INVOLVEMENT OF DIFFERENT RAB GTPᴀsᴇs IN THE TRAFFICKING OF
CXCR4 AND CCR5 HOMO- AND HETERODIMERS BETWEEN THE
ENDOPLASMIC RETICULUM AND PLASMA MEMBRANE IN HEK293 AND
JURKAT CELLS

by

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TITLE: INVOLVEMENT OF DIFFERENT RAB GTPASES IN THE
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ABSTRACT

Little is known about the outward trafficking of receptor dimers from the endoplasmic reticulum to the plasma membrane, or the role that trafficking plays in assembly, targeting and specificity of receptor signalling. Bimolecular fluorescence complementation was used to follow prescribed receptor homo/heterodimers in Jurkat cells and clarify the trafficking itineraries those receptors follow to reach the plasma membrane. Chemokine receptors CXCR4 and CCR5 were chosen due to their implication in numerous pathologies including, HIV and cancer, and their ability to form homo and hetero-oligomers. This study demonstrates that although the individual receptors composing heterodimeric complexes are the same as in homodimeric complexes, the heterodimer traffics and signals independently of its constituent homodimers. The presence of CD4 affects the trafficking of CCR5 containing dimers but not the CXCR4 homodimer. These observations demonstrate the importance of considering receptor heterodimers as distinct signalling entities that should be more carefully and individually characterized.

LIST OF ABBREVIATIONS AND SYMBOLS USED

6'-GNTI	6'-Guanidinonaltrindole
7TMR	Seven Transmembrane Receptor
$A_{2A}R$	Adenosine α_{2A} Receptor
AC	Adenylyl Cyclase
ADP	Adensine Diphosphate
AIDS	Acquired Immunodeficiency Syndrome
APJ	Apelin Receptor
AT1R	Angiotensin II Type 1 Receptor
BiFC	Bimolecular Fluorescence Complementation
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
CCL5	C-C Motif Ligand 5
CCR2	C-C Motif Chemokine Receptor Type 2
CCR5	C-C Motif Chemokine Receptor Type 5
CD34	Cluster of Differentiation 34
CD4	Cluster of Differentiation 4
CD8	Cluster of Differentiation 8
cDNA	Complementary Deoxyribonucleic Acid
Co-IP	Co-Immunoprecipitation
COP	Coat Protein Complex
β1-AR	β1 Adrenergic Receptor
β2-AR	β2 Adrenergic Receptor
C-terminus,C-tail	carboxy(l)-terminus
CXCL12	C-X-C Motif Ligand 12
CXCR4	C-X-C Motif Chemokine Receptor Type 4
D1R	Dopamine D1 Receptor
D2R	Dopamine D2 Receptor
DAG	Diacylgylcerol
DMEM	Dulbecco's Modified Eagle's Medium

DN	Dominant Negative
DOR	δ(delta)-Opioid Receptor
DRiP78	Dopamine Receptor Interacting Protein, 78KD
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic Reticulum
ERGIC	ER-Golgi Intermediate Compartment
FAK	Focal Adhesion Kinase
FDA	US Food and Drug Administration
FRET	Fluorescence Resonance Energy Transfer
G protein	Guanine Nucleotide-Binding Protein
$GABA_B$	Type B γ-aminobutyric Acid
GAP	GTPase-Activating Protein
GDI	Guanine Nucleotide Dissociation Inhibitor
GDP	Guanosine Diphosphate
GEF	Guanine Nucleotide Exchange Factor
GFP	Green Fluorescent Protein
GlcNAc	N-acetylglucosamine
GnHR	Gonadotropin-Releasing Hormone Receptor
gp120	Envelope Glycoprotein GP120
gp41	Envelope Glycoprotein GP41
GPCR	G-Protein Coupled Receptor
GTP	Guanosine Triphosphate
HAART	Highly Active Antiretroviral Therapy
HEK293	Human Embryonic Kidney 293 Cells
HeLa	Cervical Cancer Cell Line Derived from Henrietta Lacks
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
IB	Immunoblot
IP3	Inositol Triphosphate
JAK	Janus Kinase

κOR	κ(Kappa)-Opioid Receptor
LHR	Lutenizing Hormone Receptor
mGluR	Metabotropic Glutamate Receptor
MHC-II	Major Histocompatibility Complex Class II
NHERF-1	Na/H exchange regulatory factor-1
N-terminus	Amino-terminus
OR	Opioid Receptor
p130Cas	Crk-associated substrate
PBS	Phosphate Buffer Saline
PIP2	Phosphatidylinosityl-4,5-bisphosphate
PLC-β	Phospholipase Cβ
PM	Plasma Membrane
PTX	Pertussis Toxin
Pyk-2	Proline-Rich Kinase-2
Rab	Ras-Related Protein in Brain
RAMP	Receptor-Activity-Modifying Protein
RANTES	Regulated on Activation Normal T Cell Expressed and Secreted
REP	Rab Escort Proteins
RIPA	Radioimmune Precipitation Assay Buffer
RPMI-1640	Roswell Park Memorial Institute Medium-1640
RSRR	C-terminal Arginine-Based ER retention/Retrieval Signal
SDF-1	Stromal Derived Factor-1
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
shRNA	Small Hairpin Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
SKFF83959	Selective D1 Receptor Synthetic Benzine Derivative
SNSR-4	Sensory Neuron-Specific Receptor-4
$SSTR_1$	Somatostatin Receptor 1
$SSTR_{2A}$	Somatostatin Receptor 2A
$SSTR_5$	Somatostatin Receptor 5
STAT	Signal Transducer and Activator of Transcription

TGN	Trans-Golgi Network
V1aR	Vasopressin Type 1a Receptor
V2R	Vasopressin Type 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)
VFT	Venus Fly Trap Domain
WHIMS	Warts, Hypogammaglobulinemia, Infections, Myelokathexis Syndrome
WT	Wild-type
YFP	Yellow Fluorescent Protein

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Chapter 1 : INTRODUCTION

1.1 G-Protein- Coupled Receptors

G-protein-coupled receptors (GPCRs) are a superfamily of integral membrane proteins constituting 3-5% of the mammalian genome, making them the largest family of cellular signalling proteins [1]. The sheer size of the GPCR family, their ability to mediate responses to an array of structurally diverse stimuli including peptides, ions, biogenic amines, hormones and lipids, and their central role in nearly all physiological processes make GPCRs very important pharmaceutical targets. GPCRs have a long history as clinically effective drug targets for wide range of disorders including those of the cardiovascular, gastro-intestinal and central nervous systems. Today, drugs targeting GPCRs account up to 30% of the current drug market [2]. Over the past 30 years enormous academic and industrial efforts has been invested into understanding the mechanisms by which GPCRs function and signal but much less is known about the exact structure of most GPCRs or the processes contributing to their membrane expression [3, 4]. Understanding the process by which a newly synthesized receptor is assembled and trafficked to the plasma membrane could offer new avenues for therapeutic intervention in diseases associated with the mature receptor.

1.2 Basic GPCR Structure

Despite vast ligand diversity and low primary amino acid sequence homology, all GPCRs have a remarkably similar global architecture. Basic GPCR topology consists of a seven transmembrane α-helical core with alternating intracellular and extracellular

loops bounded by an extracellular amino (N) terminus and an intracellular carboxy (C)

terminus [5]. This common structure accounts for the superfamily's alternative name,

Seven Transmembrane Receptors (7TMRs). While no single motif is shared by all

GPCRs, the transmembrane region shows the most sequence homology while the size

and structure of the termini and loops can vary widely both between and among the

different GPCR families [4, 6]. The N-terminus is the most variable region of the GPCR

sequence and ranges from only seven amino acids in the Adenosine A_2 receptor to a

large, multi-domain structure as in the case of the immunoglobulin, lectin or laminin

receptors [1]. The third intracellular loop and the C-terminus are also areas of great

variability even within a particular GPCR class, and unique motifs within the C-terminus

are thought to contribute to ligand-dependent receptor signalling, internalization and

desensitization. These structural divergences also confer differences in receptor

pharmacology by altering binding and signalling properties [6].

