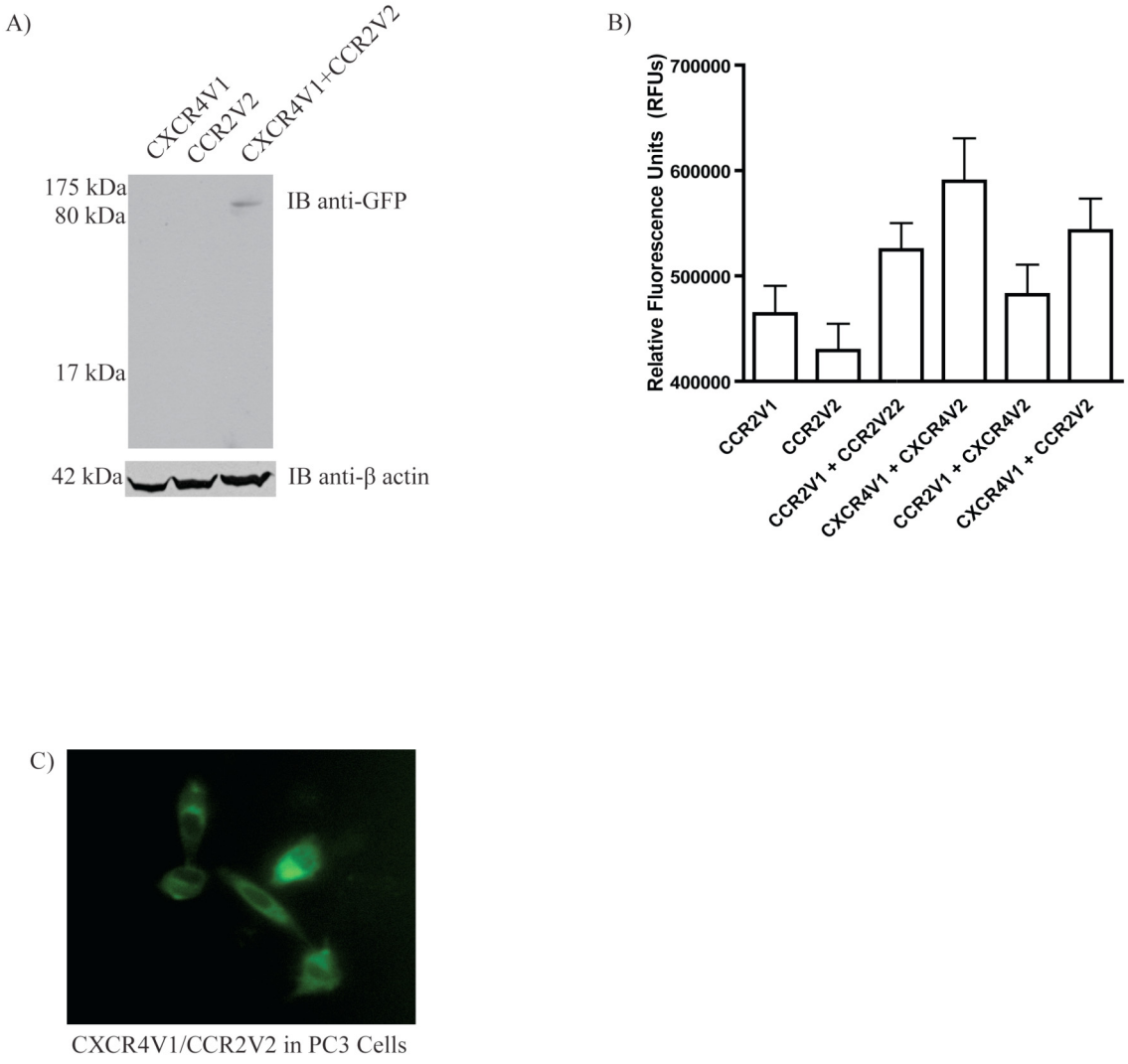


Figure 3.2 Specificity of the anti-GFP antibody to the CXCR4V1/CXCR4V2 heterodimer, bioluminescence fluorescence complementation (BiFC) and immunofluorescence of the CXCR4V1/CCR2V2 heterodimer in PC3 cells. (A) Cells were transfected with CXCR4V1, CCR2V2 or CXCR4V1/CCR2V2 and 48 h post transfection, cells were lysed in RIPA buffer and western blot analysis was completed with anti-GFP (1:1000) to indicate the presence of the YFP variant, Venus. (B) The histogram shows levels of fluorescence observed by combination of various pairs of BiFC constructs. (C) Sample fluorescence microscopy demonstrating the fluorescence of the CXC4V1/CCR2V2 heterodimer. Venus was excited at 488 nm and the image was acquired at 525 nm. Results are representative of 3 independent experiments (Gillies et al. (2013) [180], Figure and text extract from Cellular Physiology and Biochemistry by S./KARGER AG. Reproduced with permission of S./KARGER AG in the format reuse in a thesis/dissertation via Copyright Clearance Center).

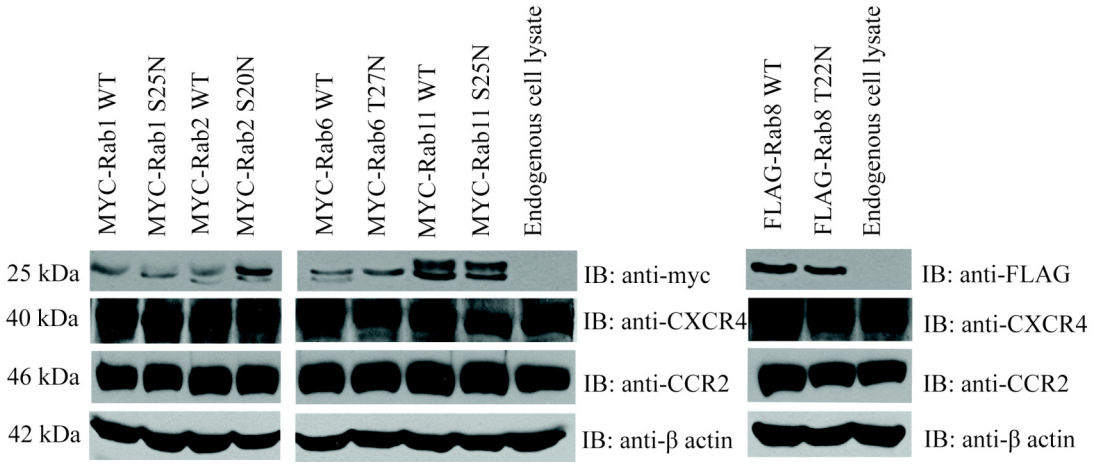


Figure 3.3 Expression of Rab GTPases, as well as total endogenous CXCR4 and CCR2 receptors, in PC3 cells transfected with WT and DN Rab constructs. 48 h post-transfection, cells were harvested and lysed with RIPA biffer and processed with SDS-PAGE. Membranes were probed with anti-myc (1:1000), anti-flag (1:1000), anti-CXCR4 (1:1000), anti-CCR2 (1:1000) or anti-β actin (1:5000). Results are representative of 3 independent experiments (Gillies et al. (2013) [180], Figure and text extract from Cellular Physiology and Biochemistry by S./KARGER AG. Reproduced with permission of S./KARGER AG in the format reuse in a thesis/dissertation via Copyright Clearance Center).

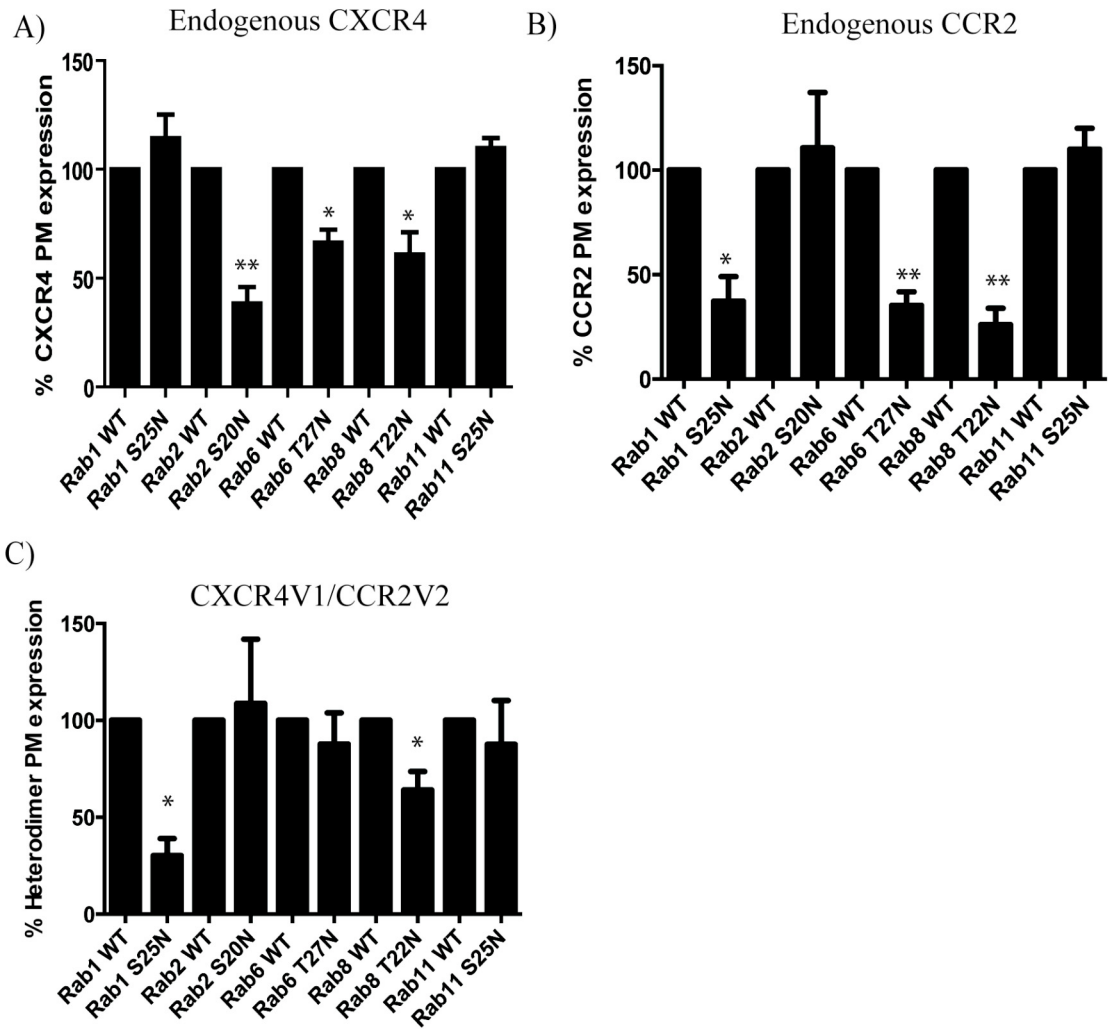


Figure 3.4 Effects of DN Rab GTPase mutants on cell surface expression of CXCR4, CCR2 and the overexpressed CXCR4V1/CCR2V2 heterodimer in PC3 cells. PC3 cells expressing endogenous receptors were transfected with either RabGTPase WT or the DN mutant and subsequent cell surface expression of (A) endogenous CXCR4, (B) endogenous CCR2 and (C) the over expressed CXCR4V1/CCR2V2 heterodimer was measured with biotin-streptavidin cell surface assays, followed by SDS-PAGE. Immunoblots were probed with anti-CXCR4 (1:1000), anti-CCR2 (1:1000) or anti-GFP (1:1000). Results are expressed as means ± SEM. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ when compared with the Rab WT samples, using a two-tailed unpaired Student's t test. Results are representative of at least 3 independent experiments (Gillies et al. (2013) [180], Figure and text extract from Cellular Physiology and Biochemistry by S./KARGER AG. Reproduced with permission of S./KARGER AG in the format reuse in a thesis/dissertation via Copyright Clearance Center).

Figure 3.5 Effects of DN Rab GTPase mutants on FAK activation in both the CCR2 and CCR4 receptors individually and the CXCR4V1/CCR2V2 heterodimer in PC3 cells. (A) PC3 cells expressing endogenous receptors were transfected with either Rab GTPase WT or DN constructs and stimulated with SDF-1α at 30 ng/ml for 15 min at 37°C in 5% CO_2, or vehicle. These cells were then lysed and subjected to western blot analysis against p-FAK levels (1:1000). The same membrane was then reprobed for FAK levels (1:1000), as a control. (B) Cells transfected the same way as in (A) were stimulated with MCP-1 at a concentration of 20 ng/ml for 15 min at 37°C in 5% CO_2, or vehicle. The cells were then lysed and subjected to western blot analysis. (C) PC3 cells were transfected with the CXCR4V1/CCR2V2 heterodimer and stimulated with SDF-1 at 30 ng/ml for 15 min at 37°C in 5% CO_2, or vehicle. Western blot analysis was carried out as previously described. (D) PC3 cells transfected with the CXCR4V1/CCR2V2 heterodimer were stimulated with MCP-1 at 20 ng/ml for 15 min at 37°C in 5% CO_2, or vehicle. The samples were then examined using western blot analysis. Results are expressed as means ±SEM. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$ when compared with the Rab WT stimulated samples, using a two-tailed Student's *t* test. Results are representative of at least 3 independent experiments (Gillies et al. (2013) [180], Figure and text extract from Cellular Physiology and Biochemistry by S./KARGER AG. Reproduced with permission of S./KARGER AG in the format reuse in a thesis/dissertation via Copyright Clearance Center). (E) Representative blot of FAK levels with transfected WT and DN Rab GTPase isoforms, either non-stimulated (NS) or stimulated (S) with SDF-1α and endogenous CXCR4 expression.