GPCR are notoriously difficult to crystallize, hampered by the many problems

maintaining the stability of integral membrane proteins outside of their native

environment. As a result the crystal structures of only 6 GPCRs have been solved to date.

The first was Rhodopsin in 2000 [7]. Since then the structures of Opsin [8, 9], the β_2

adrenergic receptor [10], the β_1 adrenergic receptor [11], the adenosine A_{2A} receptor [12],

the dopamine D3 receptor [13] and the CXCR4 chemokine receptor [14] bound to

various agonists and antagonists have also been solved. The pace of structural discoveries

has quickened over the last decade due to new techniques that increase the

crystallizability of GPCRs, adjustments in purification regimes and new approaches, such

as fusion proteins and mutants, to stabilize GPCRs during crystallization. It is predicted

that in 2011 alone three more unique crystal structures and up to a dozen more ligand-bound structures related to known base structures may be produced.

1.3 Classification of GPCRs

Originally, GPCRs were classified into one of six groups, A through F, on the basis of overall sequence homology, but a surge in the number of GPCR sequences provided by genome projects necessitated a more organized system based on both genetics and structural homology of the heptahelical domain [15]. Now, most vertebrate GPCRs can be classified into one of 3 main families or classes [16].

Class A, the Rhodopsin-like GPCRs, constitutes roughly 80 percent of the entire GPCR superfamily and contains the receptors for odorants, several important neurotransmitters, neuropeptides, chemokines and glycoprotein hormones [17]. Class A GPCRs have a characteristic tilted structure due to several helix distorting amino acids in the transmembrane regions and a disulphide bridge linking the first and second transmembrane domains. Class B secretin GPCRs are a relatively small subset of GPCRs and serve as receptors for many hormones, such as glucagon, secretin and parathyroid hormone. They usually have a relatively long N-terminus with a number of cysteines that engage in a series of disulphide bridges [17]. The Class C GPCR family consists of eight different receptor subtypes able to transduce signals from glutamate, Ca^{2+} ions, sweet and umami tastes and basic amino acids. Class C GPCRs have two significant features: a large, well-defined extracellular domain known as the Venus Fly Trap (VFT) which is thought to be able to open and close around a bound agonist, and a long cytoplasmic tail. The remaining GPCRs can be grouped into one of two smaller GPCR families, frizzled/taste2 or adhesion.

To date there are nearly 100 orphan GPCRs for which natural ligands remain to be identified [18, 19]. Many of these orphans exhibit too little sequence homology with known GPCRs to be classified into a specific receptor family. In fact, some show much more homology with other orphan receptors, offering the possibility that they may represent new sub-families of GPCRs for potentially novel ligands and a diverse range of function [20].

1.4 GPCR Activation and Signal Transduction

Communication between individual cells within a multicellular organism is obligatory for coordinated function; however, most signals do not enter the cell. G-protein-coupled receptors form a physical interface between the extracellular and intracellular compartments by transducing extracellular signals across the cell membrane and trigging a cascade of intracellular events involving second messengers, enzymes and ion channels, which ultimately elicit a biological response [21]. These intracellular changes can be both short-term effects, such as changes in intracellular calcium levels, as well as long-term effects involving changes in gene transcription.

Ligand binding to a GPCR elicits a series of conformational changes in the receptor, which in most cases promotes guanine nucleotide exchange in the α unit of a receptor-associated heterotrimeric guanine nucleotide-binding protein (G protein). The G protein then dissociates into a $\beta\gamma$ dimer complex and an α monomer, to which the GTP is bound. G proteins have been broadly classified into four families on the basis of sequence similarity of the Gα subunit: G_s (G_s, G_{olf}), G_i (G_{i1-3}, G_t, G_g, G_o, G_z), G_q (G_q, G_{11}, G_{14}, $G_{15/16}$) or G_{12} (G_{12}, G_{13}), and while there are notable exceptions, this subunit classification

has also served to define receptor-effector coupling as well [22]. Briefly, the Gαs subunit stimulates adenylyl cyclase (AC) while Gαi inhibits adenylyl cyclase. The Gαq family acts through a different effector, phospholipase Cβ (PLC-β), which cleaves phosphatidylinositol-4,5-bisphosphate (PIP2) into second messengers that mobilize intracellular Ca^{2+} stores and further activate a number of protein kinases. Like the Gαq subunit, the G$\beta\gamma$ subunit also triggers PLC activation, diacylgylcerol (DAG) and inositol triphosphate (IP3) second messenger generation and Ca^{2+} mobilization [23]. Although less is known about the Gα_{12} mediated pathway, it has been shown to be associated with the Ras, RasGAP and Brunton's tyrosine kinase (Btk) and may stimulate phospholipase-D and protein kinase-C [22]. Receptor-G-protein coupling defines receptor specificity by allowing a particular extracellular signal to be routed to specific G proteins and downstream pathways.

1.5 GPCR Traffic Between the Endoplasmic Reticulum and the Plasma Membrane

While the GPCR signal termination processes of endocytosis, recycling and degradation after agonist stimulation have been extensively studied [24, 25], our understanding of the assembly and trafficking of GPCR complexes to the plasma membrane is a relatively neglected area of study. The balance between endocytic and exocytic trafficking plays a large part in regulating receptor expression at the cell surface and thus dictates the magnitude cellular response to a given signal [26]. Furthermore, forward trafficking could be potentially connected with the assembly of GPCR complexes, and would in turn have consequences for specificity and rapidity of subsequent signalling events.

GPCR biosynthesis and folding occurs in the rough endoplasmic reticulum (ER) after which receptors must be transported to the plasma membrane where they can interact with the extracellular space (PM). Early folding and the recruitment and packaging of receptors into COPII-coated vesicles in the ER is the rate-limiting step for export of the δ-opioid receptor (DOR) [27]. The efficient assembly of transmembrane proteins exclusively into COP-II vesicles may involve the presence of several critical ER export signals within the C-termini of these proteins with components of the vesicle complexes [26].

1.6 Involvement of the ER and Golgi

The eukaryotic anterograde transport pathway is composed of an elaborate endomembrane system that connects several independent organelles to one another in sequential fashion [28]. Each compartment contributes a specialized microenvironment in which various stages of protein biosynthesis, modification and sorting occur.

The endoplasmic reticulum (ER) serves as the start point for newly synthesized proteins. In the process of protein biosynthesis ribosomes 'dock' with a Sec61 translocation site in the ER membrane and the newly generated protein is released into the ER lumen, where facilitated protein folding and disulphide bond formation occur. An ER quality control system excludes proteins from ER-derived transport vesicles until they have been released from ER chaperones and folding factors in a correctly folded conformation [26]. Although it was previously believed that ligand binding recruited signalling molecules to the activated receptor, there is mounting evidence that signalling complexes are in fact formed in the ER, early in biosynthesis [29]. Proper folding and

association of receptors with effector proteins such as Gα and Gβγ and molecular chaperones, is often necessary for recruitment into transport vesicles leaving the ER [30].

The next major stops for a protein on route to the plasma membrane are the ER-Golgi intermediate complex (ERGIC), the Golgi apparatus and the trans-Golgi network (TGN). Within the Golgi stack, each cisterna houses a separate set of enzymes; for example, the early, or *cis*, Golgi contains mannosidase I, the medial Golgi contains *N*-acetylglucosamine (GlcNAc) transferase I and the late, or *trans,* Golgi houses galactosyl transferase [31]. During the highly regulated transport between these compartments, receptors undergo a number of post-translational modifications necessary to attain proper maturation [26]. After processing by the Golgi, the *trans*-Golgi then functions as a sorting station, directing proteins either to the cell membrane or to additional compartments within the endomembrane system for further modification. Once expressed at the plasma membrane, the receptor is available for ligand binding. Binding of a ligand to the receptor induces retrograde trafficking involving phosphorylation of the receptor, recruitment of arrestin and endocytosis. The receptor is then either recycled back to the plasma membrane or degraded by lysosomes.

1.7 Role of Rab GTPases in Trafficking

A subset of GTPases called Rab proteins, formally known as Ras-related proteins in brain, are important in the regulation of membrane protein traffic between the various intracellular compartments along both the anterograde and retrograde pathways. To date over 70 Rab and Rab-like GTPases have been identified [32]. Despite their small size, only 20-25kDa, Rab proteins possess multiple surfaces through which they interact with

both regulatory and effector molecules. A post-translationally modified carboxy-terminal cysteine residue determines membrane localization and directs Rab escort protein delivery to the appropriate membrane [33]. These multiple associations allow Rabs to function as protein scaffolds within a single organelle and act in concert with their effectors to regulate all stages of membrane traffic, including cargo delivery and membrane recycling [31].

The key to Rab protein function is their ability to cycle between an active GTP-bound form and an inactive GDP-bound form. Rab proteins in their GTP bound form are carried by transport vesicles but after membrane fusion with their target organelles, GTP hydrolysis converts the Rab proteins to their inactive, GDP-bound form. Rab escort proteins (REPs) can then associate with the inactive Rab proteins and recycle them from their fusion targets back to their original membrane. The GDP bound Rabs are then reactivated by the actions of Rab-specific guanine nucleotide exchange factors (GEFs) and are then ready participate in another round of transport [34].

Co-localization of mistrafficked GPCRs, including the βARs and the AT1R, with ER and Golgi markers after Rab1 knockdown has established a specific role for Rab1 in anterograde trafficking of proteins between the ER and the Golgi and within compartments of the Golgi complex [35, 36]. Similarly, it is known that Rab2 directs retrograde transport between the ER and the *cis*-Golgi, Rab 6 functions within the Golgi, Rab 8 shuttles proteins between the TGN and the PM and Rab 11 is involved in both exocytic and endocytic shuttling between the PM and Golgi via recycling endosomes [33] (Figure 1.1). Dominant negative versions of most Rab GTPases, which either lock the proteins in their inactive GDP-bound form or prevent guanine nucleotide exchange,

blocking protein transport along a given route, have been developed as tools to study the specific transport requirements of various receptors.

1.8 GPCR Dimerization: Early Evidence for GPCR Oligomerization

Classically GPCRs were believed to exist and function as monomers but, while many receptors are capable of activating their cognate G proteins in a monomeric form, significant evidence supports the paradigm where dimer or higher order oligomers constitute the basic functional unit of a GPCR [37].

The possibility of physical interactions between monomeric receptors was first hypothesized in the late 1970s due to unexplained cooperativity in ligand binding assays and the realization that some receptor complexes exhibited unexpected large size estimates on gel filtration columns [38]. Over the next 20 years this hypothesis was explored using a variety of experimental approaches including radiation inactivation, photoaffinity labeling, cross linking, and gel filtration [19]; however, it has only been in the last 15 years that substantiative support for these initial findings has been produced.

The first clear evidence for GPCR dimerization and its functional implications came from the Family C $GABA_B$ receptor. In 1997, the newly isolated $GABA_B$R1 gene was found to be non-functional when expressed alone due to inefficient trafficking to the cell surface. Shortly after, the $GABA_B$R2 gene was also discovered but curiously did not bind known $GABA_B$ ligands. The co-expression of both $GABA_B$R1 and $GABA_B$R2, however, resulted in the formation of a functional $GABA_B$ receptor that both trafficked to the PM as well as exhibited proper responses and properties for the $GABA_B$ receptor. It has since been determined that this phenomenon is due to the masking of an RSRR

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carboxy terminal ER retention motif within the $GABA_BR1$ via the interaction with

$GABA_BR2$ [39]. In the time since this initial evidence for obligate GPCR dimerization

Family C $T1R_{1-3}$, mGluR and calcium-sensing receptors have also been shown to require

dimerization, where only dimers are capable of proper signal transduction [21].

Understanding the occurrence and implications of dimerization for Class A and B

GPCRS is now an area of substantial research [16].

1.9 Techniques to Observe Dimerization

Extensive evidence supporting the formation and dynamics of GPCR dimers has

been produced, initially by western blotting and co-immunoprecipitation experiments

[40] and now through more complex approaches such as functional complementation,

bimolecular fluorescence complementation (BiFC), fluorescence resonance energy

transfer (FRET), Bioluminescence resonance energy transfer (BRET) and atomic force

microscopy [41-43] .

Most evidence for GPCR dimerization uses an *ex vivo* approach, and has been

criticized because its heavy reliance on transfected cell systems may not realistically

reflect dimerization in a native context. However, these *ex vivo* results are now being

corroborated with an increasing amount of *in vivo* data. One convincing example of

receptor dimerization in native tissues was provided using atomic force microscopy on

isolated murine disc membranes, which revealed organized rows of paired rhodopin

receptors [44]. A landmark study of the lutenizing hormone receptor (LHR), a Family A

GPCR, has shown that coexpression of a binding-deficient and signalling-deficient form

of the LHR could re-establish normal lutenizing hormone actions via intermolecular

functional complementation in living transgenic mice from an LHR knockout background [21]. The complementation phenomenon implies that the binding of a ligand to a single monomer of a di/oligomer is capable of producing a conformational change in the other receptor(s) of the complex and could be a useful future research tool. Time-resolved FRET has been used to examine the existence of oxytocin receptor dimers and/or oligomers in native mammary gland tissues based on radiolabeling with selective fluorescent ligands, successfully overcoming previous challenges in applying the FRET technique to native tissues [45].

Unfortunately, current research techniques cannot properly discriminate between the possibilities of GPCR dimers or oligomers. Consequently, there is a possibility that current understanding of receptor assembly, trafficking, signalling and internalization may have to be expanded to include receptor oligomerization in the future [46]. It has even been speculated that some GPCRs, including perhaps chemokine receptors, may be part of large oligomeric complexes reminiscent of arrays observed for rhodopsin and the D2 dopamine receptors [47].

1.10 Heterodimerization Generates New Pharmacological Properties

GPCR oligomerization can have profound effects on the pharmacology of the constituent receptors because the properties of a given molecule can be influenced by the range of dimers or oligomers to which its target receptor belongs [47]. While many GPCRs have been thoroughly investigated in terms of signalling and drug interactions, the recognition of GPCR oligomerization has introduced a new level of complexity into our understanding of GPCR related processes. For hetero-dimerization or hetero-

oligomerization of GPCRs to have physiological relevance the receptors in question must be co-expressed in the same cell in spatio-temporal synchronization [46]. There is a need to understand the expression patterns of a range of GPCRs in different native tissues as it may illuminate genomic predispositions for oligomerization between two receptors [46, 48].

Even at the earliest stages of the GPCR lifecycle there is evidence that heterodimerization can have profound effects on receptor biology (Figure 1.2a). The work presented in this thesis has focused primarily on the possible alterations in GPCR trafficking promoted by dimerization. As previously mentioned, PM protein trafficking out of the ER is strictly regulated by a ER/Golgi quality control system, a strategy used to permit the export of only correctly folded complexes and excluded potentially dangerous incorrectly folded complexes. Dimerization may be a basic requirement for the maturation and function of some receptors, by allowing them to attain correctly folded status and escape the degradation pathway [15, 17, 19]. So far it has been demonstrated that $GABA_B$ receptor dimers and Class A CXCR4, CCR5, vasopressin, oxytocin and β_2AR receptor dimers are formed in a ligand-independent process within the ER early after biosynthesis [49-52]. Certain effectors along the exocytic pathway may preferentially interact with homo- and heterodimers of certain receptors. For example, the molecular chaperone Erp57 is important for the dimerization of AT1R homodimers and the β_2AR-AT1R heterodimer but played no role in the homo-dimerization of the β_2AR. Preliminary results from our lab indicate that the molecular chaperone DRiP78 interacts with the CXCR4 and CCR5 chemokine receptors but not the heterodimer formed by those receptors. The inefficient targeting of GPCR oligomers in vivo has been

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shown to contribute to some pathophysiologies, emphasizing the importance of proper receptor maturation, assembly and trafficking to the PM [53-55]; but there is currently very little known about which specific pathways receptor dimers use to reach the PM and if receptor homo- or hetero-dimerization will effect these requirements. A greater understanding of GPCR ER export will contribute to the understanding of how GPCR signalling complexes are formed, how their acquire aspects of their specificity and may identify new therapeutic targets.