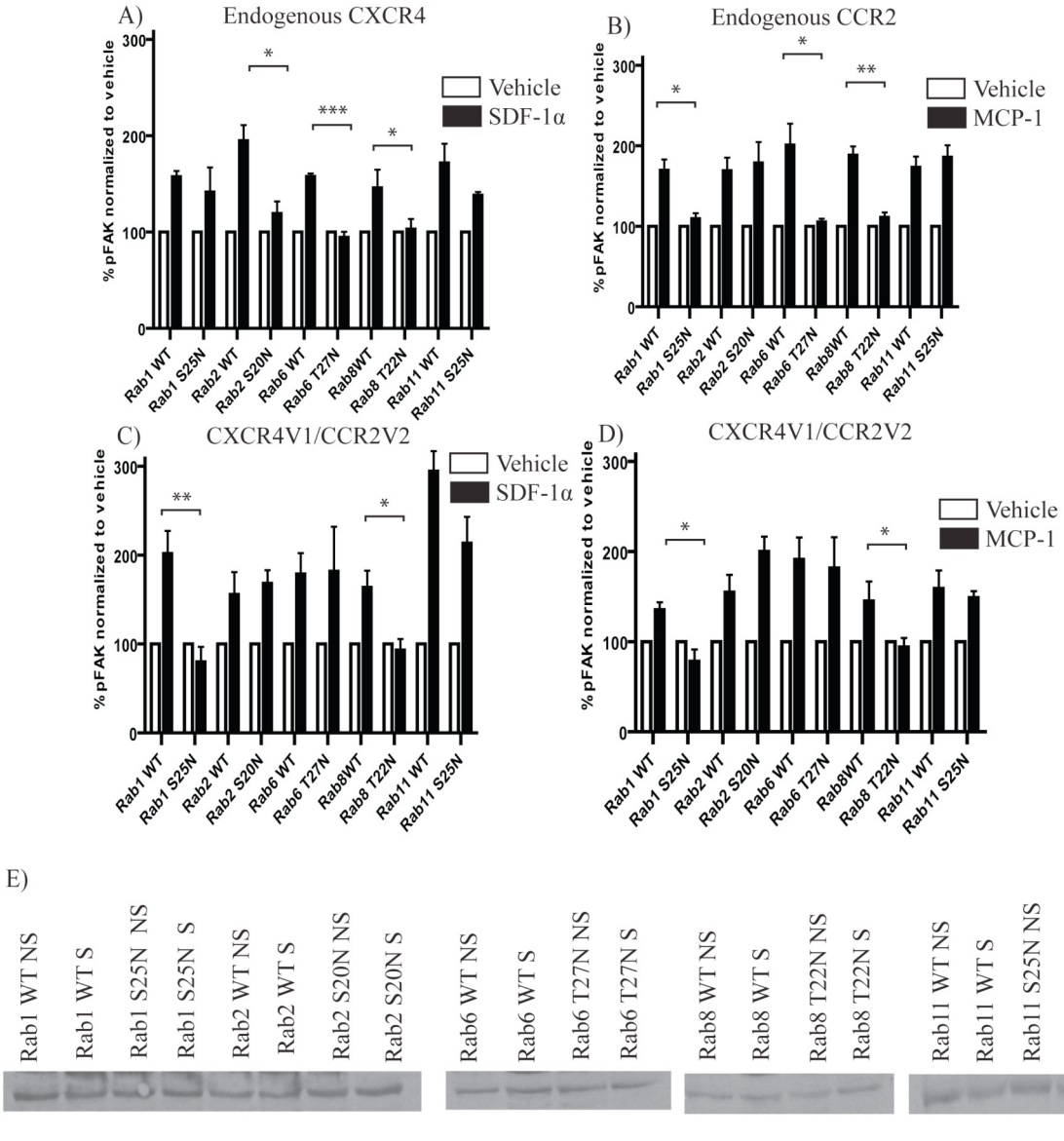
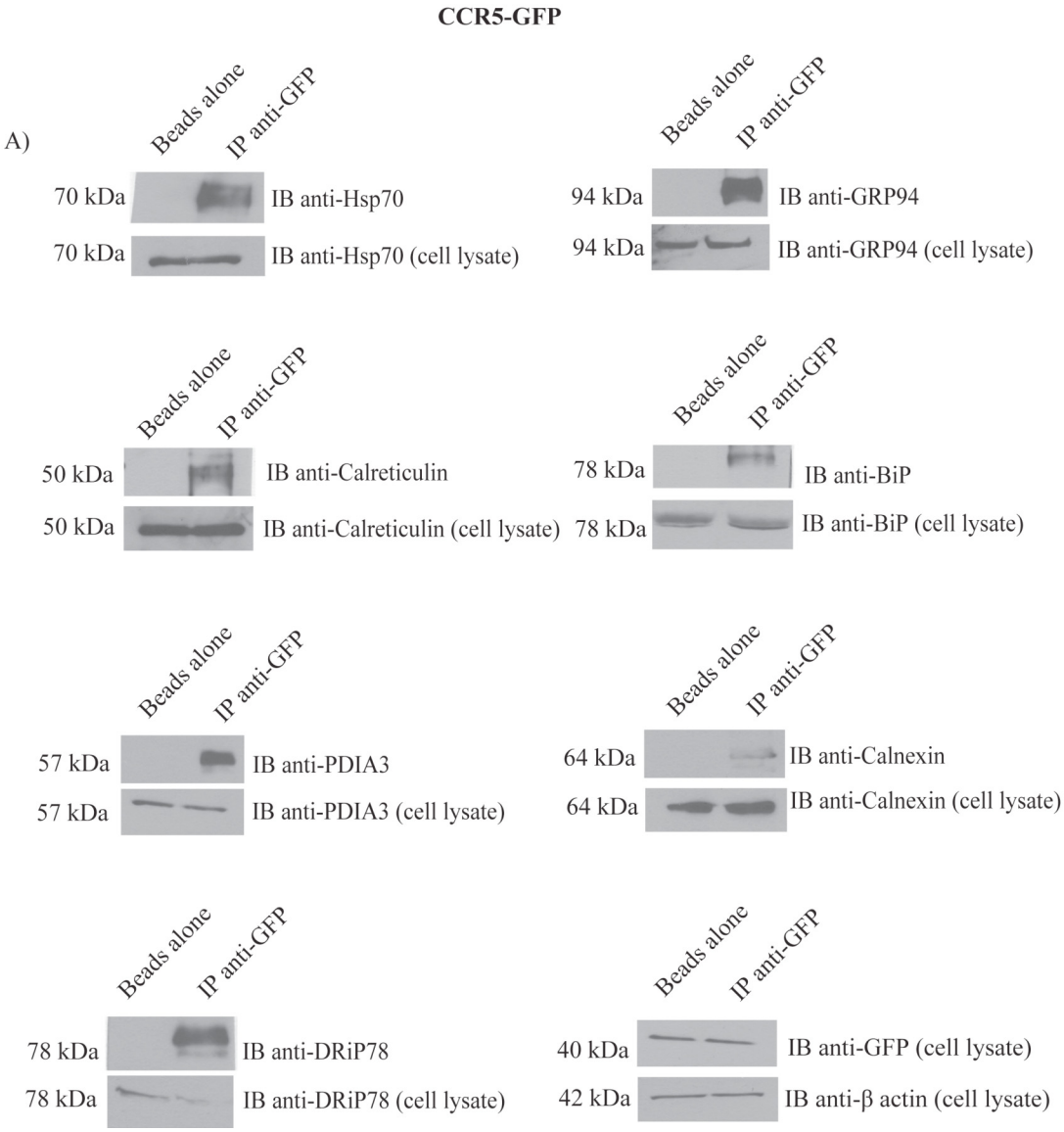
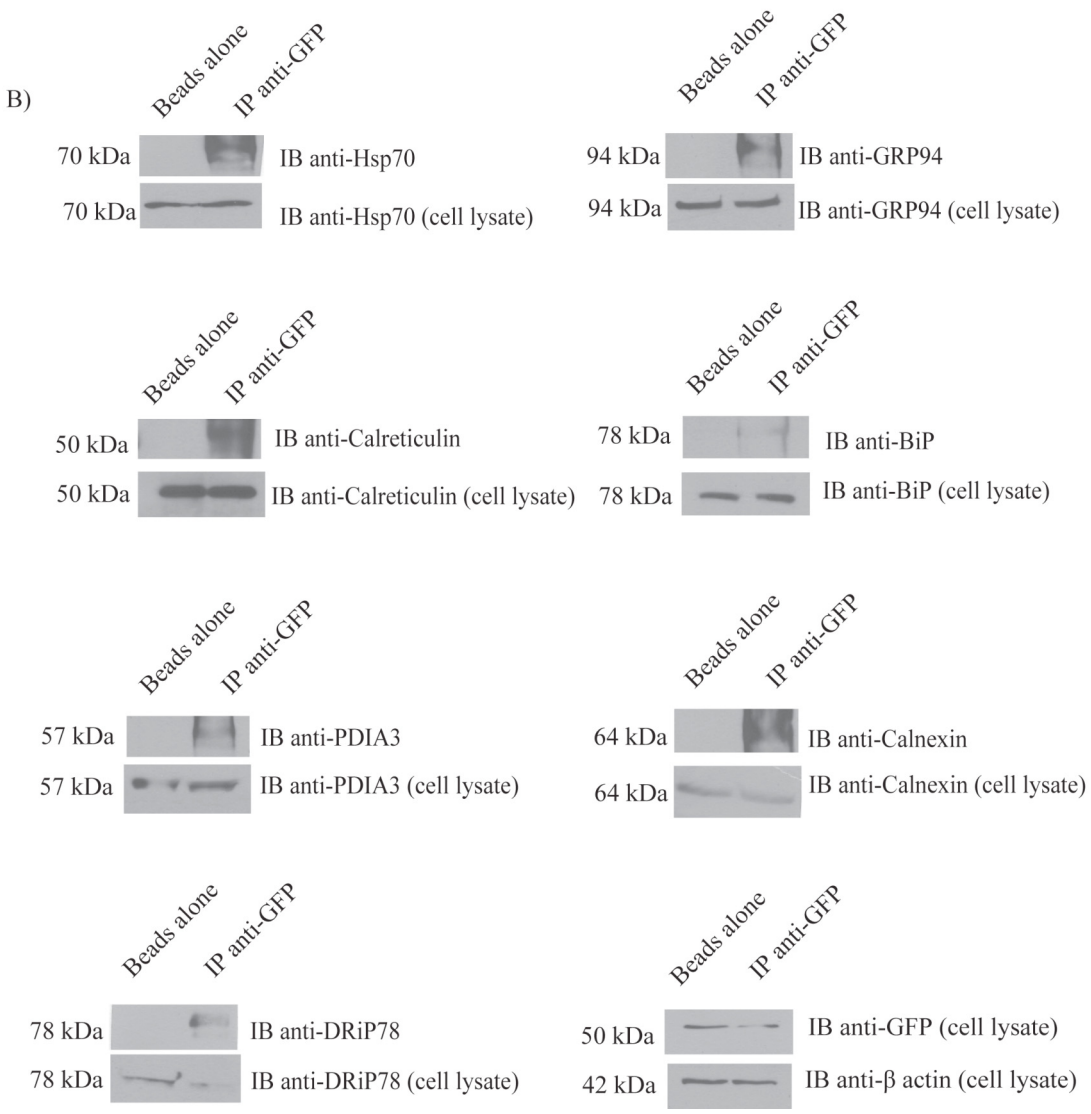

A)
Endogenous CXCR4
% pFAK normalized to vehicle
Vehicle
SDF-1α
Rab1 WT
Rab1 S25N
Rab2 WT
Rab2 S20N
Rab6 WT
Rab6 T27N
Rab8WT
Rab8 T22N
Rab11 WT
Rab11 S25N
B)
Endogenous CCR2
Vehicle
MCP-1
C)
CXCR4V1/CCR2V2
Vehicle
SDF-1α
D)
CXCR4V1/CCR2V2
Vehicle
MCP-1
E)
Rab1 WT NS
Rab1 WT S
Rab1 S25N NS
Rab1 S25N S
Rab2 WT NS
Rab2 WT S
Rab2 S20N NS
Rab2 S20N S
Rab6 WT NS
Rab6 WT S
Rab6 T27N NS
Rab6 T27N S
Rab8 WT NS
Rab8 WT S
Rab8 T22N NS
Rab8 T22N S
Rab11 WT NS
Rab11 WT S
Rab11 S25N NS
Rab11 S25N S
IB anti-FAK, 125 kDa

Figure 3.6 Interaction of molecular chaperones with CCR5 and CD4 receptors and dimers. HEK293A cells expressing endogenous chaperones, but overexpressing (A) CCR5-GFP, (B) CD4-GFP, (C) CCR5V1/CCR5V2 or (D) CCR5V1/CD4V2 constructs were lysed and immunoprobed with anti-GFP (1:1000). Samples were processed with SDS-PAGE and immunoblotted with antibodies against each chaperone of interest (1:1000). Lysates were immunoblotted with anti-GFP (1:1000), anti-β actin (1:5000) and antibodies against each chaperone of interest (1:1000). Results are representative of 3 independent experiments.


CCR5-GFP
A)
Beads alone
IP anti-GFP
70 kDa
IB anti-Hsp70
70 kDa
IB anti-Hsp70 (cell lysate)
94 kDa
IB anti-GRP94
94 kDa
IB anti-GRP94 (cell lysate)
50 kDa
IB anti-Calreticulin
50 kDa
IB anti-Calreticulin (cell lysate)
78 kDa
IB anti-BiP
78 kDa
IB anti-BiP (cell lysate)
57 kDa
IB anti-PDIA3
57 kDa
IB anti-PDIA3 (cell lysate)
64 kDa
IB anti-Calnexin
64 kDa
IB anti-Calnexin (cell lysate)
78 kDa
IB anti-DRiP78
78 kDa
IB anti-DRiP78 (cell lysate)
40 kDa
IB anti-GFP (cell lysate)
42 kDa
IB anti-β actin (cell lysate)

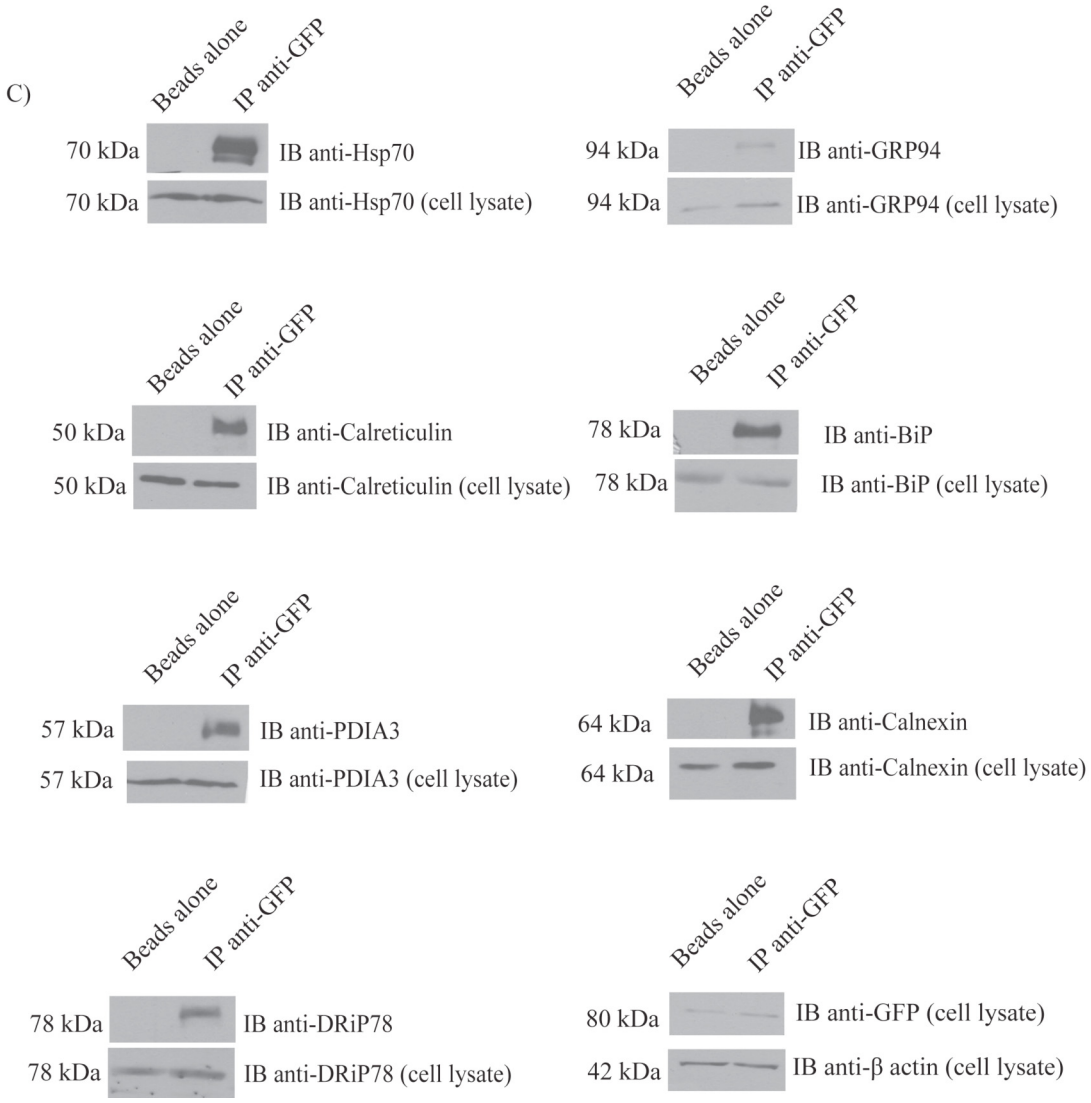
CD4-GFP

B)