Heterodimerization can also alter the G protein activation profile of some receptors through allosteric modulation between the two protomers of the heterodimer (Figure 1.2b). In this phenomenon, the binding of a ligand to its receptor is influenced by another ligand that binds to the second protomer in the dimeric complex. The μ opioid receptor (OR) provides an excellent example of allosteric modulation. When the μOR assiociates with the δOR there is a decrease in responsiveness to μ ligands [56]. Alternatively, interaction of the μOR with the $A_{2A}R$ increases responsiveness to μ ligands while association with the CB1 cannabinoid receptor has no effect on responsiveness to μ ligands [57, 58]. It is also possible for heterodimerization to create new ligand-binding properties through the formation of a novel binding site. Some ligands may prefer receptors in either their homodimeric or heterodimeric form. For example, 6'-GNTI is a selective agonist for the δOR-κOR heterodimer, but does not activate the homodimeric κOR and δOR counterparts [59]. Similarly, SKF-83959 specifically binds to the D1R/D2R heterodimer [48].

Recent evidence supports the view that a receptor dimer or oligomer will only bind a single G protein [60]. Thus, a GPCR heteromer composed of receptors typically

coupled to different G proteins will then couple to either one of the G proteins, or in some cases a completely new G protein (Figure 1.2c) [37]. The μOR and δOR both couple to Gαi when expressed individually, however, the μOR-δOR heterodimer couples to Gαz in a ligand-independent fashion. When the δOR and SNSR-4 receptors are individually stimulated by their respective ligands they couple to the Gαq and Gαi/o G proteins, respectively. When both protomers were simultaneously stimulated, either by a mixed agonist or by two receptor-specific agonists, there is a switch from a Gαi/o-mediated signalling pathway to one mediated by Gαq. This phenomenon has also been shown for dopamine D1-D2 receptor heteromers. Alterations in G protein coupling have been shown to account for the ability of a D1R specific agonist, SKF83959, to activate D2R mediated signalling pathways [59]. It also follows that small molecular antagonists could be capable of inhibiting the function of receptors to which they do not directly bind as a result of heterodimerization [47]. The selective blockade of βARs within constitutively expressed AT1R and βAR heteromers in mice cardiomyocytes resulted uncoupling of the AT1 receptor from Gαq protein and trans-inhibition of angiotensin-induced responses [61]. It is becoming increasingly apparent that association between GPCRs of different types results in the formation of complexes that often have their own unique signalling and functional properties and has far-reaching *in vivo* implications for drug specificity and efficacy. The effect of heterodimerization on the potency of GPCR agonists and antagonists also has direct implications for the efficacy of ligand-screening approaches, which often test a GPCR in isolation [46].

In addition to the potentiation or attenuation of G protein mediated signals, heterodimerization can also affect receptor internalization and desensitization (Figure

1.2d). β-arrestins are important proteins of the retrograde receptor trafficking pathway

that bind to phosphorylated GPCRs after their activation and promote uncoupling of the

signal transduction pathway from the receptor [62]. β-arrestins also serve as scaffolding

proteins and recruit many of the required effectors and accessory proteins needed for

clathrin-mediated receptor endocytosis [63]. Many studies have shown that stimulation of

only one protomer within a dimer was sufficient to promote the co-internalization of both

receptors for a number of different GPCRs including somatostatin $SSTR_1$-$SSTR_2$ receptor

dimers [64], δ-opioid/β2AR dimers [65], $α_{2A}$/β1AR dimers [66], A_{2A} adenosine/D2

dopamine receptor dimers [67] and $SSTR_{2A}$ somatostatin/μ-opioid receptor dimers [68].

In the case of the vasopressin GPCRs, the V1aR is rapidly recycled while the V2R is

more slowly returned to the membrane due to differences in the speed of β-arrestin

dissociation from the receptors in endosomes. In heterodimeric complexes with the V2R,

the more rapid recycling of the V1aR is inhibited, conferring the dimers with a slower,

V2R-like recycling pattern [69]. In the reverse fashion, heterodimerization between the

$SSTR_2$ and $SSTR_5$ causes increased recycling of $SSTR_2$ and results in reduced receptor

desensitization [70].

1.11 Heterodimers as Pharmacological Targets

Many GPCRs are ubiquitous, meaning they are expressed in a wide variety of cell

types and tissues. Often it is difficult to target these receptors in a specific tissue, and the

activation/inactivation of these receptors in non-targeted tissues can lead to complex and

dangerous side effects. The existence of GPCR heterodimers or oligomers with new

pharmacological properties generates many new drug targets. It is now known that

heterodimerization can be tissue- or disease-specific, making heteromers attractive drug targets. The intermolecular interactions between β-adrenergic receptor protomers within β_1AR-β_2AR heterodimers have been shown to result in enhanced signalling efficiency in response to agonist stimulation in cardiac myocytes, suggesting that compounds targeting the β_1AR-β_2AR complex instead of the individual receptors may specifically affect cardiomyocytes and optimize β-adrenergic modulation of cardiac contractility [71, 72]. Similarly, the AT1R-APJ (apelin) receptor dimer is known to occur in atherosclerosis and AT1R-APJ-selective compounds could possibly target the heterodimeric angiotensin receptors specifically involved in the disease while leaving homodimeric receptors unaffected [72, 73]. In addition to ligands selective only for certain heterodimers, there may be many agonists and antagonists that have been previously characterized as selective for one receptor type that may in fact be capable of activating/inhibiting other receptors through heteromerization. This cross-inhibition could in some cases be a therapeutic benefit, while in others it may be responsible for the unexplained, and occasionally detrimental, side effects associated with some drugs. A better understanding of tissue- or disease-specific heterodimers and a thorough characterization of the effectors and signalling partners associated with these dimers at various stages of the GPCR lifecycle may allow for the development of more selective therapeutics, reducing unwanted side effects at receptor homodimers. Heterodimerization could potentially offer a new, more efficient way to regulate GPCR function and may generate more pharmacologically attractive therapeutics than those currently available.

1.12 Chemokines and Chemokine Receptors

Chemokines are a family of small, 14-18 kDa, secreted cytokines so named for their ability to induce the directional migration of leukocytes to sites of inflammation and infection [14]. While chemokines were likely mediators of innate immune cell trafficking and chemotaxis during morphogenesis in early vertebrates, the function of chemokines in more advanced phyla is not limited solely to control of immune surveillance and response [74]. They have also been shown to participate in a wide range of physiological processes including T cell differentiation and polarization, granule exocytosis, gene transcription, organogenesis, hematopoiesis, cerebellar development, wound healing, angiogenesis and apoptosis [74-77]. As with most host defense systems, in addition to the evolution of normal protective and developmental functions, the chemokine system has also been the subject of several maladaptive responses, which can result in harm to the host. Chemokines and their receptors have been implicated in the pathogenesis of tumour growth, allograft rejection, asthma, atherosclerosis, arthritis and HIV infection [14, 75, 78].

To date 44 chemokines have been described which bind to a specialized subset of only 21 Class A GPCR chemokine receptors. The unique selective pressures imposed on host defenses have likely created a robust system of overlapping function and redundancy within the chemokine system [79]. This system may also aid in fine-tuning and directing very specific chemokine-mediated biological responses. Chemokines are commonly classified into one of four groups, C, CC, CXC or CX_3C, depending on the relative positions of the first two cysteines in a highly conserved N-terminal tetracysteine motif. They can also be further classified as either inflammatory/inducible or

homeostatic/constitutive based on the conditions and location of their production. Constitutive chemokines guide basal leukocyte traffic and form the architecture of secondary lymphoid organs [74]. Inducible chemokines serve as an emergency response by recruiting leukocytes in response to a physiological stressor or immunological insult and can be increased to levels of over 300-fold in only a few hours after activation [74]. Ligands for a given receptor are almost always restricted to the same chemokine subclass and chemokines receptors are in turn classified into four families depending on which of the chemokine families they bind [75]. Typically CC chemokines bind CC receptors (CCRs), CXC chemokines bind CXC chemokine receptors (CXCRs), XC chemokines bind XC chemokine receptors (XCRs) and CX_3CL1 binds the only CX_3C receptor, CX3CR1 [80].