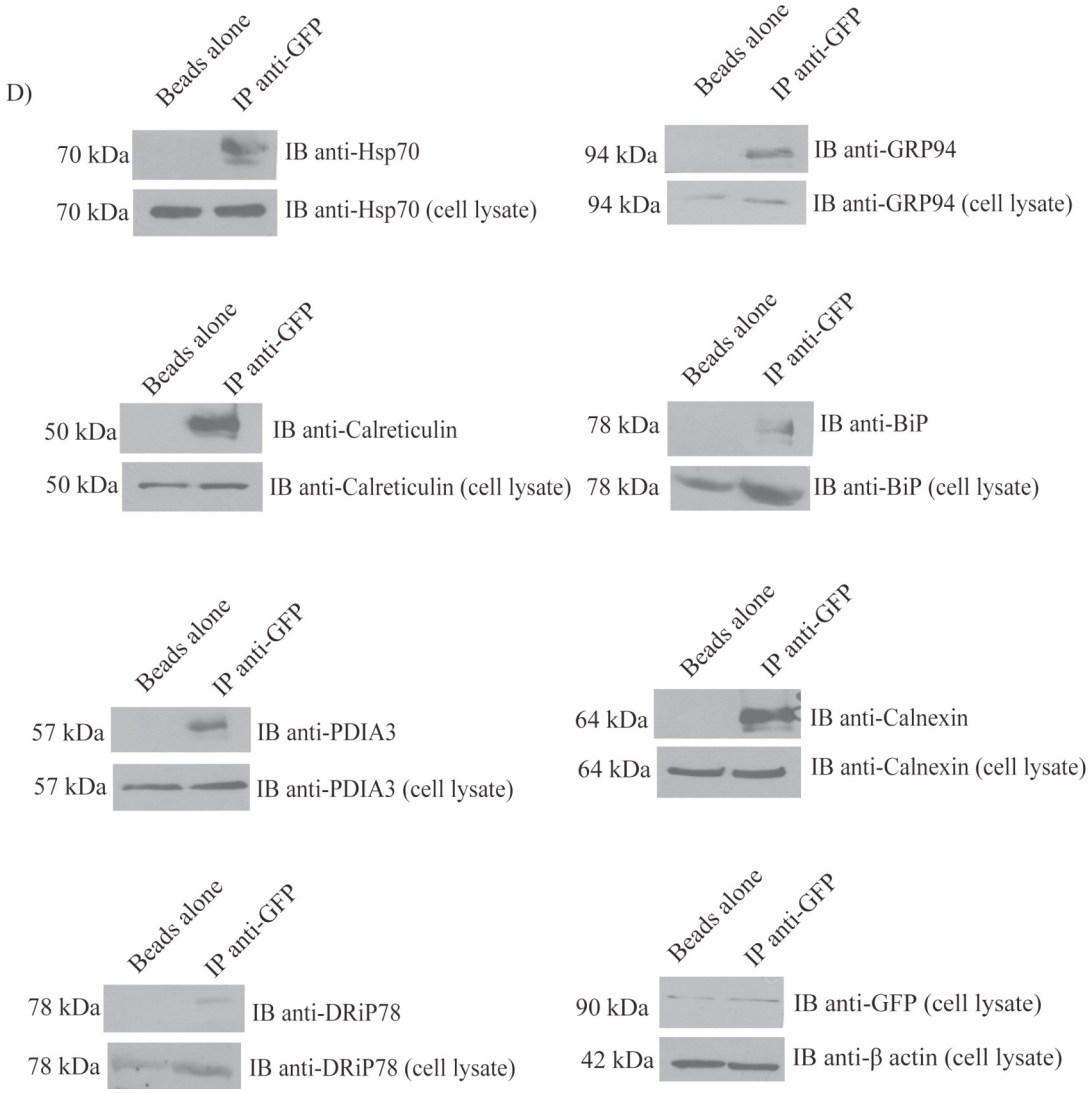

Beads alone
IP anti-GFP
70 kDa
IB anti-Hsp70
70 kDa
IB anti-Hsp70 (cell lysate)
Beads alone
IP anti-GFP
94 kDa
IB anti-GRP94
94 kDa
IB anti-GRP94 (cell lysate)
Beads alone
IP anti-GFP
50 kDa
IB anti-Calreticulin
50 kDa
IB anti-Calreticulin (cell lysate)
Beads alone
IP anti-GFP
78 kDa
IB anti-BiP
78 kDa
IB anti-BiP (cell lysate)
Beads alone
IP anti-GFP
57 kDa
IB anti-PDIA3
57 kDa
IB anti-PDIA3 (cell lysate)
Beads alone
IP anti-GFP
64 kDa
IB anti-Calnexin
64 kDa
IB anti-Calnexin (cell lysate)
Beads alone
IP anti-GFP
78 kDa
IB anti-DRiP78
78 kDa
IB anti-DRiP78 (cell lysate)
Beads alone
IP anti-GFP
50 kDa
IB anti-GFP (cell lysate)
42 kDa
IB anti-β actin (cell lysate)

CCR5V1/CCR5V2

C)



CCR5V1/CD4V2



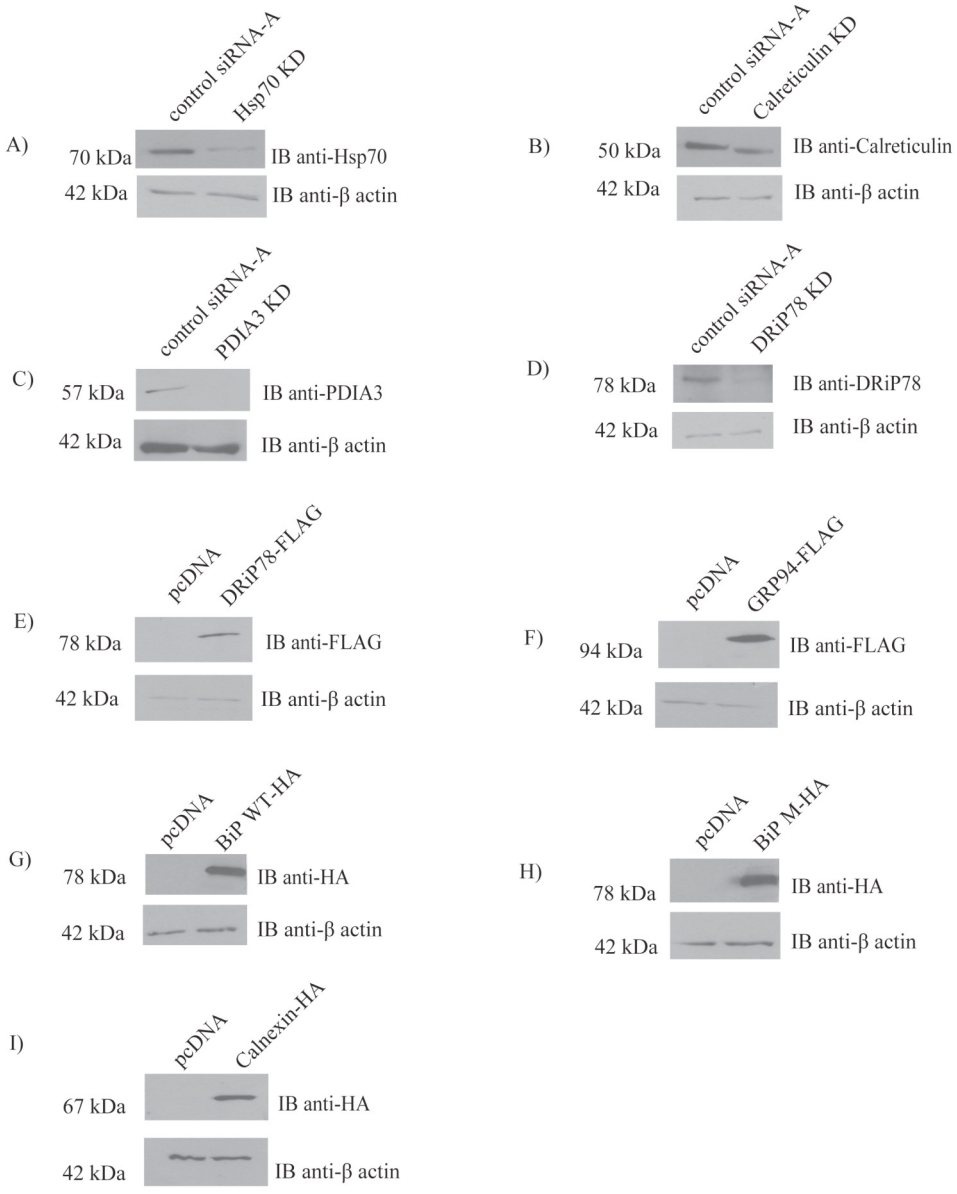


Figure 3.7 Expression of DRiP78, GRP94, BiP WT, BiP T37G and calnexin molecular chaperone constructs and knockdown of endogenous Hsp70, Calr3, PDIA3 and DRiP78. HEK293A cells were transfected with DRiP78-FLAG, GRP94-FLAG, BiP WT-HA, BiP T37G-HA or Calnexin-HA constructs. HEK293A cells were also transfected with Hsp70, calreticulin, PDIA3 siRNA or DRiP78 shRNA. Construct transfections were accompanied by transfection of pcDNA3.1 as a control, whereas siRNA and shRNA tranfections were accompanied by transfection with control siRNA-A. 48 h post-transfection, cell lysates were processed with SDS-PAGE. Immunoblots were probed with anti-FLAG (1:1000), anti-HA (1:1000), anti- Hsp70 (1:1000), anti-Calr3 (1:1000), anti-PDIA3 (1:1000), anti-DRiP78 (1:1000) or anti-β actin (1:5000). Results are representative of 3 independent experiments.

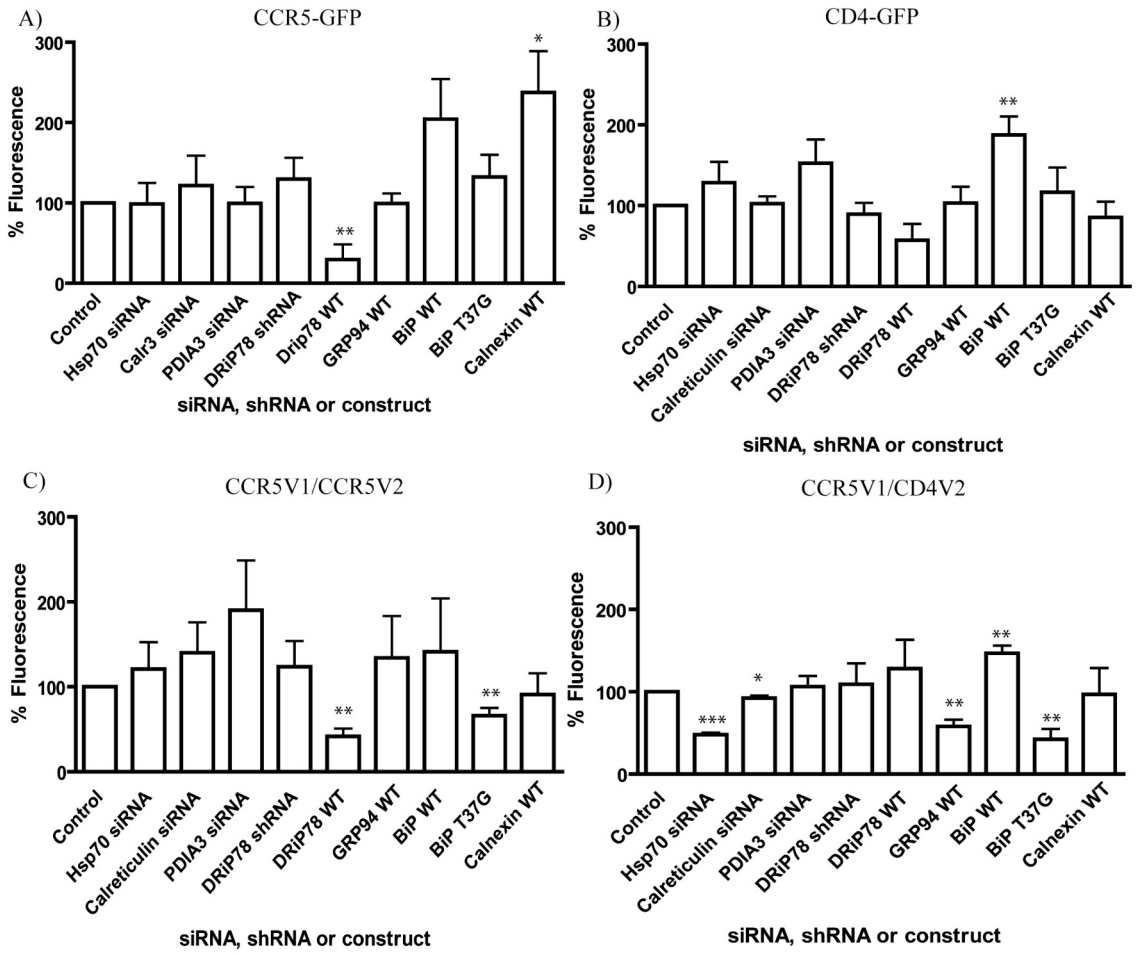


Figure 3.8 Effects of molecular chaperones on receptor maturation and dimerization. HEK293A cells were cotransfected with each construct, siRNA or shRNA, as well as (A) CCR5-GFP, (B) CD4-GFP, (C) CCR5V1/CCR5V2, (D) CCR5V1/CD4V2. 48 h post-transfection, cells were harvested in cold PBS and fluorescence was measured using a Perkin Elmer Wallac EnVision 2104 Multilabel plate reader. Fluorescence values were corrected for using cell counts. Background fluorescence of cells expressing empty pcDNA was subtracted from each sample's fluorescence reading. All fluorescence values were normalized to pcDNA samples and corrected for cell counts. Results are representative of at least 3 independent experiments.

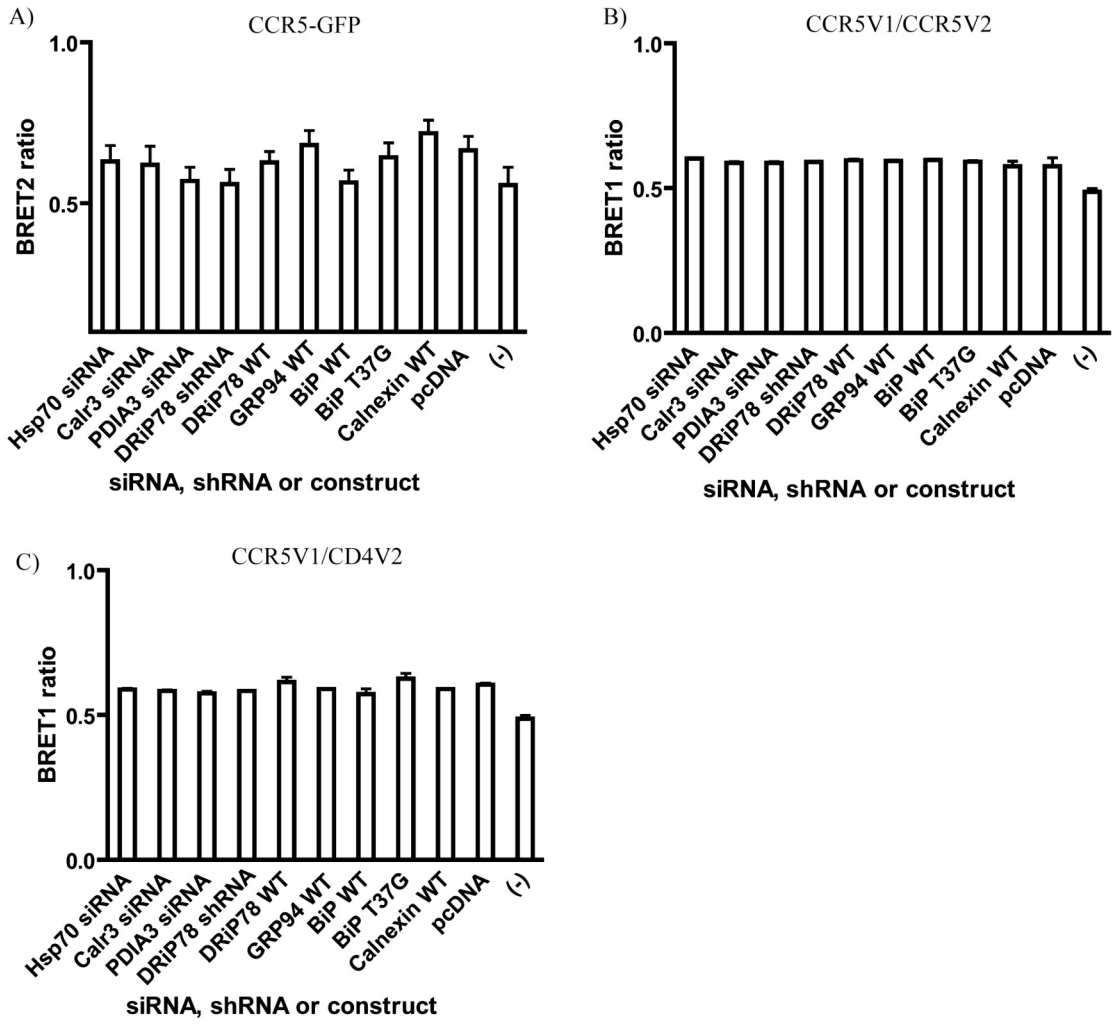


Figure 3.9 BRET experiments investigating the effects of molecular chaperones on G-protein coupling to receptors. Gβ1-RLuc and Gγ2 were transfected into HEK293A cells that were also transfected with each construct, siRNA or shRNA, as well as (A) CCR5-GFP (BRET2), (B) CCR5V1/CCR5V2 (BRET1), and (C) CCR5V1/CD4V2 (BRET1). Emission from V1/V2 or GFP was read at 520 nm with using a FLUOstar Omega BMG Labtech plate reader a plate reader. Results are representative of at least 3 independent experiments.

CHAPTER 4 DISCUSSION

4.1 Requirements for CXCR4 and CCR2 Anterograde Trafficking in a Prostate Cancer Cell Line

4.1.1 Importance and Overview

Although there have been significant advances in tems of prostate cancer diagnosis and treatment [188], prognoses associated with this disease remain poor [188,189]. The fact that approximately 90% of cancer-related deaths are due to metastasis of the primary tumour [190] justifies the study of receptors associated with the metastatic phenotype, along with signal transduction cascades that result from activation such receptors.

A variety of pharmacological avenues have tried to exploit ways in which cell surface expression of receptors (especially GPCRs) involved in disease processes can be reduced. For example, small molecule antagonists [191], blocking peptides and antibodies [192], as well as siRNAs [193], are being evaluated for their usefulness in delaying the progression of numerous pathaological states. Therefore, it is evident that the targeting of GPCRs to attenuate disease conditions has been a popular area of research in recent years. Unfortunately, these therapies target both diseased and nondiseased tissues. However, both tissue-specific and disease-specific heterodimers are evident [194,195]. It is possible that pharmacological inhibition of only the heterodimer may inhibit signaling through its constituent receptors of the diseased tissues, while the non-diseased tissues are not affected by this inhibition. Relatively fewer side effects would arise due to this mode of targeting only diseased tissues.