1.13 Chemokine Receptor CXCR4

CXCR4, represented by a two dimensional topographic diagram in Figure 1.3a, is one of the best-studied chemokine receptors, and currently the only chemokine receptor for which the crystal structure has been determined. It is widely expressed on a number of different cell types including most cells of the immune system (CD34+ hematopoietic stem cells, T-lymphocytes, B-lymphocytes, monocytes, macrophages, neutrophils and eosinophils) and cells of the brain (microglia, astrocytes and neuronal cells), lung, colon, heart, kidney, spleen, thymus and liver [23]. CXCR4 is also expressed on endothelial cells and pericytes in hypoxically injured or pathological tissues and on a number of different progenitor cells (embryonic pluripotent stem cells, and several types of tissue-committed stem cells) [23, 81]. The only known endogenous ligand for CXCR4 is the

chemokine CXCL12, also designated as stromal derived factor- 1 (SDF-1). Cells that express CXCR4 can migrate or invade along CXCL12 gradients, toward areas of high chemokine concentration. CXCR4 is essential for murine development as knockout of either the CXCR4 or CXCL12 genes in mice leads to defects in vascular and CNS development and impaired trafficking of hematopoietic stem cells, proving to be embryonic lethal in late gestation [14, 23, 82, 83].

1.14 Chemokine Receptor CCR5

Unlike CXCR4, CCR5, depicted in Figure 1.3b, is a highly promiscuous receptor with multiple known agonists including CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES; Regulated on activation normal T cell expressed and secreted), CCL8 (MCP-2), CCL11 (Eotaxin), CCL14a (HCC-1) and CCL16 (NCC-4), and a number of selective antagonists [84]. CCR5 is expressed on both CD4+ and CD8+ T-lymphocytes, natural killer T cells, monocytes, macrophages and immature dendritic cells. Within various human populations there exist a large number of CCR5 variants that have assisted in the elucidation of the multiple mechanisms for regulation of the receptor [85]. Several of these mutations involve changes within the coding sequence of the protein that decrease the cell surface expression of CCR5. Of note is the CCR5Δ32 mutant, present in 10% of North American populations but rarely in African and Asian populations, which has a well-characterized 32 base pair deletion that sequesters the truncated receptor within the ER. The deletion removes a membrane-proximal basic domain within the carboxyl-tail required for proper transport of the receptor to the cell surface [85]. CCR5 deficient mice develop normally and humans with the CCR5Δ32 allele are not known to have any health

impairments or deficits in reproductive fitness, likely due to compensation for the lack of the CCR5 receptor by redundancy in the chemokine system [50, 86]. The unperturbed health of those with the CCR5Δ32 mutation also suggests promise for CCR5 antagonists or other therapies that disrupt CCR5 expression at cell surface.

1.15 Role of CXCR4 and CCR5 in HIV Infection

HIV-1 enters cells via a fusion process that is mediated by binding of the HIV-1 envelope glycoprotein gp120 to the cell surface protein CD4, the main receptor for the virus. However, HIV-1 binding to CD4 alone is not sufficient to allow for viral entry. In 1996 it was recognized that CXCR4 and CCR5 were the co-receptors for entry of the human immunodeficiency virus (HIV-1) into lymphocytes (X4) or macrophages (R5) respectively, sparking great therapeutic interest in the chemokine receptors CXCR4 and CCR5. The process of viral entry is now well understood (Figure 1.4). The initial binding of gp120 to CD4 induces conformational changes in both CD4 and gp120 and exposes CD4-induced structural elements which allow the CD4-gp120 complex to then interact with either CXCR4 or CCR5. Binding of the virus to a chemokine co-receptor initiates another series of complex conformational changes that allows for insertion of the N-terminal fusion peptide subunit of gp41 into the target cell membrane. A gp41 six helix bundle then forms, bringing the cell and viral membranes in close enough proximity to allow fusion [87, 88]. R5 HIV viruses are preferentially transmitted over X4 variants. Early, acute infection primarily occurs through the R5 HIV-1 virus and targets predominantly macrophages. During the course of infection the virus can evolve to dual-tropic (X4R5) or mixed (X4 and R5) populations which infect macrophages, naïve CD4+

T-cells and memory CD4+ T-cells. At late stages of the disease X4 variants emerge in 40-60% of HIV-1 positive patients [89]. The later involvement of the X4 and X4R5 variants results in expanded cell tropism, an increase in viral replication rate and the progression of the disease from HIV to acquired immunodeficiency syndrome (AIDS).

In addition to its central role in HIV-1 infection, CD4 also plays an important role in the activation of T lymphocytes through the amplification of major histocompatibility complex (MHC) class II generated signals from antigen-presenting cells [90] and assists in the priming of CD8+ memory cells [91]. Studies have demonstrated that CCR5 and CD4 interact at the plasma membrane in living cells [92], an association that forms within the ER and contributes to increased cell surface expression of CCR5 [93]. In the same study, CD4 was not able to affect the cellular distribution of CXCR4.

The central role of CXCR4 and CCR5 in HIV-1 infection is further evidenced by the apparent antiviral activity antagonists for these receptors show [94, 95] as well as by the fact that the aforementioned intracellularly sequestered CCR5Δ32 mutant confers resistance to infection by the HIV virus. Heterozygotes with the CCR5Δ32 mutation can become infected by the virus but exhibit a slower progression of the disease, while homozygous mutations show near complete resistance to M-tropic infection [85, 86].

Current highly active antiretroviral HIV therapies (HAART) have been highly successful at slowing disease progression and increasing the health and well being in the majority of AIDS patient; however, as these drugs target only two viral proteins, the emergence of HIV strains capable of evading the drug-selective pressure is becoming an increasing problem [50]. Furthermore, these therapies require strict adherence and are often used in complicated regimens with other drugs, contributing to adverse and cross-

drug reactions. Additional avenues to regulate the expression of chemokine receptors at the cell surface are still of great therapeutic interest.

1.16 Role of CXCR4 and CCR5 in Cancer

The migration of cancer cells from an original tumour to selective organs distant in the body, also known as metastasis, exhibits many of the same properties as normal leukocyte traffic. The orderly, multi-step process is the majority cause of mortality in most cancers [96]. It is now widely believed that a balance between CXCL12 and CXCR4 has a large influence on tumour biology [82]. High tissue levels of CXCL12 are believed to play a role in directing angiogenesis, metastasis and survival in cancer pathology and may account for the bias of organ-specific metastases in tissues that highly express CXCL12, such as the lymph nodes, lungs, liver and bone [23]. Furthermore, there is evidence that blockade of CXCR4-CXCL12 signalling suppresses prostate cancer cell proliferation invasion and metastasis [23].

1.17 CXCR4 and CCR5 Involvement in other diseases

Both CXCR4 and CCR5 have been implicated in a number of other significant human diseases, summarized in Table 1.1, for which they could possibly be therapeutic targets. Excessive production of chemokines in response to normally harmless stimuli or oversensitivity of chemokine receptors to chemokine production can result in excessive recruitment of inflammatory cells, tissue damage and chronic inflammatory states. In such cases it is desirable to interrupt the aberrant activation and associated signalling

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pathways. Using antagonists for these chemokine receptors to accomplish this has often been the focus of pharmaceutical research; however, the functional redundancy of the chemokine system has hampered clinical attempts to block the deleterious effects of specific chemokines and chemokine receptors by this approach [97]. Several other processes, including the prevention of receptor traffic to the cell surface, contribute to cell surface expression of chemokine receptors and are also viable possibilities for further research. There is an urgent need for further research into the basic biological organization and regulation of chemokine receptor expression at the cell surface of immune cells. This will provide a better understand how these receptors function *in vivo* may identify unique pharmacological properties that would permit the development of more effective therapeutics.