Nevertheless, much of this research considers GPCRs only in the view of individual receptors and little attention has been given to the role of the heterodimer. This observation is especially true for the receptors of interest to this portion of the project – CXCR4 and CCR2. Therefore, the work of this portion of the thesis presents another avenue by which the cell surface expression of these chemokine receptors can be decreased, but also highlights the importance careful consideration of the resultant heterodimer. Whereas pharmacological properties of the heterodimer may be different from those of its constituent receptors [15], the trafficking requirements of the heterodimer may also be different from each of its constituent receptors [114].

4.1.2 CXCR4 and CCR2 Receptors Interact in PC3 Cells

Along with the aforementioned studies that CXCR4 and CCR2 are expressed endogenously by PC3 cells [136,151], PC3 cells also have a highly metastatic phenotype [196]. Additionally, anti-CXCR4 antibodies have been shown to decrease PC3 cell migration and invasion [197]. Therefore, this cell line was a suitable choice to study anterograde trafficking requirements of CXCR4 and CCR2. Furthermore, observations relating to the endogenous expression of these chemokine GPCRs in PC3 cells [136,151] indicate that CXCR4 and CCR2 could participate in cellular processes that favor the progression of prostate cancer.

Not only has this project confirmed endogenous expression of these chemokine GPCRs in PC3 cells, co-IP experiments have confirmed that CXCR4 and CCR2 interact with each other at endogenous levels [180]. More specifically, an interaction between these two receptors was observed upon immunoprecipitation of either receptor [180]. This acts to strengthen the co-IP results and points to a true protein-protein interaction

[198]. These results are also strengthened by the observation that studying protein-protein interactions at endogenous expression levels helps eliminate the possibility of false-positives that can result from overexpression of the proteins of interest [199]. Because it was previously discussed that cellular signaling resulting from activation of a receptor in a heterodimer complex may be different from what is predicted for the individual receptor [34], further investigation of the CXCR4/CCR2 signaling complex in PC3 cells was carried out.

4.1.3 Rab GTPases Have Differential Effects on the Cell Surface Expression of CXCR4, CCR2, and CXCR4/CCR2

Before evaluating the effects of Rab GTPase constructs on the cell surface expression of CXCR4, CCR2 or the resultant heterodimer, it was important to demonstrate successful expression of each Rab GTPase construct in the PC3 cell line [180]. As evident in Figure 3.3, results obtained from this line of investigation confirmed that each Rab GTPase construct was expressed and that these constructs were expressed at relatively similar levels to one another [180]. Therefore, any variations in expression levels of CXCR4, CCR2 or the CXCR4/CCR2 heterodimer were not due to different expression levels of individual Rab GTPases [180]. Total endogenous levels of CXCR4 and CCR2 were also assessed following overexpression of each Rab GTPase construct [180]. Because these levels did not vary with upon Rab GTPase overexpression, variations in expression levels of these chemokine GPCRs can be attributed to how these Rab GTPases regulate receptor trafficking [180].

Previous studies have investigated the anterograde trafficking pathways of a variety of GPCRs. Much like the present work, these previous studies evaluated which Rab GTPases played a role in the cell surface expression of different receptors. For

example, transfection with DN mutants or siRNA of Rab1 GTPase in HEK293 cells was used to discover that this Rab GTPase was required for plasma membrane expression of both the AT1R and β2-AR [112]. Signaling cascades resulting from activation of each receptor were hindered upon blockade of Rab1 GTPase activity [112]. In another study of the β2-AR, HEK293 cells were used to determine that the Rab6, Rab8 and Rab11 GTPases were required for β2-AR cell surface expression [113]. Additionally, cardiac myocytes were used as a model to demonstrate the reliance of the AT1R on Rab1 GTPase for expression and function at the cell surface [106].

A study carried out with Jurkat cells (a cell line of human T lymphocytes [200]) examined the effects of Rab GTPases on CXCR4 cell surface expression. By overexpressing Rab2 S20N, Rab6 T27N and Rab8 T22N DN constructs, it was found that endogenous CXCR4 cell surface expression was negatively affected by these DN isoforms [114]. Additionally, expression of either the Rab1 S25N or Rab11 S25N DN construct did not influence endogenous CXCR4 cell surface expression [114]. Therefore, the results of this study mirror those that were found in the current project. In regards to PC3 cells, endogenous CXCR4 expression was significantly decreased upon overexpression of Rab2 S20N, Rab6 T27N and Rab8 T22N DN constructs and there was no effect of either the Rab1 S25N or Rab11 S25N DN construct. This suggests that the effects of Rab GTPases on the trafficking requirements of CXCR4 are not unique to only the PC3 cell line, but that they may be conserved across multiple cell lines.

In regards to CCR2, the present study found that Rab1 S25N, Rab6 T27N and Rab8 T22N significantly decreased the amount of receptor expressed at the plasma membrane [180]. Furthermore, neither Rab2 S20N nor Rab11 S25N appeared to alter the

anterograde trafficking of CCR2 [180]. Currently, it cannot be assessed whether this trafficking pathway is unique to the PC3 cell line or if the Rab GTPase isoforms found to affect CCR2 cell surface expression in PC3 cells also affect the cell surface of this receptor in other cell lines because no study of this kind can be found in the literature.

Unlike CXCR4, decreased cell surface expression of the CXCR4/CCR2 heterodimer was evident upon coexpression with Rab1 S25N [180]. Additionally, similar to both of its component receptors, the heterodimer also exhibited decreased cell surface expression when it was coexpressed with Rab8 T22N [180]. This is the first study to evaluate the effects of a subset of Rab GTPases on cell surface expression of the CXCR4/CCR2 heterodimer. However, this study does agree with that of Charette et al. (2011) [114]. Charette et al. (2011) [114] used Jurkat cells to determine the Rab GTPases required for the trafficking of CXCR4 and CCR5 homo- and heterodimers. The CXCR4 homodimer required Rab2, Rab 6 and Rab8 for proper trafficking to the plasma membrane and trafficking requirements did not change in the presence of CD4 [114]. The CCR5 homodimer required Rab1 and Rab11 when expressed without CD4, but required Rab1 and Rab8 in the presence of CD4 [114]. Additionally, the CXCR4/CCR5 heterodimer required Rab1, Rab2 and Rab11 when expressed without CD4, but only Rab1 was necessary for proper heterodimer trafficking in the presence of CD4 [114]. Therefore, this study and the work of this thesis both support the importance of recognizing heterodimers as having unique trafficking requirements when compared to their constituent receptors.

Rab8 was required for the trafficking of endogenous CXCR4, endogenous CCR2 and the overexpressed CXCR4V1/CD4V2 heterodimer. Therefore, this Rab GTPase may

be essential for the trafficking of receptors from between the Golgi complex and plasma membrane. In terms of endogenous CXCR4 and CCR2, Rab6 was required for the shuttling of these receptors within the Golgi complex. However, the overexpressed CXCR4V1/CD4V2 heterodimer did not rely on Rab6 for trafficking within the Golgi. Therefore, further study of the Rab GTPases required for trafficking of the heterodimer within the Golgi complex may reveal the Rab GTPases responsible for this portion of the heterodimer's anterograde trafficking pathway.

4.1.4 Rab GTPases Affect Both Receptor Cell Surface Expression and Signaling

Investigation of a signal transduction cascade known to be initiated through the activation of either CXCR4 or CCR2 can act to confirm Rab GTPase regulation in the anterograde trafficking of these receptors [180]. It would also corroborate data obtained by cell surface assays, as was done in by previous studies [106,112].

When CXCR4 was studied, cells that expressed the DN mutants of Rab2, Rab6 and Rab8 exhibited significant decreases in relation to both the expression of CXCR4 at the plasma membrane and FAK activation after stimulation with SDF-1α [180]. Similarly, expression of the DN mutant constructs that impaired CCR2 cell surface expression (Rab1, Rab6 and Rab8) also impaired FAK activation following MCP-1 stimulation [180].

Upon SDF-1α or MCP-1 stimulation of the CXCR4/CCR2 heterodimer, it was apparent that the same RabGTPase DN constructs hindering cell surface expression of the heterodimer also decreased its activation by either ligand [180]. Therefore, these results support those found in the cell surface assays because proper expression of functional Rab1 and Rab8 GTPases was required for expression of this

heterodimer at the cell surface [180]. Because of altered cell surface expression of the heterodimer upon transfection with Rab1 and Rab8 DN constructs, signaling through the heterodimer was negatively affected with each of these constructs [180].

4.1.5 Future Work

It is possible to extend the work of this project further by exploring several different aspects and consequences of the anterograde trafficking requirements of CXCR4, CCR2 and the resultant heterodimer. Because these receptors are involved in many cancers other than just prostate cancer [135,149,150], it would be interesting to determine whether they retain the same anterograde trafficking requirements of the Rab GTPases in other cancer cell lines as those found in the PC3 cell line. If these receptors relied on the same Rab GTPases in a wide array of cancer cell lines, it would indicate that their trafficking pathways may not be tissue specific and that therapeutics targeting one or more of these Rab GTPases could be useful to treat a myriad of cancer types. Although one might suggest confirming the results of the present study through siRNA or shRNA knockdown of the Rab GTPase isoforms studied, knockdown of Rab GTPase function in other studies garnered the same results transfection with DN mutant constructs. An example of this is evident in the study by Wu et al. (2003) [112].

Because FAK is associated with integrin-mediated migration [201], it would be interesting to relate decreased FAK activation to changes in the migratory or invasive properties of PC3 cells. A variety of methods such as scratch assays, along with transwell migration and invasion assays, have been developed to study migration and invasion of cells under different conditions and treatments [202]. This data would add to the observation that by affecting the anterograde trafficking of receptors, functional

consequences associated with such receptors are also affected. CXCR4 and CCR2 signaling pathways associated with signaling mediators other than FAK could also be explored to further add to this evidence of functional consequences relating to the anterograde trafficking of these chemokine receptors.

This work can also be extended in a more general sense than the options listed above. By discussing how Rab GTPases may play key roles in the development of novel therapeutics to treat a variety of disease states, especially cancer, Cheng et al. (2005) noted that some human tumours exhibit upregulation of Rab GTPase gene expression, resulting in increased Rab GTPase expression at the mRNA and possibly protein level [203]. Therefore, we are not the first group to recognize the importance of Rab GTPases in regulatory processes that could lead to the progression of disease states.

Any study of the Rab GTPases required for the cell surface expression of a receptor requires knowledge of how Rab GTPases transport vesicles between membranes of origin and target membranes. By acting in concert with a variety of effector molecules, each Rab GTPase cycles between its active GTP-bound form and inactive GDP-bound form to move vesicular cargo. These effector molecules include GEFs, GAPs and REPs [105]. However, there are also GDP-dissociation inhibitors (GDIs) that may interact with GDP-bound Rabs, hindering activation [105]. Therefore, it is possible to limit the cell surface expression of a receptor by manipulation of the effector molecules required for proper Rab GTPase function.

The manipulation of these effector molecules by pharmacological compounds may reveal novel therapeutic strategies to disrupt expression of receptors associated with disease progression. By reviewing the roles of such compounds in the schematic

presented by Figure 1.4, it is apparent that these compounds could either hinder GEF activity or act like GDIs to prevent the activation of GDP-bound Rabs. These compounds could also mimic GAPs to inactivate GTP-bound Rabs. They could also commandeer REP function such that vesicle cargo could be mistrafficked.

There are many examples of how manipulation of these effector molecules has already been carried out by bacterial pathogens. For instance, many proteins of *Legionella pneumophila*, a human pathogen, modify Rab proteins. The defect in Rab1 recruitment A protein has been found to have GEF activity towards Rab1 GTPase [204], while the type 1 signal peptidase is known to have GAP activity in relation to its effect on Rab1 GTPase [205].

Rab effectors are also present in mammalian cells. For example, Rab8 interacting protein has GEF activity towards Rab8 [206] and a large scale screening of Rab binding proteins has demonstrated that there is a variety of Rab effectors with the ability to associate with more than one Rab GTPase isoform [207]. Therefore, the present study acts to enhance what is known about how specific Rab GTPases alter the trafficking of chemokine GPCRs to the plasma membrane, but it may also lead to the identification of portential targets for future pharmacological interventions.

Cheng et al. (2005) also indicated that Rab GTPases may also play a role in the transduction of cellular cascades promoting cancer cell survival [203]. This is due to the association of signaling mediators with vesicles requiring Rab GTPases for transport to designated areas throughout the cell [203]. Consequently, more detailed studies of the specific Rab GTPases associated with cellular signaling and not necessarily anterograde trafficking may also be useful to elucidate their role in disease states.

4.1.6 Significance of CXCR4 and CCR2 Trafficking by Rab GTPases

It has been shown that CXCR4 requires Rab2, Rab6 and Rab8 for expression at the cell membrane [180]. CXCR4 also requires the same subset of Rab GTPases for initiation of signaling, as measured by FAK activation, after stimulation with SDF-1α [180]. Unlike CXCR4, CCR2 relies on Rab1, Rab6 and Rab8 for expression at the plasma membrane [180]. Due to the decreased cell surface expression of CCR2 that results from expression of Rab1, Rab6 and Rab8 DN constructs, these constructs also dampened FAK activation [180]. The CXCR4/CCR2 heterodimer differed from each of its constituent receptors in regards to the Rab GTPases necessary for its anterograde trafficking to the plasma membrane [180]. Rab1 and Rab8 are necessary for plasma membrane expression of the CXCR4/CCR2 heterodimer [180]. Coexpression of the heterodimer with Rab1 and Rab8 DN constructs resulted in decreased FAK activation after stimulation with either SDF-1α or MCP-1 [180].

The results of the present study indicate that individual receptors have different trafficking requirements than the heterodimer. Although the reasons for the unique trafficking requirements of each receptor and the heterodimer are not yet known, this work supports the belief that heterodimeric receptors must be viewed as having unique properties when compared their constituent receptors. Because the receptors of this project play central roles in initiating the signaling cascades responsible for many processes associated with cancer progression, factors that regulate their anterograde trafficking to the plasma membrane represent an important area of cancer metastasis research.

4.2 Exploratory Work Relating to Molecular Chaperones Guiding Maturation of CCR5 and CD4 Receptors

4.2.1 Importance and Overview

Many aspects of the GPCR life cycle are relatively well known. These aspects include processes that control signal termination processes, most notably desensitization and endocytosis. Conversely, processes governing both the maturation of newly synthesized receptors, assembly of receptor signaling complexes and their trafficking to the plasma membrane are generally not well characterized. The first portion of this discussion explored results relating to how a subset of Rab GTPases are important in regulating the anterograde trafficking of chemokine GPCRs. However, this portion of the discussion will examine an exploratory approach that was taken in an effort to study the interaction of molecular chaperones whose constructs or inhibitory RNAs were already available in the Dupré lab (calnexin, calreticulin, GRP94, Hsp70, BiP, PDIA3 and DRiP78) with CCR5 and CD4, two receptors heavily implicated in HIV infection. By studying different aspects of the maturation and trafficking of GPCR, processes associated with the proper transport of these receptors from the ER to the plasma membrane can be determined. Important mediators of these processes can also be identified with the ultimate goal of identifying potentially druggable targets.

Although this portion of the project is rather removed from identifying druggable targets, the need to expand this area of research is apparent upon examination of the current state of therapeutics aimed at HIV treatment. Once diagnosed with HIV, individuals are prescribed a cocktail of antiretroviral drugs in an attempt to limit infection [208]. Each antiretroviral agent is classified depending on the viral life-cycle phase it

attempts to inhibit [208]. Employing several different classes of antiretroviral agents at one time is referred to as highly active antiretroviral therapy (HAART) [208]. While HAART has been shown to decrease viral load, slow disease progression and help maintain the integrity of the immune system [209], HIV strains are evolving to escape mechanisms of action associated with antitretroviral agents. For example, due to mutations in gp41, HIV has developed resistance to enfuvirtide, a first entry inhibitor that binds to gp41 to hinder pore formation and prevent the virus from entering the cell [210]. AMD3100 was originally developed to target CXCR4, preventing its association HIV [191]. However, due to its contribution to countless physiological processes, indication of AMD3100 to limit HIV infection was halted [191]. Furthermore, CCR5 antagonists were also tried in attempts to hinder HIV infection. These drugs also resulted in severe side effects, such as liver toxicities [211]. As a result, only one of these CCR5 antagonists (maraviroc) is still indicated for clinical use in particular subsets of HIV-1 patients [212]. Unfortunately, resistance to maraviroc has also been documented [212]. Consequently, studying new ways to hinder cell surface expression of HIV co-receptors is still therapeutically relevant in light of the pitfalls of ligand-based therapies.

4.2.2 Co-IP Experiments Indicate a Possible Interaction Between Each Receptor and Molecular Chaperone Investigated

The fact that the CCR5Δ32 mutation is associated with a phenotype that is resistant to HIV infection underlines the role of this co-receptor in viral pathology [167,184]. Complete resistance to R5 virus strains is conferred to individuals homozygous for the CCR5Δ32 mutation, while heterozygotes harboring the CCR5Δ32 mutation experience slow disease progression [184,213]. The health of individuals harboring the CCR5Δ32 mutation is not normally affected by this mutation [170].