1.18 Signalling of Chemokines:

While some GPCR signalling pathways have been extensively studied, the association of chemokine receptors with trafficking partners has been largely uncharacterized. Experiments using pertussis toxin (PTX), a bacterial toxin that catalyzes the ADP-ribosylation of the $G\alpha i$ subunit and prevents G-protein coupled signalling, have revealed that most chemokine receptors generate predominantly $G\alpha i$-mediated signal transduction [98]. However, recent evidence suggests chemokine receptors may also associate with other G proteins including $G_{q/11}$ and G_{16} [99]. CCR5 and the closely related CCR2 receptor can induce PTX-insensitive tyrosine phosphorylation events after ligand binding. CXCR4 coupling may not be limited to $G\alpha i$, and the receptor may interact with other $G\alpha$ proteins such as $G\alpha q$, $G\alpha o$, and $G\alpha s$. Until the complexities of

chemokine receptor signal transduction have been further classified it will be difficult to mediate the function and cellular responses to chemokines [80].

In general, activation of cell surface chemokine receptors generates active Rho-family GTPases and PIP2, which with other cellular components catalyze the formation of actin filament branching. The rapid growth of this actin network pushes the plasma membrane of these cell forward at the leading edge of the cell while extension is suppressed at its sides and trailing end. Both the Gβγ and the Gα subunits are capable of activating PI3K, which results in phosphorylation of several focal adhesion components including proline-rich kinase-2 (Pyk-2), Crk-associated substrate (p130Cas), focal adhesion kinase (FAK), pailin, Nck, Crk, and Crk-L. These adhesion proteins with key roles in cell migration and invasion are up-regulated after exposure to CXCL12. Binding of CXCL12 to the CXCR4 receptor initiates a number of intracellular signalling processes that have divergent responses such as chemotaxis, cell survival and/or cell proliferation (Figure 1.5) [23]. The exact constitution and nature of these pathways may be tissue-dependent and therefore may differ among cell types [100].

1.19 Dimerization of Chemokine receptors

The chemokine receptors CXCR4 and CCR5, as well as CCR2 and CXCR2 and CXCR7, have been shown to form constitutive, ligand-independent homodimers by a variety of techniques [49, 83, 101-103]. Recently spontaneous heterodimerization has also been demonstrated between CXCR4 and CCR5 [104]. In addition, both receptors form dimers with the closely related chemokine receptor CCR2 and heterooligomers of

all three receptors have been reported [47]. CXCR4 also dimerizes with the non-chemokine δ-opioid receptor as well, an association that may suppress signalling of both receptors in a dominant-negative fashion [105]. The suppression of chemokine receptor signalling via heterodimerization could have significant repercussions for pathological processes such as inflammation, pain sensation and HIV-1 infection [105].

1.20 New Ideas

Due to their prominent roles as fusion cofactors for the HIV virus, significant research has been conducted concerning the structure, function and mechanisms regulating cell surface expression and signalling of CXCR4 and CCR5. However, much of this research has focused on the removal of these receptors from the membrane after stimulation. There remain many questions concerning transport of CXCR4 and CCR5 between the endoplasmic reticulum to the membrane. Little is known about the specific Rab GTPases involved in chemokine receptor transport to the plasma membrane after biosynthesis. Furthermore, the recent recognition that GPCRs dimerize introduces a new layer of complexity requiring additional study. To our knowledge there are no other studies of how dimerization may alter the outward trafficking of CXCR4 and CCR5 despite exocytic trafficking controlling a crucial step of cell surface expression. A more complete characterization of the effectors involved in the exocytic transport of dimers of CXCR4 and CCR5 between the endoplasmic reticulum to the plasma membrane could reveal novel targets for the regulation of cell surface expression of these important receptors. Additionally, if dimer-, tissue- or cell-type specific differences in Rab GTPase requirements exist in the trafficking itineraries of CXCR4 or CCR5, it could reveal new,

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more specific pharmacological targets for treatment of a range of disease states associated with these receptors.

1.21 Hypotheses

This project can be divided into three main hypotheses:

1) The involvement of Rab GTPases will differ between the anterograde pathways of homodimers and heterodimers of CXCR4 and CCR5 (the trafficking itineraries)

2) The presence of CD4 will affect the trafficking requirements of CCR5-containing receptor dimers.

3) Using dominant-negative versions of the Rab GTPases to interfere with cell surface expression of dimers of CXCR4 and CCR5 will have functional consequences on cell migration in Jurkat cells.
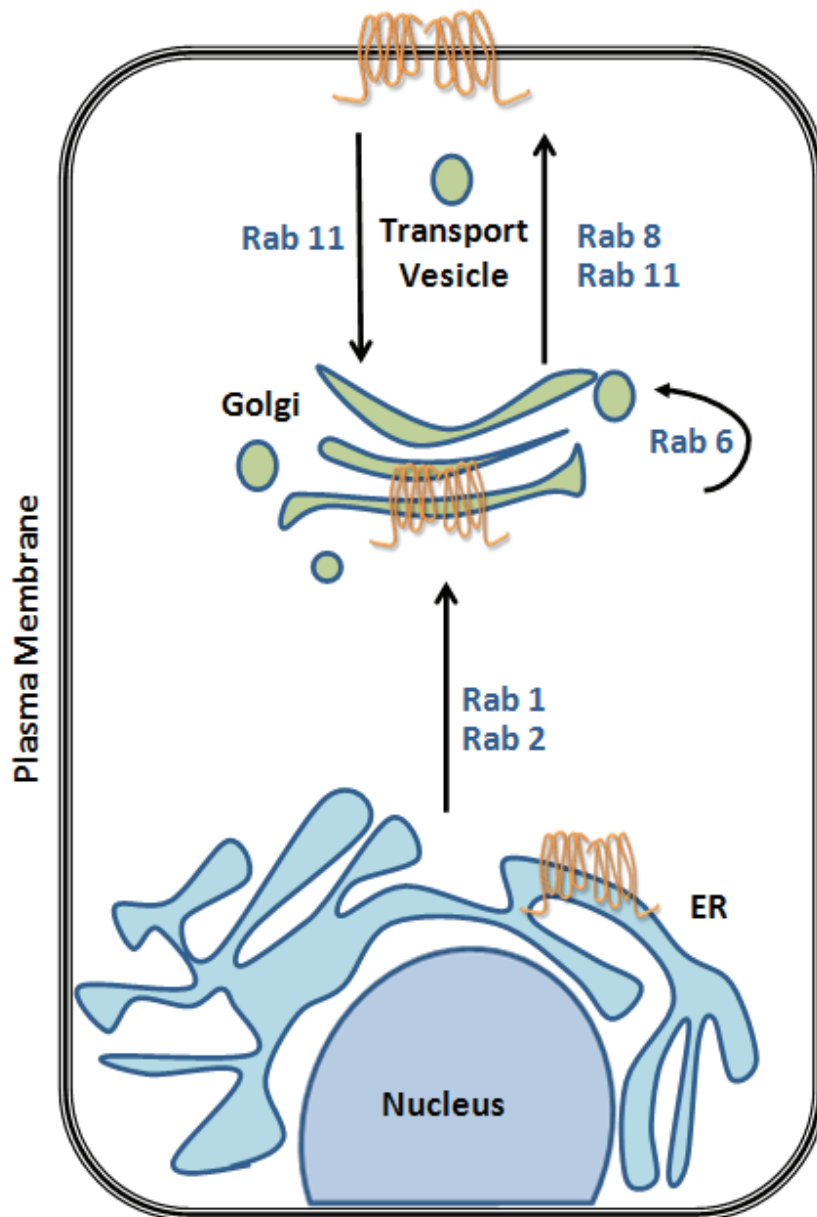
Figure 1.1 Rab GTPase Involvement in Anterograde Transport
Like other integral membrane proteins, GPCRs are transported by a specific subset of
Rab GTPases from the ER to the plasma membrane. There is evidence that at least some
GPCRs can dimerize and/or assemble with signalling partners before exit from the ER.
The exact components of this pathway can be elucidated by using dominant negative
versions of the Rab GTPases and looking at effects on the downstream expression of that
receptor at the plasma membrane.