Therefore, studying how molecular chaperones may differentially interact with CCR5 homo- and heterodimers can introduce novel ways in which this receptor's plasma membrane expression can be limited. Interfering with CCR5 plasma membrane expression could someday prove as a useful anti-HIV therapeutic strategy.

Co-IP experiments were conducted to determine the interaction of each chaperone with CCR5-GFP, CD4-GFP, the CCR5 homodimer and the CCR5/CD4 heterodimer. CCR5-GFP and CD4-GFP were included to examine how each chaperone may interact with these receptors individually and if such interactions would be maintained upon dimer formation. These experiments indicated an interaction between each receptor and receptor dimer investigated.

A variety of databases (for example, STRING (string-db.org), BioGRID (thebiogrid.org), IntAct (ebi.ac.uk/inact) and PubMed (http://www.ncbi.nlm.nih.gov/pubmed/)) were searched to determine if the chaperones studied in this project were previously shown to interact with either CCR5 or CD4. CCR5 has been found to associate with microbial Hsp70 [214]. Both HA-CCR5 WT and the CCR5 homodimer can interact with DRiP78 [215]. BiP and calnexin were also shown to colocalize with CCR5 in a Chinese hamster ovary cell line [216]. In terms of CD4, a proteomic screen of interaction partners indicated that CD4 can interact with isoforms of PDIs and heat shock proteins in human primary macrophages [217]. Therefore, these references serve to indicate that the interactions noted in this study may indeed be occurring.

The present co-IP experiments did not indicate a differential interaction between chaperones and the individual receptors, CCR5 homodimer or CCR5/CD4 heterodimer.

However, a previous study noted that DRiP78 interacts with both CCR5 and CXCR4, but does not interact with the CCR5/CXCR4 heterodimer [215]. Another study noted that chaperones showed differential interaction amongst AT1R homodimers, β2AR homodimers and the AT1R/β2AR heterodimer [100]. It is possible that by employing different techniques to examine protein-protein interactions, the chaperones studied in this project may be shown to interact with individual receptors, but not the dimers.

4.2.3 Molecular Chaperones Have Differential Effects on the Maturation of Each Receptor and Receptor Pair

Molecular chaperones have been found to play numerous roles in the maturation of a variety of GPCRs. Whereas some molecular chaperones are known to affect GPCR maturation, others are known to modulate plasma membrane expression and glycosylation of these receptors [218]. For example, as reviewed by Williams et al. (2011) [218], BiP and GRP94 assist in the proper folding of the LHR, whereas DRiP78 is known to regulate D_1R plasma membrane expression and melanocortin-2 receptor protein (MRAP) is involved in the glycosylation of the melanocortin-2 receptor.

As a way of determining how different chaperones contribute to the maturation of GPCR homo- and heterodimers, levels of these chaperones were altered in the cell and fluorescence readings of receptor constructs were used as a measure of receptor maturation or dimer formation, with results summarized in Table 4.1. It was found that DRiP78 WT overexpression resulted in a significant decrease in observed fluorescence when cotransfected with CCR5-GFP, as was similarly observed by Kuang et al. (2012) [215]. Interestingly, DRiP78 WT had no effect on the heterodimer, while it negatively affected CCR5 maturation. Furthermore, overexpression of calnexin WT resulted in a significant increase in observed fluorescence of CCR5-GFP. Therefore, DRiP78 WT

may retain the receptor within the ER during the folding and maturation steps required by CCR5-GFP. Because CCR5-GFP maturation was negatively affected by this ER chaperone, it is possible that DRiP78 WT overexpression hindered proper folding of CCR5-GFP and prevented either the further modification of CCR5-GFP by other chaperones or export of this receptor from the ER. Because calnexin WT overexpression had the opposite effect of DRiP78 WT overexpression, calnexin may be involved in the proper folding of CCR5-GFP and overexpression of this chaperone could have promoted CCR5-GFP folding and maturation.

Overexpression of BiP WT resulted in increased fluorescence when cotransfected with CD4-GFP, demonstrating a possible role of this chaperone in the folding of CD4. Therefore, much like how calnexin WT may promote the folding of CCR5-GFP, BiP WT may promote the folding of CD4-GFP.

By studying homo- or heterodimerization with either the CCR5V1/CCR5V2 or CCR5V1/CD4V2 receptor pairs, it is possible to examine how molecular chaperones may influence the assembly of two receptors in a dimeric complex. In terms of the CCR5V1/CCR5V2 homodimer, overexpression of DRiP78 WT and that of the BiP M significantly decreased observed fluorescence. The DRiP78 WT result coincides with that of a previous study [215] and may be due to this chaperone's ability to limit CCR5 expression at the plasma membrane. Therefore, the proper folding of this receptor by DRiP78 WT may represent a required step prior to the formation of a homodimer with its complementary receptor. Whereas BiP M overexpression did not affect the folding of CCR5-GFP, it negatively affected the assembly of the CCR5V1/CCR5V2 heterodimer.

The effect of the BiP M used in this study suggests that this construct prevented proper CCR5V1/V2 assembly in the ER.

Unlike the homodimer, knockdown of Hsp70 and calreticulin decreased fluorescence associated with the CCR5V1/CD4V2 heterodimer. Whereas knockdown of Hsp70 did not affect the folding of each constituent receptor in the heterodimer, it is possible that this chaperone is required for the association of CCR5 and CD4 into a dimeric complex. Because Hsp70 is primarly found in the cytoplasm [89], this result indicates that a proportion of heterodimeric complexes may form upon export from the ER. While BiP WT resulted in an increased fluorescence, both its mutant (BiP T37G) and GRP94 WT resulted in significantly decreased fluorescence. As a result, overexpression of BiP M negatively affected the assembly of both homo- and heterdimers. Although overexpression of GRP94 WT did not influence homodimer formation, it hindered assembly of the heterodimer. Conversely, BiP WT overexpression promoted heterodimer assembly, possibly due to its positive effects on the folding of individual CD4 receptors.

It is evident that the molecular chaperones affecting maturation of the homodimer differed from those that affected maturation of the heterodimer (Table 4.1). It is possible that as receptors interact with one another, potential sites of interaction with molecular chaperones become masked, leading to different patterns of molecular chaperone interaction between homo- and heterodimers.

The role of BiP overexpression was examined in relation to the thyrotropin receptor. It was found that BiP overexpression resulted in enhanced degradation of this receptor [219]. Contrary to the results of this thesis work, BiP has been shown to

negatively influence receptor maturation as it may promote retrograde translocation to the ER and subsequent protein degradation [219]. Therefore, this "destabilizing effect" [219] of BiP was not found in this thesis work. Receptor interactions with calreticulin and calnexin, and later interaction with PDIA3, have been shown to stabilize newly synthesized receptors and enhance folding of these receptors [219]. Only modulation of calreticulin was sufficient to decrease CCR5/CD4 receptor interaction. Consistent with the observations that Hsp70 promotes receptor folding and maturation [220], it was found that knockdown of this molecular chaperone negatively affected CCR5/CD4 heterodimer formation. Nevertheless, it would be prudent to confirm these findings with western blot analysis.

Since it was found that effects on recepotor folding and subsequent assembly into dimeric complexes was most often affected by overexpression of molecular chaperone constructs and not knockdown with inhibitory RNAs, it is possible that protein knockdown levels were not sufficient to elicit biological effects. It is also possible that while the knockdown was performed with high efficiency, thus preventing the synthesis of new chaperones, the natural degradation process of the chaperones remained incomplete. Consequently, high levels of endogenous chaperones in the experimental cells synthesized prior to transfection may have remained throughout the experiment. Therefore, subsequent experiments may benefit from examining knockdown at a time point that extends beyond that of 48 h to ensure chaperone levels are maximally depleted from the cells.

4.2.4 BRET Experiments Do Not Indicate Molecular Chaperone Effects on the Preasssembly of Signaling Complexes

Several studies have demonstrated that GPCRs become associated with members of their respective signaling complexes prior to being expressed at the plasma membrane [113,221]. Therefore, signaling complex assembly may not require ligand activation [222]. Because chaperones influence early synthesis and maturation of receptors, preliminary BRET experiments were conducted to determine if chaperones could guide the association of G-proteins with CCR5, the CCR5 homodimer or CCR5/CD4 heterodimer. The G-proteins coexpressed with each receptor or receptor pair included Gβ1 and Gγ2. Similar to a previous study that examined the effect of chaperones on the association of G-proteins with AT1R/β2AR homo- and heterodimers [100], no significant differences are apparent with any of the receptors or receptor dimers tested.

A study of AT1R/β2AR homo- and heterodimers tested possible effects of BiP, calreticulin and PDIA3 on G-protein association with receptor dimers, but also examined the coupling of Gαs and Gαi with these dimers [100]. Therefore, it is possible that the chaperones studied in this thesis may be required for the coupling of Gαi and Gαq with CCR5, the CCR5 homodimer or CCR5/CD4 heterodimer and this would require further testing with BRET experiments. It is well established that CCR5 can couple with Gαi and Gαq [223]. It is also likely that chaperones other than those studied in this thesis may influence the association of G-proteins with receptor dimers. As a result, a more rigorous investigation into possible interaction partners must be conducted. Such an investigation can be accomplished by the variety of means by which researchers study protein-protein interactions, including two-hybrid screening or more sophisticated mass

spectrometry techniques [199].

4.2.5 Future Work

Because this project took an exploratory approach on the chaperones that guide the maturation of CCR5 and CD4 receptors, several different avenues of investigation can be explored. In the aforementioned study of proteins that interacted with CD4, a variety of chaperones were reported [217]. Therefore, a more extensive list of chaperones can be studied in terms of how they may influence the maturation of individual receptors or the resultant dimers (the CCR5 homodimer or the CCR5/CD4 heterodimer). Chaperone interactions could also be studied in other cell lines. One cell line of interest is a human osteosarcoma cell line that can stably express CCR5 and CD4 [224]. This would allow each receptor to be studied without the need for overexpression.

There are also a variety of different methods by which chaperone effects receptor maturation can be measured. While the present study looked at whole cell fluorescence as a measure of receptor present, florescence activated cell sorting, as well as cell surface biotin-labeling assays can be used to determine chaperone involvement in the trafficking of each receptor to the plasma membrane. It can also be investigated whether or not glycosylation (a key step in the maturation of many receptors) of CCR5 and CD4 dictate the nature of their chaperone interactions. Because it was determined that non-glycosylated mutants of a dimer's constituent receptors can dictate a dimer's cell surface expression [100], a construct could be designed such that a point mutation would be introduced into the O-glycosylation site of CCR5 at serine 6 in the N-terminus [225]. It is possible that the chaperones tested in this study may show different interaction patterns with dimers containing the non-glycosylated CCR5 mutant, when compared to those with

the WT form of this receptor. If this was the case, confocal microscopy could be completed to determine where in the cell these dimers were retained if they were not properly expressed at the plasma membrane.

4.2.6 Significance of the Exploratory Study Relating to Molecular Chaperones

This portion of the thesis ventured to provide preliminary insight into the mechanisms required for the export of the GPCR chemokine receptor CCR5 from the ER. CCR5 was studied in terms of the CCR5 homodimer, but also in terms of its formation of a heterodimer with CD4. By trying to establish which chaperones may be interacting with CCR5 or dimers of this chemokine GPCR, it was determined that Hsp70, calreticulin, GRP94 and BiP may play a role in the maturation of the CCR5/CD4 heterodimer. Unlike the CCR5/CD4 heterodimer, DRiP78 and BiP influenced CCR5 homodimer formation. This highlights the importance of specificity of these chaperones in terms of homo- and heterodimer formation. The chaperones investigated in BRET experiments did not influence G-protein coupling to receptors. However, this does not rule out the possibility that chaperones may indeed be involved in the coupling of G-proteins to receptors. A more exhaustive list of chaperones could be tested in an attempt to characterize their effects on G-protein coupling to receptors, while also examining their effects on CCR5 homo- and heterodimer formation.

4.3 Overall Conclusion

Both projects of this thesis attempt to examine complementary requirements for the expression of chemokine GPCRs and their dimers at the plasma membrane. Whereas each dimer is formed of constituent receptors, it is important to keep in mind that the trafficking and maturation requirements of these constituent receptors do not necessarily

dictate those of the dimer. By identifying essential components required for receptor trafficking and maturation, a broader understanding of a relatively unknown aspect of the GPCR life cycle can be gained. Knowledge relating to anterograde pathways of CXCR4, CCR2 and CCR5 dimers may one day aid in the development of novel pharmaceuticals as each of these GPCRs have been implicated in multiple disease states.

Table 4.1 Summary of results relating to the effects of molecular chaperones on the maturation of CCR5-GFP, CD4-GFP, CCR5V1/CCR5V2 and CCR5V1/CD4V2.

	Hsp70 siRNA	Calr3 siRNA	PDIA3 siRNA	DRiP78 shRNA	DRiP78 WT	GRP94 WT	BiP WT	BiP T37G	Caln WT
CCR5-GFP	---	---	---	---	↓	---	---	---	↑
CD4-GFP	---	---	---	---	---	---	↑	---	---
CCR5/CCR5 (V1/V2)	---	---	---	---	↓	---	---	↓	---
CCR5/CD4 (V1/V2)	↓	---	---	---	---	↓	↑	↓	---

BIBLIOGRAPHY

1. Lefkowitz RJ. (2000) The superfamily of heptathelical receptors. Nature Cell Biology 2: E133-E136.

2. Nathans J, Hogness DS. (1983) Isolation, sequence analysis, and intron-exon arrangement of the gene encoding bovine rhodopsin. Cell 34: 807-814.

3. Dixon RAF, Kobilka BK, Strader DJ, Benovic JL, Dohlman HG, et al. (1986) Cloning of the gene and cDNA for mammalian β-adrenergic receptor and homology with rhodopsin. Nature 321: 75-79.

4. Kroeze WK, Sheffler DJ, Roth BL. (2003) G-protein-coupled receptors at a glance. J Cell Sci 116: 4867-4869.

5. Lattin J, Zidar DA, Schroder K, Kellie S, Hume DA, et al. (2007) G-protein-coupled receptor expression, function, and signaling in macrophages. J Leukoc Biol 82: 16-32.

6. Vassilatis DK, Hohmann JG, Zeng H, Li F, Ranchalis JE, et al. (2003) The G protein-coupled receptor repertoires of human and mouse. Proc Natl Acad Sci U S A 15: 4903-4908.

7. Insel PA, Tang CM, Hahntow I, Michel MC. (2007) Impact of GPCRs in clinical medicine: Genetic variants and drug targets. Biochim Biophys Acta 1768: 994-1005.

8. Hopkins AL, Groom CR. (2002) The druggable genome. Nat Rev Drug Discov 1: 727-730.

9. Fillmore D. (2004) It's a GPCR world. MDD 11: 24-28.

10. Overington JP, Al-Lazikani B, Hopkins AL. (2006) How many drug targets are there? Nat Rev Drug Discov 5: 993-996.

11. Fredriksson R, Lagerström MC, Lundin LG, Schiöth HB. (2003) The G-protein-coupled receptors in the human genome form five main families, phylogenetic analysis, paralogon groups, and fingerprints. Mol Pharmacol 63: 1256-1272.

12. Atwood TK, Findlay JB. (1994) Fingerprinting G-protein-coupled receptors. Protein Eng 7: 195-203.

13. Kolakowski LF. (1994) GCRDb: A G-protein-coupled receptor database. Recept Channels 2: 1-7.

14. Kobilka BK. (2007) G protein coupled receptor structure and activation. Biochim Biophys Acta 1768: 794-807.

15. Ellis C. (2004) The state of GPCR research in 2004. Nat Rev Drug Discov 3: 577-626.

16. Davies MN, Secker A, Haling-Brown M, Moss DS, Freitas AA, et al. (2008) GPCRTree: Online hierarchical classification of GPCR function. BMC Res Notes 1: 67.

17. Kniazeff J, Prézeau L, Rondard P, Pin JP, Goudet C. (2011) Dimers and beyond: The functional puzzles of class C GPCRs. Pharmacol Ther 130: 9-25.

18. Pellissier LP, Barthet G, Gaven F, Cassier E, Trinquet E, et al. (2011) G protein activation by serotonin type 4 receptor dimers: Evidence that turning on two protomers is more efficient. J Biol Chem 286: 9985-9997.

19. Oldham WM, Hamm HE. (2008) Heterotrimeric G protein activation by G-protein-coupled receptors. Nat Rev Mol Cell Biol 9: 60-71.

20. Neves SR, Ram PT, Iyengar R. (2002) G protein pathways. Science 296: 1636-1639.

21. Hein P, Rochais F, Hoffmann C, Dorsch S, Nikolaev VO, et al. (2006) Gs activation is time-limiting in initiating receptor-mediated signaling. J Biol Chem 281: 33345-33351.