a Ontogenesis **b** Pharmacological diversity **c** Signal transduction **d** Internalization

Golgi

ER

Transport vesicle

Nature Reviews | Drug Discovery
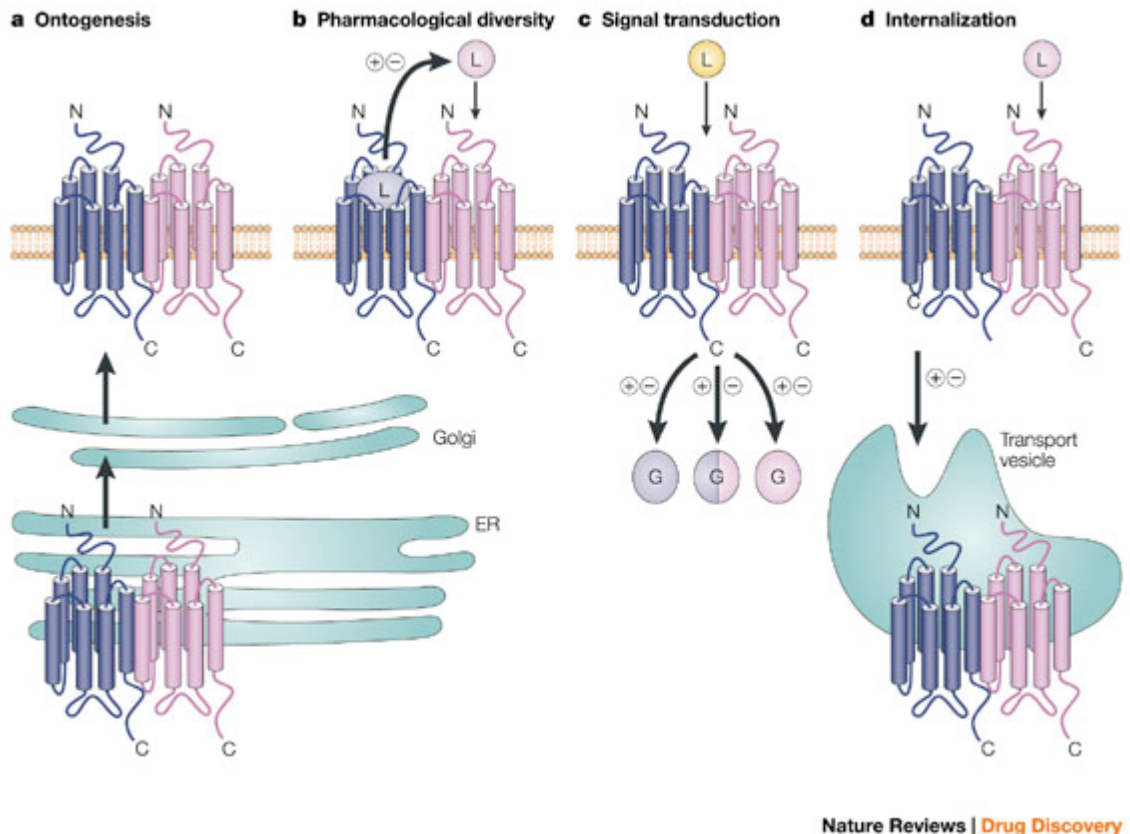
Figure 1.2 Potential Roles for GPCR Heterodimerization
a) Heterodimerization can play a role in the folding, quality control, and membrane targeting of newly synthesized GPCRs. b) Many studies have show that ligand binding to one receptor (blue) can positively (+) or negatively (-) influence the binding of another ligand to the other receptor in the dimer (red). c) Heterodimerization can alter signal transduction by potentiating (+) or attenuating (-) G protein (G) signalling or even changing G protein selectivity. d) Heterodimerization can impact receptor internalization. Ligand binding to one protomer can cause the co-internalization of both receptors (+), or an internalization-resistant protomer can inhibit agonist-promoted endocytosis of the heterodimer complex (-). G, G protein ; L, ligand [Ellis, *Nature Reviews Drug Discovery 2004* 3:577-626, with permission].
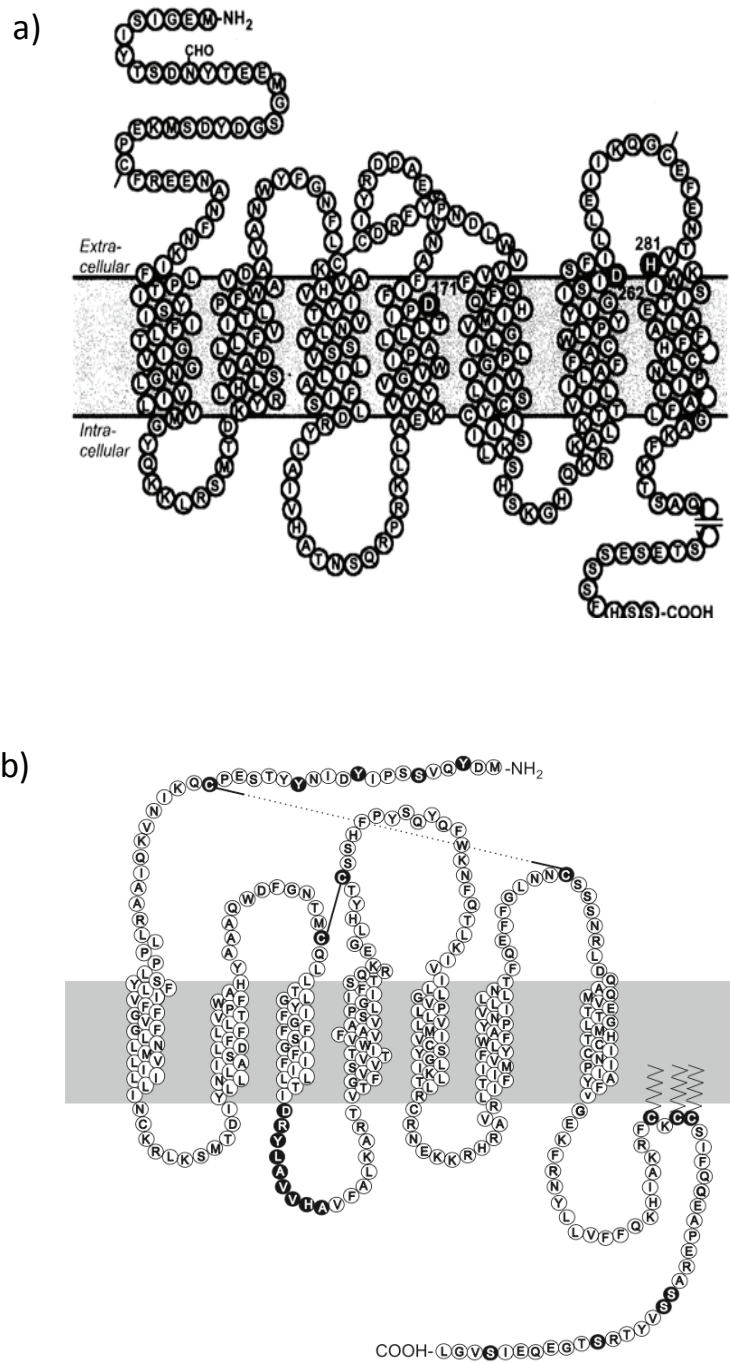
Figure 1.3 The Structures of CXCR4 and CCR5