22. Balla T. (2010) Putting G protein-coupled receptor-mediated activation of phospholipase C in the limelight. J Gen Physiol 135: 77-80.

23. Vroon A, Heijen CJ, Kavelaars A. (2006) GRKs and arrestins: Regulators of migration and inflammation. J Leukoc Biol 80: 1214-1221.

24. Xiao K, McClatchy DB, Shukla AK, Zhao Y, Chen M, et al. (2007) Functional specialization of beta-arrestin interactions revealed by proteomic analysis. Proc Natl Acad Sci U S A 104: 12011-12016.

25. Maggio R, Vogel Z, Wess J. (1993) Coexpression studies with mutant muscarinic/adrenergic receptors provide evidence for intermolecular "cross-talk" between G-protein-linked receptors. Proc Natl Acad Sci U S A 90: 3103-3107.

26. Margeta-Mitrovic M, Jan YN, Jan LY. (2000) A trafficking checkpoint controls GABA(B) receptor heterodimerization. Neuron 27: 97-106.

27. White JH, Wise A, Main MJ, Green A, Fraser NJ, et al. (1998) Heterodimerization is required for the formation of a functionl GABA(B) receptor. Nature 392: 679-682.

28. Hebert TE, Moffett S, Morello JP, Loisel TP, Bichet DG, et al. (1996) A peptide derived from a beta2-adrenergic receptor transmembrane domain inhibits both receptor dimerization and activation. J Biol Chem 271: 16384-16392.

29. Salim K, Fenton T, Bacha J, Urien-Rodriguez H, Bonnert T, et al. (2002) Oligomerization of G-protein-coupled receptors shown by selective co-immunoprecipitation. J Biol Chem 277: 15482-15485.

30. Kuner R, Köhr G, Grünewald S, Eisenhardt G, Bach A, et al. (1999) Role of heterodimer formation in GABAB receptor function. Science 283: 74-77.

31. Kerppola TK. (2008) Bimolecular fluorescence complementation: Visualization of molecular interactions in living cells. Methods Cell Biol 85: 431-470.

32. Rivero-Müller A, Chou Y-, Ji I, Lajic S, Hanyaloglu AC, et al. (2009) Rescue of defective G protein-coupled receptor function in vivo by intermolecular complementation. Proc Natl Acad Sci U S A 107: 2319-2324.

33. Milligan G, Bouvier M. (2005) Methods to monitor the quaternary structure of G protein-coupled receptors. FEBS Journal 272: 2914-2925.

34. Sohy D, Yano H, de Nadai P, Urizar E, Guillabert A, et al. (2009) Hetero-oligomerization of CCR2, CCR5, and CXCR4 and the protean effects of "selective" antagonists. Journal of Biological Chemistry 284: 31270-31279.

35. Villemure J-, Adam L, Bevan NJ, Gearing K, Chénier S, et al. (2005) Subcellular distribution of GABAB receptor homo- and hetero-dimers. Biochemical Journal 388: 47-55.

36. Terrillon S, Durroux T, Mouillac B, Breit A, Ayoub MA, et al. (2003) Oxytocin and vasopressin V1a and V2 receptors form constitutive homo- and heterodimers during biosynthesis. Molecular Endocrinology 17: 677-691.

37. Chen JD, Bai X, Yang AG, Cong Y, Chen SY. (1997) Inactivation of HIV-1 chemokine co-receptor CXCR-4 by a novel intrakine strategy. Nature Medicine 10: 1110-1116.

38. Issafras H, Angers S, Bulenger S, Blanpain C, Parmentier M, et al. (2002) Constitutive agonist-independent CCR5 oligomerization and antibody-mediated clustering occurring at physiological levels of receptors. J Biol Chem 277: 34666-34673.

39. Vilardaga JP, Nikolaev VO, Lorenz K, Ferrandon S, Zhuang Z, et al. (2008) Conformational cross-talk between alpha2A-adrenergic and mu-opioid receptors controls cell signaling Nat Chem Biol 4: 126-131.

40. Monnier C, Tu H, Bourrier E, Vol C, Lamarque L, et al. (2011) Trans-activation between 7TM domains: Implication in heterodimeric GABAB receptor activation. EMBO J 30: 32-42.

41. Gomes I, Filipovska J, Jordan BA, Devi LA. (2002) Oligomerization of opioid receptors Methods 27: 358-365.

42. Jordan BA, Devi LA. (1999) G-protein-coupled receptor heterodimerization modulates receptor function Nature 399: 697-700.

43. Waldhoer M, Fong J, Jones RM, Lunzer MM, Sharma SK, et al. (2005) A heterodimer-selective agonist shows in vivo relevance of G protein-coupled receptor dimers Proc Natl Acad Sci U S A 102: 9050-9055.

44. Breit A, Gagnidze K, Devi LA, Lagace M, Bouvier M. (2006) Simultaneous activation of the delta opioid receptor (deltaOR)/sensory neuron-specific receptor-4 (SNSR-4) hetero-oligomer by the mixed bivalent agonist bovine adrenal medulla peptide 22 activates SNSR-4 but inhibits deltaOR signaling Mol Pharmacol 70: 686-696.

45. Hasbi A, Nguyen T, Fan T, Cheng R, Rashid A, et al. (2007) Trafficking of preassembled opioid mu-delta heterooligomer-gz signaling complexes to the plasma membrane: Coregulation by agonists Biochemistry 46: 12997-13009.

46. Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, et al. (2000) Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers J Biol Chem 275: 7862-7869.

47. Jordan BA, Trapaidze N, Gomes I, Nivarthi R, Devi LA. (2001) Oligomerization of opioid receptors with beta 2-adrenergic receptors: A role in trafficking and mitogen-activated protein kinase activation Proc Natl Acad Sci U S A 98: 343-348.

48. Hillion J, Canals M, Torvinen M, Casado V, Scott R, et al. (2002) Coaggregation, cointernalization, and codesensitization of adenosine A2A receptors and dopamine D2 receptors J Biol Chem 277: 18091-18097.

49. Pfeiffer M, Koch T, Schroder H, Laugsch M, Hollt V, et al. (2002) Heterodimerization of somatostatin and opioid receptors cross-modulates phosphorylation, internalization, and desensitization J Biol Chem 277: 19762-19772.

50. Xu J, He J, Castleberry AM, Balasubramanian S, Lau AG, et al. (2003) Heterodimerization of alpha 2A- and beta 1-adrenergic receptors J Biol Chem 278: 10770-10777.

51. Terrillon S, Barberis C, Bouvier M. (2004) Heterodimerization of V1a and V2 vasopressin receptors determines the interaction with beta-arrestin and their trafficking patterns Proc Natl Acad Sci U S A 101: 1548-1553.

52. Cooper GM. (2000) The endoplasmic reticulum. In: Anonymous The Cell: A Molecular Approach. Sunderland (MA): Sinauer Associates.

53. Cooper GM. (2000) Translation of mRNA. In: Anonymous The Cell: A Molecular Approach. Sunderland (MA): Sinauer Associates.

54. Talal N, Kaltreider HB. (1968) Functional and structural studies of membrane-bound and free ribosomes from rat spleen. J Biol Chem 243: 6504-6510.

55. David M, Richer M, Mamarbachi AM, Villeneuve LR, Dupre DJ, et al. (2006) Interactions between GABA-B1 receptors and kir 3 inwardly rectifying potassium channels Cell Signal 18: 2172-2181.

56. Salshpour A, Angers S, Mercier J-, Lagacé M, Marullo S, et al. (2004) Homodimerization of the β2-adrenergic receptor as a prerequisite for cell surface targeting. J Biol Chem 279: 33390-33397.

57. Benkirane M, Jin D-, Chun RF, Koup RA, Jeang K-. (1997) Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. J Biol Chem 272: 30603-30606.

58. Davare MA, Avdonin V, Hall DD, Peden EM, Burette A, et al. (2001) A beta-2 adrenergic receptor signaling complex assembled with the Ca2+ channel Cav1.2. Science 293: 98-101.

59. Lavine N, Ethier N, Oak JN, Pei L, Lui F, et al. (2002) G protein-coupled receptors form stable complexes with inwardly rectifying potassium channels and adenylyl cyclase. J Biol Chem 277: 46010-46019.

60. Barki-Harrington L, Luttrell LM, Rockman HA. (2003) Dual inhibition of beta-adrenergic and angiotensin II receptors by a single antagonist: A functional role for reeptor-receptor interaction in vivo. Circulation 108: 1611-1618.

61. Wu G. (2013) Identification of endoplasmic reticulum export motifs for G protein-coupled receptors. Methods Enzymol 521: 189-202.

62. Petaja-Repo UE, Hogue M, Laperriere A, Walker P, Bouvier M. (2000) Export from the endoplasmic reticulum represents the limiting step in the maturation and cell surface expression of the human delta opiod receptor. J Biol Chem 275: 13727-13736.

63. Appenzeller-Herzog C, Hauri H-. (2006) The ER-golgi intermediate compartment (ERGIC): In search of its identity and function. J Cell Sci 119: 2173-2183.

64. Gu F, Crump CM, Thomas G. (2006) Trans-golgi network sorting. Cell Mol Life Sci 58: 1067-1084.

65. Xu D, Esko JD. (2009) A golgi-on-a-chip for glycan synthesis. Nat Chem Biol 5: 612-613.

66. Zhang K, Kaufman RJ. (2004) Singlaing the unfolded protein response from the endoplasmic reticulum. J Biol Chem 279: 25935-25938.

67. Irvine GB, El-Agnaf OM, Shankat GM, Walsh DM. (2008) Protein aggregation in the brain: The molecular basis for alzheimer's and parkinson's diseases. Mol Med 14: 451-464.

68. Arraste M, Finkbeiner S. (2012) Protein aggregates in huntington's disease. Exp Neurol 238: 1-11.

69. Good MC, Zalaton JG, Lim WA. (2011) Scaffold proteins: Hubs for controling the flow of cellular information. Science 332: 680-686.

70. Nagradova N. (2007) Enzymes catalyzing protein folding and their cellular functions. Curr Protein Pept Sci 8: 273-282.

71. Hebert DN, Molinari M. (2007) In and out of the ER: Protein folding, quality control, degradation, and related human diseases. Physiol Rev 87: 1377-1408.

72. Vembar SS, Brodsky JL. (2008) One step at a time: Endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol 9: 944-957.

73. Camacho P, John L, Li Y, Paredes RM, Roderick HL. (2000) Calnexin and calreticulin, ER associated modulators of calcium transport in the ER. Madame Curie Bioscience Database [Internet] 2013.

74. Corbett EF, Oikawa K, Francois P, Tessier DC, Kay C, et al. (1999) Ca2+ regulation of interactions between endoplasmic reticulum chaperones. J Biol Chem 274: 6203-6211.

75. Torres M, Encina G, Soto C, Hetz C. (2011) Abnormal calcium homeostasis and protein folding stress at the ER. Commun Integr Biol 4: 258-261.

76. Fliegel L, Burns K, MacLennan DH, Reithmeier RA, Michalak M. (1989) Molecular cloning of the high affinity calcium-binding protein (calreticulin) of skeletal muscle sarcoplasmic reticulum. J Biol Chem 264: 21522-21528.

77. Wada I, Rindress D, Cameron PH, Ou WJ, Doherty JJ, et al. (1991) SSR alpha and associated calnexin are major calcium binding proteins of the endoplasmic reticulum membrane. J Biol Chem 266: 19599-19610.

78. Danilczyk UG, Williams DB. (2001) The lectin chperone calnexin utilizes polypeptide-based interactions to associate with many of its substrates *in vivo*. J Biol Chem 276: 25532-25540.

79. Swanton E, High S, Woodman P. (2003) Role of calnexin in the glycan-independent quality control of proteolipid protein. EMBO J 22: 2948-2958.

80. Bedard K, Szabo E, Michalak M, Opas M. (2005) Cellular functions of endoplasmic reticulum chaperones calreticulin, calnexin, and ERp57. Int Rev Cytol 245: 91-121.

81. Peterson JR, Helenius A. (1999) In vitro reconstitution of calreticulin-substrate interactions. J Cell Sci 112: 2775-2784.

82. Schrag JD, Bergeron JJ, Li Y, Borisova S, Hahn M, et al. (2001) The structure of calnexin, an ER chaperone involved in quality control of protein folding. Mol Cell 8: 633-644.

83. Kato K, Kamiya Y. (2007) Structural views of glycoprotein-fate determination in cells. Glycobiol 17: 1031-1044.

84. Kozlov G, Bastos-Aristizabal S, Maatanen P, Rosenauer A, Zheng F, et al. (2010) Structural basis of cyclophilin B binding by the calnexin/calreticulin P-domain. J Biol Chem 285: 35551-35557.

85. Wang CC, Tsou CL. (1993) Protein disulfide isomerase is both an enzyme and a chaperone. FASEB J 7: 1515-1517.

86. Flynn GC, Pohl J, Flocco MT, Rothman JE. (1991) Peptide-binding specificity of the molecular chaperone BiP. Nature 24: 726-730.

87. Bukau B, Horwich AL. (1998) The Hsp70 and Hsp60 chaperone machines. Cell 92: 351-366.

88. Mayer MP, Kukau B. (2005) Hsp70 chaperones: Cellular functions and molecular mechanism. Cell Mol Life Sci 62: 670-684.

89. Michels AA, Kanon B, Konings AWT, Ohtsuka K, Bensaude O, et al. (1997) Hsp70 and Hsp40 chaperone activities in the cytoplasm and the nucleus of mammalian cells. J Biol Chem 272: 33283-33289.

90. Gething MJ. (1999) Role and regulation of the ER chaperone BiP. Semin Cell Dev Biol 10: 465-472.

91. Plemper RK, Böhmler S, Bordallo J, Sommer T, Wolf DH. (1997) Mutant analysis links the translocon and BiP to retrograde protein transport for ER degradation. Nature 388: 891-895.

92. Melnick J, Dul JL, Argon Y. (1994) Sequential interaction of the chaperones BiP and GRP94 with immunoglobulin chains in the endoplasmic reticulum. Nature 370: 373-375.

93. Kelley WL. (1998) The J-domain family and the recruitment of chaperone power. Trends Biochem Sci 23: 222-227.

94. Bermak JC, Li M, Bullock C, Zhou QY. (2011) Regulation of transport of the dopamine D1 receptor by a new membrane-associated ER protein. Na Cell Biol 3: 492-498.

95. Leclerc PC, Auger-Messier M, Lanctot PM, Escher E, Leduc R, et al. (2001) A polyaromatic caveolin-binding-like motif in the cytoplasmic tail of the type 1 receptor for angiotensin II plays an important role in receptor trafficking and signaling. Endocrinology 143: 4702-4710.

96. Baker AK, Colley NJ, Zuker CS. (1994) The cyclophilin homolog NinaA functions as a chaperone, forming a stable complex in vivo with its protein target rhodopsin. EMBO J 13: 4886-4895.

97. Ferreira PA, Nakayama TA, Pak WL, Travis GH. (1996) Cyclophilin-related protein RanBP2 acts as chaperone for red/green opsin. Natre 383: 637-640.

98. Free RB, Hazelwood LA, Cabrera DM, Spalding HN, Namkung Y, et al. (2007) D1 and D2 dopamine receptor expression is regulated by direct interaction with the chaperone protein calnexin. J Bio Chem 282: 21285-21300.

99. Abd AJ, Reeck K, Langer A, Streichert T, Quitterer U. (2009) Calreticulin enhances B2 bradykinin receptor maturation and heterodimerization. Biochem Biophys Res Commun 387: 186-190.

100. Hammad MH, Dupré DJ. (2010) Chaperones contribute to G protein coupled receptor oligomerization, but do not participate in assembly of the G ptoein with the receptor signaling complex. J Mol Signal 5: 1-13.

101. Pereira-Lea J, Seabra MC. (2001) Evolution of the rab family of small GTP-binding proteins. J Mol Biol 313: 889-901.

102. Schwatrz SL, Cao C, Pylypenko O, Rak A, Wandinger-Ness A. (2007) Rab GTPases at a glance. J Cell Sci 120: 3905-3910.

103. Stenmark H, Olkkonen VM. (2001) The RabGTPase family. Genome Biol 2: reviews3007-reviews3007.7.

104. Hutagalung AH, Novick PJ. (2011) Role of RabGTPases in membrane traffic and cell physiology. Physiol Rev 91: 119-149.

105. Stenmark H. (2009) Rab GTPase functions in vesicle trafficking. Nat Rev Mol Cell Biol 10: 513-525.

106. Filipeanu CM, Zhou F, Claycomb WC, Wu G. (2004) Regulation of the cell surface expression and function of angiotensin II type 1 receptor by Rab1- mediated endoplasmic reticulum-to-golgi transport in cardiac myocytes. J Biol Chem 279: 41077-41084.

107. Filipeanu CM, Zhou F, Fugetta EK, Wu G. (2006) Differential regulation of the cell-surface targeting and function of beta- and alpha1--adrenergic receptors by Rab1 GTPase in cardic myocytes. Mol Pharmacol 69: 1571-1578.

108. Tisdale EJ, Balch WE. (1996) Rab2 is essential for the maturation of pre-golgi intermediates. J Biol Chem 271: 29372-29379.

109. Martinez O, Schmidt A, Salaméro J, Hoflack B, Roa M, et al. (1994) The small GTP-binding protein rab6 functions in tnra-golgi transport. J Cell Biol 127: 1575-1588.

110. Huber LA, Pimplikar S, Parton RG, Virta H, Zerial M, et al. (1993) Rab8, a small GTPase involved in vesicular traffic between the TGN and the basolateral plasma membrane. J Cell Biol 123: 35-45.

111. Takahashi S, Kubo K, Waguri S, Yabashi A, Shin H-, et al. (2012) Rab11 regulates exocytosis of recycling vesicles at the plasma membrane. J Cell Sci 125: 4049-4057.

112. Wu G, Zhao G, He Y. (2003) Distinct pathways for the trafficking of angiotensin II and adrenergic receptors from the endoplasmic reticulum to the cell surface: Rab1-independent transport of a G protein-coupled receptor. J Biol Chem 278: 47062-47069.

113. Dupré DJ, Robitaille M, Ethier N, Villeneuve LR, Mamarbachi AM, et al. (2006) Seven transmembrane receptor core signaling complexes are assembled prior to plasma membrane trafficking. J Biol Chem 281: 34561-34573.

114. Charette N, Holland P, Frazer J, Allen H, Dupré DJ. (2011) Dependence on different Rab GTPases for the trafficking of CXCR4 and CCR5 homo or heterodimers between the endoplasmic reticulum and plasma membrane in jurkat cells. Cell Signal 23: 1738-1749.

115. Dinarello CA. (2011) Historical review of cytokines. Eur J Immunol 37: S34-S45.

116. Rossi D, Zlotnik A. (2000) The biology of chemokinesand their receptors. Ann Rev Immunol 18: 217-242.

117. Raman D, Sobolik-Delmaire T, Richmond A. (2012) Chemokines in health and disease. Exp Cell Res 317: 575-589.

118. Graves DT, Jiang Y. (1995) Chemokines a family of chemotactic cytokines. Crit Re Oral Biol Med 6: 109-118.

119. Mantovani A. (1999) The chemokine system: Redundancy for robust outputs. Immunol Tolday 20: 254-257.

120. Teicher BA, Fricker SP. (2010) CXCL12(SDF-1)/CXCR4 pathway in cancer. Clin Cancer Res 16: 2927-2931.

121. Baggiolini M, Dewald B, Moser B. (1997) Human chemokines: An update. Ann Rev Immunol 15: 675-705.

122. Murdoch C. (2000) CXCR4: Chemokine receptor extraordinaire. Immunol Rev 177: 175-184.

123. Busillo JM, Benovic JL. (2007) Regulation of CXCR4 signaling. Biochim Biophys Acta 1768: 952-963.

124. Nagasawa T, Hirota S, Tachibana K, Takakura N, Nishikawa S, et al. (1996) Defects of B-cell lumphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. Nature 382: 635-658.

125. Bleul CC, Fuhlbrigge RC, Casasnovas JM, Aiuti A, Springer TA. (1996) A highly efficacious lymphocyte chemoattractant, stromal cell-derived factor 1 (SDF-1). J Exp Med 184: 1101-1109.

126. Shirozu M, Nakano T, Inazawa J, Tashiro K, Tada H, et al. (1995) Structure and chromosomal localization of the human stromal cell-derived factor 1 (SDF1) gene. Genomics 28: 495-500.

127. Hesselgesser J, Liang M, Hoxie J, Greenberg M, Brass LF, et al. (1998) Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: Ligand binding, biological activity, and HIV-1 infectivity. J Immunol 160: 877-883.

128. Lu M, Grove EA, Miller RJ. (2002) Abnormal development of the hippocampal dentate gyrus in mice lacking the CXCR4 chemokine receptor. Proc Natl Acad Sci U S A 99: 7090-7095.

129. Nie Y, Waite J, Brewer F, Sunshine M-, Littman DR, et al. (2004) The role of CXCR4 in maintaining peripheral B cell compartments and humoral immunity. JEM 200: 1145-1156.

130. McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. (1999) Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. Dev Biol 213: 442-456.

131. Ratajczak MZ, Kucia M, Reca R, Majka M, Janowska-Wieczorek A, et al. (2003) Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells "hide out" in the bone marrow. Leukemia 18: 29-40.

132. Wald O, Pappo O, Safadl R, Dagan-Berger M, Beider K, et al. (2004) Involvement of the CXCL12/CXCR4 pathway in the advanced liver disease that is associated with hepatitis C virus or hepatitis B virus. Eur J Immunol 34: 1164-1174.

133. Walter DH, Haendeler J, Reinhold J, Rochwalsky U, Seeger F, et al. (2005) Impaired CXCR4 signaling contributes to the reduced neovascularization capacity of endothelial progenitor cells from patients with coronary artery disease. Circ Res 25: 1142-1151.

134. Fidler IJ. (2003) The pathogenesis of cancer metastasis: The "seed and soil" hypothesis revisited. Nat Rev Cancer 3: 453-458.

135. Balkwill F. (2004) The significance of cancer cell expression of the chemokine receptor CXCR4. Semin Cancer Biol 14: 171-179.

136. Sun Y-, Wang J, Shelburne CE, Lopatin DE, Chinnaiyan AM, et al. (2003) Expression of CXCR4 and CXCL12 (SDF-1) in human prostate cancers (PCa) *in vivo*. J Cell Biochem 8: 462-473.

137. Akashi T, Koizumi K, Tsuneyama K, Saiki I, Takano Y, et al. (2008) Chemokine receptor CXCR4 expression and prognosis in patients with metastatic prostate cancer. Cancer Sci 99: 539-542.

138. Darash-Yahana M, Pikarsky E, Abramovitch R, Zeira E, Pal B, et al. (2004) Role of high expression levels of CXCR4 in tumor growth, vascularization, and metastasis. FASEB J 18: 1240-1242.

139. Zhang S, Qi L, Li M, Zhang D, Xu S, et al. (2008) Chemokine CXCL12 and its receptor CXCR4 expression are associated with perineural invasion of prostate cancer. J Exp Clin Cancer Res 62.

140. Taichman RS, Cooper C, Keller ET, Pienta KJ, Taichman NS, et al. (2002) Use of the stromal cell-derived factor-1/CXCR4 pathway in prostate cancer metastasis to bone. Cancer Res 62: 1832-1837.

141. Sun Y-, Schneider A, Jung Y, Wang J, Dai J, et al. (2005) Skeletal localization and neuralization of the SDF-1(CXCL12)/CXCR4 axis blocks prostate cancer metastasis and growth in osseous sites *in vivo*. J Bone Miner Res 20: 318-329.

142. Mack M, Cihak J, Simonis C, Luckow B, Proudfoot AE, et al. (2001) Expression and characterization of the chemokine receptors CCR2 and CCR5 in mice. J Immunol 166: 4697-4794.

143. Deshmane SL, Kremlev S, Amini S, Sawaya BE. (2009) Monocyte chemoattractant protein-1 (MCP-1): An overview. J Interferon Cytokine Res 29: 313-326.

144. Tacke F, Alvarez D, Kaplan TJ, Jakubzick C, Spanbbroek R, et al. (2007) Monocyte subsets differentially emply CCR2, CCR5, and CX3CR1 to accumulate within atherosclerotic plaques. J Clin Invest 117: 185-194.

145. El Khoury J, Toft M, Hickman SE, Means TK, Terada K, et al. (2007) Ccr2 deficiency impairs microglial accumulation and accelerates progression of alzheimer-like disease Nat Med 13: 432-438.

146. Philipson O, Lord A, Gumucio A, O'Callaghan P, Lannfelt L, et al. (2010) Animal models of amyloid-beta-related pathologies in alzheimer's disease FEBS J 277: 1389-1409.

147. Huang B, Lei Z, Zhao J, Gong W, Liu J, et al. (2007) CCL2/CCR2 pathway mediates recruitment of myeloid suppressor cells to cancers Cancer Lett 252: 86-92.

148. Wolf MJ, Hoos A, Bauer J, Boettcher S, Knust M, et al. (2012) Endothelial CCR2 signaling induced by colon carcinoma cells enables extravasation via the JAK2-Stat5 and p38MAPK pathway Cancer Cell 22: 91-105.

149. Vande Broek I, Asosingh K, Vanderkerken K, Straetmans N, Van Camp B, et al. (2003) Chemokine receptor CCR2 is expressed by human multiple myeloma cells and mediates migration to bone marrow stromal cell-produced monocyte chemotactic proteins MCP-1, -2 and -3 Br J Cancer 88: 855-862.

150. Fang WB, Jokar I, Zou A, Lambert D, Dendukuri P, et al. (2012) CCL2/CCR2 chemokine signaling coordinates survival and motility of breast cancer cells through Smad3 protein- and p42/44 mitogen-activated protein kinase (MAPK)-dependent mechanisms J Biol Chem 287: 36593-36608.

151. Lu Y, Cai Z, Xiao G, Liu Y, Keller ET, et al. (2007) CCR2 expression correlates with prostate cancer progression. J Cell Biochem 101: 676-685.

152. Lu Y, Cai Z, Galson DL, Xiao G, Liu Y, et al. (2006) Monocyte chemotactic protein-1 (MCP-1) acts as a paracrine and autocrine factor for prostate cancer growth and invasion. Prostate 66: 1311-1318.

153. Stamatovic SM, Keep RF, Mostarica-Stojkovic M, Andjelkovic AV. (2006) CCL2 regulates angiogenesis via activation of ets-1 transcription factor. J Immunol 177: 2651-2661.

154. Loberg R, Ying C, Craig M, Yan L, Snyder LA, et al. (2007) CCL2 as an important mediator of prostate cancer growth *in vivo* through the regulation of macrophage infiltration. Neoplasia 9: 556-562.

155. Rodrígues-Frade JM, Vila-Coro A, Martín de Ana A, Albar JP, M artínez-A C, et al. (1999) The chemokine monocyte chemoattractant protein-1 induces functional responses through dimerization of its receptor CCR2. Proc Natl Acad Sci USA 96: 3628-3633.

156. Vila-Coro AJ, Rodrígues-Frade JM, Martín de Ana A, Moreno-Ortíz MC, Martínez -A C, et al. (1999) The chemokine SDF-1a triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. FASEB J 13: 1699-1710.

157. El-Asmar L, Springael JY, Ballet S, Andrieu EU, Vassart G, et al. (2005) Evidence for negative binding cooperativity within CCR5-CCR2b heterodimers. Mol Pharmacol 67: 460-469.

158. Percherancier Y, Berchiche YA, Slight I, Volkmer-Engert R, Tamamura H, et al. (2005) Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. J Biol Chem 280: 9895-9903.

159. Wang J, He L, Combs CA, Roderiquez G, Norcross MA. (2006) Dimerization of CXCR4 in living malignant cells: Control of cell migration by a synthetic peptide that reduces homologous CXCR4 interactions. Mol Cancer Ther 5: 2474-2483.

160. Sohy D, Parmentier M, Springael JY. (2007) Allosteric transinhibition by specific antagonists in CCR2/CXCR4 heterodimers. J Biol Chem 282: 30062-30069.

161. Wierda RJ, Kuipers HF, van Eggermond MC, Benard A, van Leeuwen JC, et al. (2012) Epigenetic control of CCR5 transcript levels in immune cells and modulation by small molecule inhibitors. J Cell Mol Med 16: 1866-1877.

162. Blanpain C, Migeotte I, Lee B, Vakili J, Doranz BJ, et al. (1999) CCR5 binds multiple CC-chemokines: MCP-3 acts as a natural antagonist. Blood 94: 1899-1905.

163. Pollok-Kopp B, Schwarze K, Baradari VK, Oppermann M. (2003) Analysis of ligand-stimulated CC chemokine receptor 5 (CCR5) phosphorylation in intact cells using phosphosite-specific antibodies. J Biol Chem 278: 2190-2198.

164. Jayadev S, Garden GA. (2009) Host and viral factors influencing the pathogenesis of HIV-associated neurocognitive disorders. J Neuroimmune Pharmacol 4: 175-189.

165. Menten P, Wuyts A, Van Damme J. (2002) Macrophage inflammatory protein-1. Cytokine Growth Factor Rev 13: 455-481.

166. Christmann BS, Moran JM, McGraw JA, Buller RM, Corbett JA. (2011) Ccr5 regulates inflammatory gene expression in response to encephalomyocarditis virus infection. Am J Pathol 179: 2941-2951.

167. Galvani AP, Novembre J. (2005) The evolutionary history of the CCR5-Delta32 HIV-resistance mutation. Microbes Infect 7: 302-309.

168. Novembre J, Galvani AP, Slatkin M. (2005) The geographic spread of the CCR5 delta32 HIV-resistance allele. PLoS Biol 3: e339.

169. Venkatesan S, Petrovic A, Ryk DI, Locati M, Weissman D, et al. (2001) Reduced cell surface expression of CCR5 in CCR5delta32 heterozygotes is mediated by gene dosage, rather than by receptor sequestration. 277: 2287-2301.

170. Eri R, Jonsson JR, Pandeya N, Purdie DM, Clouston AD, et al. (2004) CCR5-delta32 mutation is strongly associated with primary sclerosing cholangitis. Genes Immun 5: 444-450.

171. Bennetts BH, Teutsch SM, Buhler MM, Heard RN, Stewart GJ. (1997) The CCR5 deletion mutation fails to protect against multiple sclerosis. Hum Immunol 58: 52-59.

172. Suzaki Y, Hamada K, Nomi T, Ito T, Sho M, et al. (2008) A small-molecule compound targeting CCR5 and CXCR3 prevents airway hyperresponsiveness and inflammation Eur Respir J 31: 783-789.

173. Durig J, Schmucker U, Duhrsen U. (2001) Differential expression of chemokine receptors in B cell malignancies Leukemia 15: 752-756.

174. Moore JP. (1997) Coreceptors--implications for HIV pathogenesis and therapy. Science 276: 51-52.

175. Checkley M, Luttge BG, Freed EO. (2012) HIV-1 envelope glycoprotein biosynthesis, trafficking, and incorporation. J Mol Biol 410: 582-608.

176. Wilen CB, Tilton JC, Doms RW. (2012) HIV: Binding and entry. Cold Spring Harb Perspect Med 2.

177. Kawamura T, Gulden FO, Sugaya M, McNamara DT, Borris DL, et al. (2003) R5 HIV productively infects langerhans cells, and infection levels are regulated by compound CCR5 polymorphisms. Proc Natl Acad Sci U S A 100: 8401-8406.

178. Libert F, Cochaux P, Beckman G, Samson M, Aksenova M, et al. (1998) The deltaccr5 mutation conferring protection against HIV-1 in caucasian populations has a single and recent origin in northeastern europe. Hum Mol Genet 7: 399-406.

179. Achour L, Scott MG, Shirvani H, Thuret A, Bismuth G, et al. (2009) CD4-CCR5 interaction in intrcellular compartments contributes to receptor expression at the cell surface. Blood 113: 1938-1947.

180. Gillies K, Wertman J, Charette N, Dupré DJ. (2013) Anterograde trafficking of CXCR4 and CCR2 receptors in a prostate cancer cell line. Cell Physiol Biochem 32: 74-85.

181. Lappano R, Maggiolini M. (2011) G protein-coupled receptors: Novel targets for drug discovery in cancer. Nature Rev Drug Discov 10: 47-60.

182. Golubovskaya VM, Kweh FA, Cance WG. (2009) Focal adhesion kinase and cancer. Histol Histopathol 24: 503-510.

183. Thompson MA, Berg JA, Hoy JF, Telenti A, Benson C, et al. (2012) Antiretroviral treatment of adult HIV infection. JAMA 308: 387-402.

184. Benkraine M, Jin DY, Chun RF, Koup RA, Jeang KT. (1997) Mechanism of transdominant inhibition of CCR5-mediated HIV-1 infection by ccr5delta32. J Biol Chem 272: 30603-30606.

185. Hammad MM, Kuang Y-, Yan R, Allen H, Dupré DJ. (2010) Na+/H+ exchanger regulatory factor-1 is involved in chemokine receptor homodimer CCR5 internalization and signal transduction but does not affect CXCR4 homodimer or CXCR4-CCR5 heterodimer. J Biol Chem 285: 34653-34664.

186. Dupré DJ, Robitaille M, Richer M, Ethier N, Mamarbachi AM, et al. (2007) Dopamine receptor-interacting protein 78 acts as a molecular chaperone for ggamma subunits before assembly with gbeta. J Biol Chem 282: 13703-13715.

187. Lukov GL, Baker CM, Ludtke PJ, Hu T, Carter MD, et al. (2006) Mechanism of assembly of G protein betta gamma subunits by protein kinase CK2-phosphorylated phosducin-like protein and the cytosolic chaperonin complex. J Biol Chem 281: 22261-22274.

188. Brower V. (2007) Researchers tackle metastasis, cancer's last frontier. J Natl Cancer Inst 99: 109-111.

189. Budendorf L, Schopfer A, Wagner U, Sauter G, Moch H, et al. (2000) Metastatic patterns of prostate cancer: An autopsy study of 1,589 patients. Hum Pathol 31: 578-583.

190. Dolgin E. (2009) Cancer metastasis scrutinized. Nature 461: 854-855.

191. Onuffer JJ, Horuk R. (2002) Chemokines, chemokine receptors and small-molecule antagonists: Recent developments. Trends Pharmacol Sci 23: 459-467.

192. Hutchings CJ, Koglin M, Marshall FH. (2010) Therapeutic antibodies directed at G protein-coupled receptors. MAbs 2: 594-606.

193. Haochuan L, Yang W, Chen PW, Alizadeh H, Niederkorn JY. (2009) Ihibition of chemokine receptor expression on uveal melanomas by CXCR4 siRNA and its effect on uveal melanoma liver metastases. Invest Ophthalmol Vis Sci 50: 5522-5528.

194. Bouguerra F, Babron MC, Eliaou JF, Debbabi A, Clot J, et al. (1997) Synergistic effect of two HLA heterodimers in the susceptibility to celiac disease in tunisia. Genet Epiemiol 14: 413-422.

195. Levoye A, Jockers R, Ayoub MA, Delagrange P, Savaskan E, et al. (2006) Are G protein-coupled receptor heterodimers of physiological relevance? - Focus on melatonin receptors. Chronobiol Int 23: 419-426.

196. Pulukuri SM, Gondi CS, Lakka SS, Julta A, Estes N, et al. (2005) RNA interference-directed knockdown of urokinase plasminogen activator and urokinase plasminogen activator receptor inhibits prostate cancer cell invasion, survival and tumorigenicity *in vivo*. J Biol Chem 28: 36539-36540.

197. Singh S, Singh UP, Grizzle WE, Lillard JW,Jr. (2004) CXCL12-CXCR4 interactions modulate prostate cancer cell migration, metalloproteinase expression and invasion. Lab Invest 84: 1666-1676.

198. Phizicky EM, Fields S. (1995) Protein-protein interactions: Methods for detection and analysis. Microbiol Rev 59: 94-123.

199. Berggård T, Linse S, James P. (2007) Methods for the detection and analysis of protein-protein interaction. Proteomics 7: 2833-2842.

200. Yiemwattana I, Ngoenkan J, Paensuwan P, Kriangkrai R, Cheunjitkuntaworn B, et al. (2012) Essential role of the adaptor protein Nck1 in jurkat T cell activation and function. Clin Exp Immunol 167: 99-107.

201. Zhao X, Guan J-. (2003) Focal adhesion kinase and its signaling pathways in cell migration and angiogenesis. Adv Drug Deliv Rev 63: 610-615.

202. Valster A, Tran NL, Nakada M, Berens ME, Chan AY, et al. (2005) Cell migration and invasion assays. Methods 37: 208-215.

203. Cheng KW, Lahad JP, Gray JW, Mills GB. (2005) Emerging role of RAB GTPases in cancer and human disease. Cancer Res 65: 2516-2519.

204. Murata T, Delprato A, Ingmundson A, Toomre DK, Lambright DG, et al. (2006) The legionella pneumophila effector protein DrrA is a Rab1 guanine nucleotide-exchange factor. Nat Cell Biol 8: 971-977.

205. Ingmundson A, Delprato A, Lambright DG, Roy CR. (2007) Legionela pheumophila proteins that regulate Rab1 membrane cycling. Nature 450: 365-369.

206. Hattula K, Furuhjelm J, Arffman A, Peränen J. (2002) A Rab8-specific GDP/GTP exhange factor is involved in actin remodeling and polarized membrane transport. Mol Biol Cell 13: 3268-3280.

207. Fukuda M, Kanno E, Ishibashi K, Itoh T. (2008) Large scale screening for novel rab effectors reveals unexpected broad rab binding specificity. Mol Cell Proteomics 7: 1031-1042.

208. Arts EJ, Hazuda DJ. (2012) HIV-1 antiretroviral drug therapy. Cold Spring Harb Perspect Med 2: a007161.

209. Sánchez JM, Ramos Amador JT, Fernández de Miguel S, González Tomée MI, Rojo Conejo P, et al. (2003) Impact of highly active antiretroviral therapy on the morbidity and mortality in spanish human immunodeficiency virus-infected children. Pediatr Infect Dis J 22: 863-867.

210. Greenberg ML, Cammack N. (2004) Resistance to enfuvirtide, the first HIV fusion inhibitor. J Antimicrob Chemother 54: 333-340.

211. Nichols WG, Steel HM, Bonny T, Adkison K, Curtis L, et al. (2008) Hepatotoxicity observed in clinical trails of aplaviroc (GW873140). Antimicrob Agents Chemother 52: 858-865.

212. Roche M, Salimi H, Duncan R, Wilkinson BL, Chikere K, et al. (2013) A common mechanism of clinical HIV-1 resistance to the CCR5 antagonist maraviroc despote divergent resistance levels and lack of common gp120 resistance mutations. Retrovirology 10: 43.

213. Zimmerman PA, Buckler-White A, Alkhatib G, Spalding T, Kubofcik J, et al. (1997) Inherited resistance to HIV-1 conferred by an inactivating mutation in CC chemokine receptor 5: Studies in populations with contrasting clinical phenotypes, defined racial background, and quantified risk. Mol Med 3: 23-36.

214. Whittall T, Wang Y, Younson J, Kelly C, Bergmeier L, et al. (2006) Interaction between the CCR5 chemokine receptors and microbial HSP70. Eur J Immunol 36: 2304-2314.

215. Kuang YQ, Charette N, Frazer J, Holland PJ, Attwood KM, et al. (2012) Dopamine receptor-interacting protein 78 acts as a molecular chaperone for CCR5 chemokine receptor signaling complex organization PLoS One 7: e40522.

216. Achour L, Labbé-Jullié C, Scott MG, Marullo S. (2008) An escort of GPCRs: Implications for regulation of receptor density at the cell surface. Trends Pharmacol Sci 29: 528-535.

217. Raposo RAS, Thomas B, Ridlova G, James W. (2011) Proteomic-based identification of CD4-interacting proteins in human primary macrophages. PLoS One 6: e18690.

218. Williams D, Devi LA. (2011) Escorts take the lead: Molecular chaperones as therapeutic targets. Prog Mol Biol Transl Sci 91: 121-149.

219. Siffroi-Fernandez S, Giraud A, Lanet J, Franc J-. (2002) Association of the thyrotropin receptor with calnexin, calreticulin and BiP: Effects on the maturation of the receptor. Eur J Biochem 269: 4930-4937.

220. Kimmins S, MacRae T. (2000) Maturation of steroid receptors: An example of functional cooperation among molecular chaperones and their associated proteins. Cell Stress Chaperones 5: 76-86.

221. Dupré DJ, Baraglia A, Reboisb V, Éthiera N, Hébert TE. (2007) Signaling complexes associated with adenylyl cyclase II are assembled during their biosynthesis. Cell Signal 19: 481-489.

222. Nobles M, Benians A, Tinker A. (2005) Heterotrimeric G proteins precouple with G protein-coupled receptors in living cells. Proc Natl Acad Sci USA 102: 18706-18711.

223. Lin Y-L, Mettling C, Portales P, Reant B, Clot J, et al. (2005) G-protein signaling triggered by R5 human immunodeficiency virus type 1 increases virus replication efficienct in primary T lymphocytes. J Virol 79: 7938-7941.

224. Hug P, Lin H-J, Korte T, Xiao X, Dimitrov DS, et al. (2000) Glycosphingolipids promote entry of a broad range of human immunodeficiency virus type 1 isolates into cell lines expressing CD4, CXCR4, and/or CCR5. J Virol 74: 6377-6385.

225. Bannert N, Craig S, Farzan M, Sogah D, Santo NV, et al. (2001) Sialylated O-glycans and sulfated tyrosines in the NH2-terminal domain of CC chemokine receptor 5 contribute to high affinity binding of chemokines. J Exp Med 194: 1661-1673.

APPENDIX A LICENSE FOR FIGURE 1.1

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Oct 22, 2013

This is a License Agreement between Kelsie Gillies ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3250261000904
License date	Oct 15, 2013
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Drug Discovery
Licensed content title	The state of GPCR research in 2004
Licensed content author	Clare Ellis
Licensed content date	Jul 1, 2004
Volume number	3
Issue number	7
Type of Use	reuse in a thesis/dissertation
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
High-res required	no
Figures	Figure 5, page 616
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	An examination of how Rab GTPases and molecular chaperones influence plasma membrane expression of chemokine receptor dimers
Expected completion date	Dec 2013
Estimated size (number of pages)	100
Total	0.00 USD
Terms and Conditions	

APPENDIX B LICENSE FOR FIGURE 1.2

ELSEVIER LICENSE
TERMS AND CONDITIONS

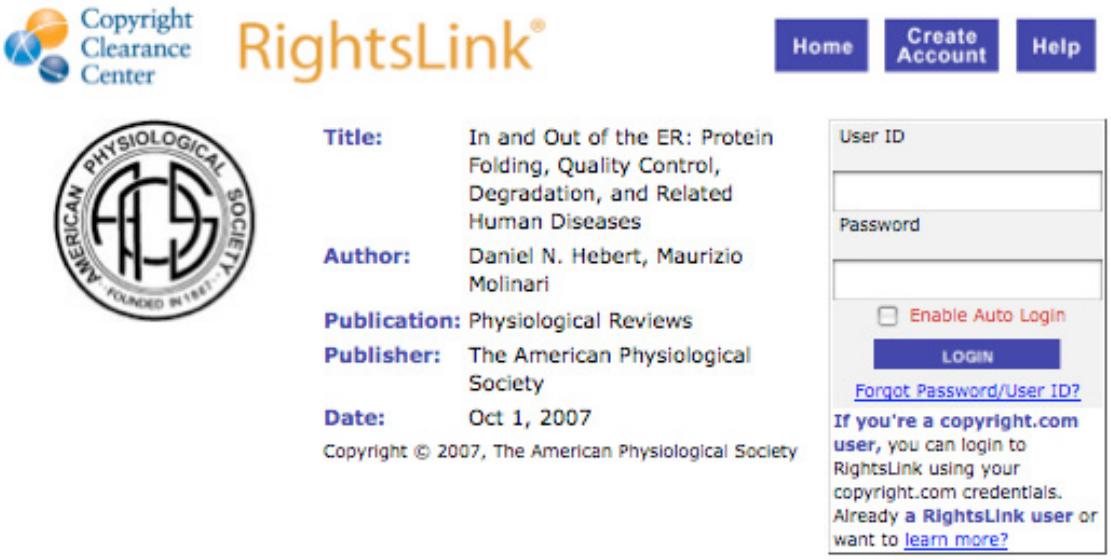
Oct 22, 2013

This is a License Agreement between Kelsie Gillies ("You") and Elsevier ("Elsevier") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Elsevier, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

Supplier	Elsevier Limited The Boulevard,Langford Lane Kidlington,Oxford,OX5 1GB,UK
Registered Company Number	1982084
Customer name	Kelsie Gillies
Customer address	105-1663 Brunswick Street Halifax, NS B0E2W0
License number	3250261390561
License date	Oct 15, 2013
Licensed content publisher	Elsevier
Licensed content publication	Trends in Pharmacological Sciences
Licensed content title	An escort for GPCRs: implications for regulation of receptor density at the cell surface
Licensed content author	Lamia Achour,Catherine Labbé-Jullié,Mark G.H. Scott,Stefano Marullo
Licensed content date	October 2008
Licensed content volume number	29
Licensed content issue number	10
Number of pages	8
Start Page	528
End Page	535
Type of Use	reuse in a thesis/dissertation
Intended publisher of new work	other
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	1
Format	both print and electronic
Are you the author of this Elsevier article?	No
Will you be translating?	No
Order reference number	
Title of your thesis/dissertation	An examination of how Rab GTPases and molecular chaperones influence plasma membrane expression of chemokine receptor dimers
Expected completion date	Dec 2013
Estimated size (number of pages)	100
Elsevier VAT number	GB 494 6272 12
Permissions price	0.00 USD
VAT/Local Sales Tax	0.0 USD / 0.0 GBP
Total	0.00 USD
Terms and Conditions	

APPENDIX C LICENSE FOR FIGURE 1.3



APPENDIX D LICENSE FOR FIGURES 1.4 and 1.5

NATURE PUBLISHING GROUP LICENSE
TERMS AND CONDITIONS

Nov 13, 2013

This is a License Agreement between Kelsie Gillies ("You") and Nature Publishing Group ("Nature Publishing Group") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Nature Publishing Group, and the payment terms and conditions.

All payments must be made in full to CCC. For payment instructions, please see information listed at the bottom of this form.

License Number	3267201162137
License date	Nov 13, 2013
Licensed content publisher	Nature Publishing Group
Licensed content publication	Nature Reviews Molecular Cell Biology
Licensed content title	Rab GTPases as coordinators of vesicle traffic
Licensed content author	Harald Stenmark
Licensed content date	Aug 1, 2009
Volume number	10
Issue number	8
Type of Use	reuse in a dissertation / thesis
Requestor type	academic/educational
Format	print and electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	2
High-res required	no
Figures	Figure 2, Figure 3
Author of this NPG article	no
Your reference number	
Title of your thesis / dissertation	An examination of how Rab GTPases and molecular chaperones influence plasma membrane expression of chemokine receptor dimers
Expected completion date	Dec 2013
Estimated size (number of pages)	100
Total	0.00 USD

APPENDIX E LICENSE FOR FIGURES 3.1, 3.2, 3.3, 3.4, 3. 5

Copyright Clearance Center

1 PAYMENT 2 REVIEW 3 CONFIRMATION

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us at 978-646-2600, M-F between 8:00 AM and 6:00 PM (Eastern), or write to us at info@copyright.com.

Confirmation Number: 11132582
Order Date: 10/23/2013

If you pay by credit card, your order will be finalized and your card will be charged within 24 hours. If you pay by invoice, you can change or cancel your order until the invoice is generated.

Payment Information

Kelsie Gillies
keagillies@gmail.com
+1 (902) 240-2254
Payment Method: n/a

Order Details

Cellular physiology and biochemistry

Order detail ID:	64090391
Order License Id:	3254761149731
Article Title:	Anterograde Trafficking of CXCR4 and CCR2 Receptors in a Prostate Cancer Cell Line
Author(s):	Gillies, Kelsie ; et al
DOI:	10.1159/000350126
Date:	Jan 01, 2013
ISSN:	1015-8987
Publication Type:	Journal
Volume:	32
Issue:	1
Start page:	74
Publisher:	S./KARGER AG

Permission Status:	Granted
Permission type:	Republish or display content
Type of use:	reuse in a thesis/dissertation
Licensed copyright line	Copyright © 2013, Copyright © 2013, Karger Publishers
Requestor type	author of requested content
Format	Print, Electronic
Portion	figures/tables/illustrations
Number of figures/tables /illustrations	5
Include Image file	no
Rights for	Main product

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 0.00**

APPENDIX F LICENSE FOR TEXT EXTRACTS

Copyright Clearance Center

1 PAYMENT 2 REVIEW 3 CONFIRMATION

Step 3: Order Confirmation

Thank you for your order! A confirmation for your order will be sent to your account email address. If you have questions about your order, you can call us at 978-646-2600, M-F between 8:00 AM and 6:00 PM (Eastern), or write to us at info@copyright.com.

Confirmation Number: 11132586
Order Date: 10/23/2013

If you pay by credit card, your order will be finalized and your card will be charged within 24 hours. If you pay by invoice, you can change or cancel your order until the invoice is generated.

Payment Information

Kelsie Gillies
keagillies@gmail.com
+1 (902) 240-2254
Payment Method: n/a

Order Details

Cellular physiology and biochemistry

Order detail ID:	64090400
Order License Id:	3254770039121
Article Title:	Anterograde Trafficking of CXCR4 and CCR2 Receptors in a Prostate Cancer Cell Line
Author(s):	Gillies, Kelsie ; et al
DOI:	10.1159/000350126
Date:	Jan 01, 2013
ISSN:	1015-8987
Publication Type:	Journal
Volume:	32
Issue:	1
Start page:	74
Publisher:	S./KARGER AG

Permission Status:	**Granted**
Permission type:	Republish or display content
Type of use:	reuse in a thesis/dissertation
Licensed copyright line	Copyright © 2013, Copyright © 2013, Karger Publishers
Requestor type	author of requested content
Format	Print, Electronic
Portion	text extract
Number of text extract requested	5
Rights for	Main product
Duration of use	Life of current edition/presentation

Note: This item will be invoiced or charged separately through CCC's **RightsLink** service. More info **$ 0.00**