a) The 352 amino acid sequence and seven transmembrane configuration of CXCR4. The grey box depicts the approximate location of the membrane when the receptor is inserted in the plasma membrane. [Hatse S. et al., *Molecular Pharmacology* 2001, 60(1): 164-173, with permission]. b) The 352 amino acid sequence of CCR5. Filled circles indicate amino acids known to be critical to the function of CCR5. The grey box depicts the approximate location of the lipid bilayer [Opermann M. *Cellular Signalling* 2004, 16(11): 1201-1210, with permission].
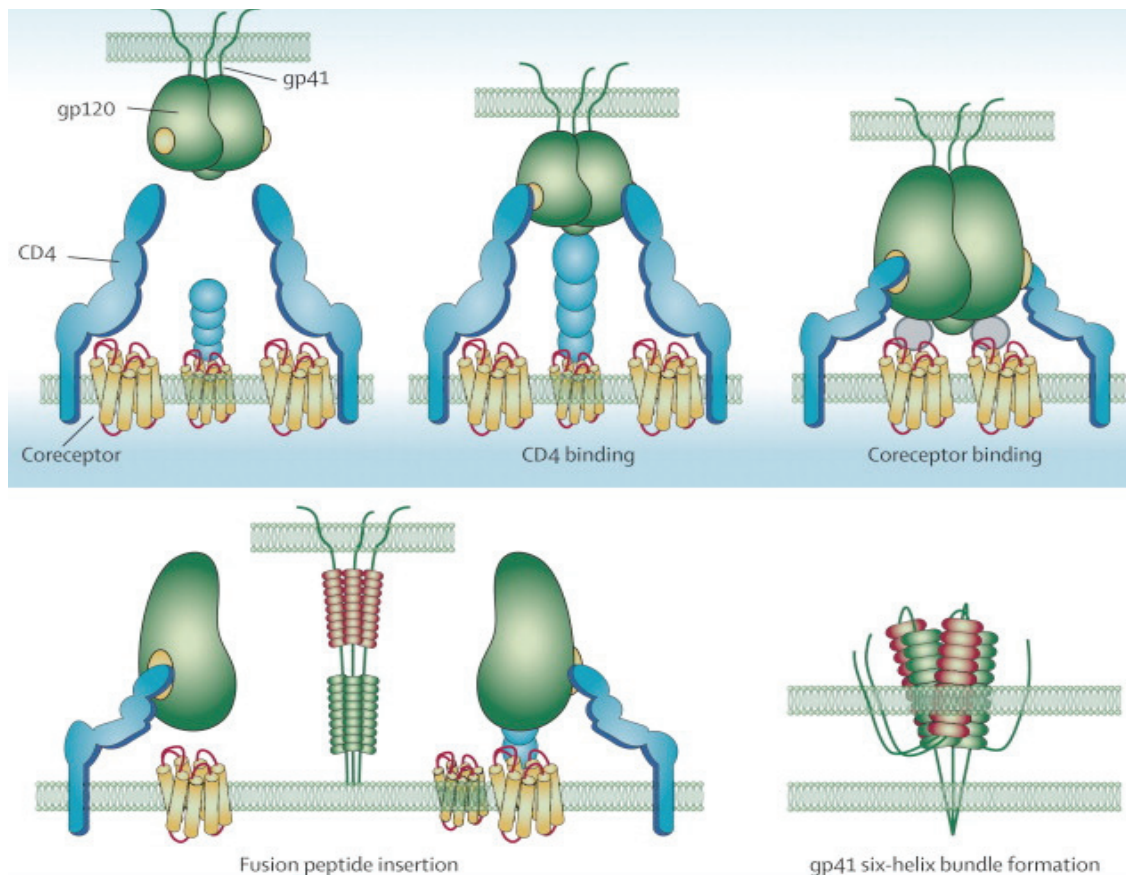
Figure 1.4 Mechanism of HIV Entry
The viral entry process involves series of cooperative sequential interactions between multiple components of both the viral envelope and host cell surface membrane. First, the viral envelope protein gp120 binds to the receptor CD4, revealing new CD4-induced epitopes on the virus capable of binding to either CXCR4 or CCR5. Co-receptor binding initiates another series of conformational changes allowing the insertion of the N-terminal portion of gp41into the host cell membrane. The subsequent formation of a gp41 six-helix bundle formation allows for the close approximation of the host and viral membranes and membrane fusion. [Este and Telenti, *Lancet* 2007, 370(9581): 81-88, with permission].
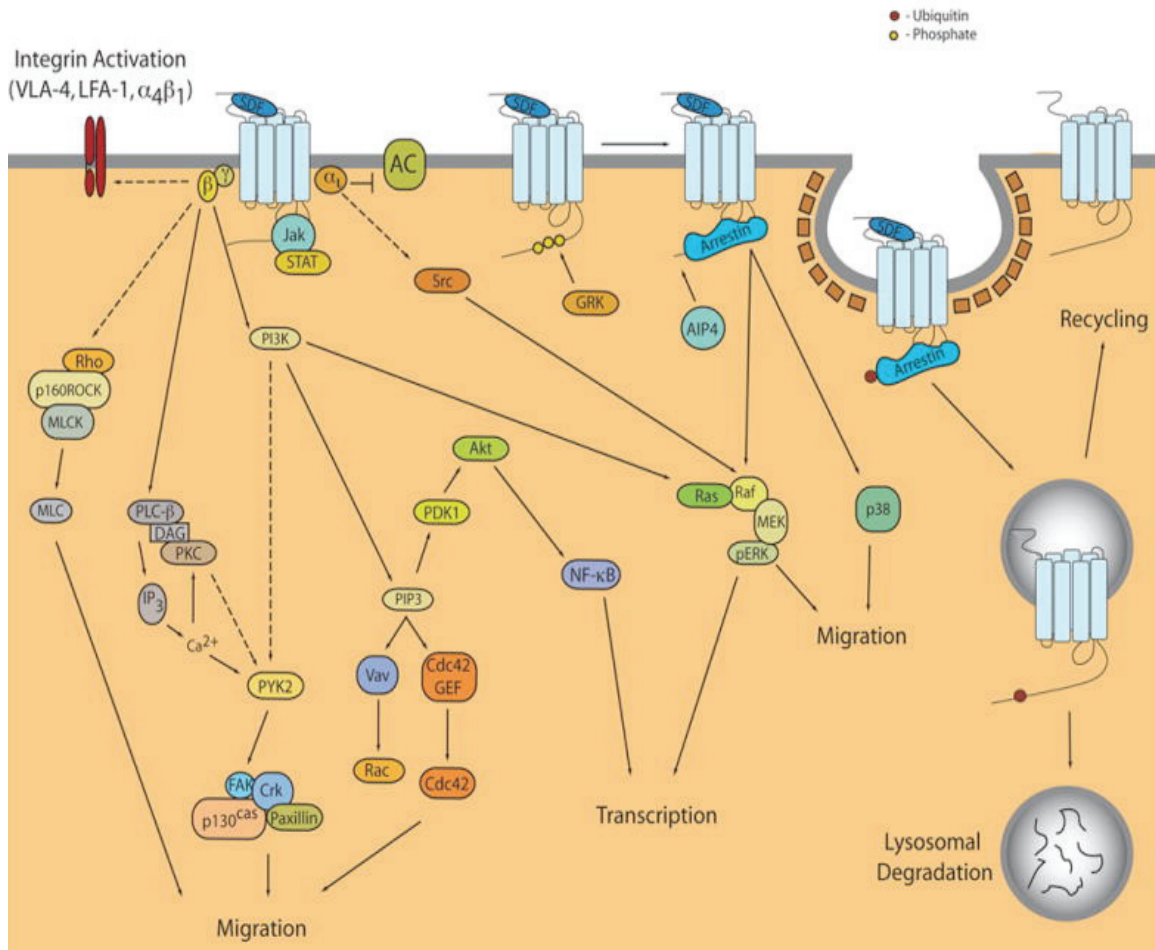
Figure 1.5 Key Intracellular Signal Transduction Pathways Coupled to CXCR4
Activation of CXCR4 by its natural ligand CXCL12 initiates a complex cascade of
intracellular messengers. G-protein mediated signals are involved in a wide range of
processes that have both immediate effects, such as the mobilization of intracellular
calcium stores, as well as long term effects such as alterations in transcription and gene
expression, cell proliferation and survival. CXCR4 is also believed to signal through a g-
protein independent JAK/STAT signalling pathway [Busillo and Benovic 2007,
Biochimica et Biophysica Acta, 1768(4): 952-963, with permission].

Table 1.1 Diseases in which CXCR4 and CCR5 have been implicated

Receptor	Disease	References	Implication
CXCR4	HIV	[106]	Virus co-receptor
	Rheumatoid Arthritis	[107]	Inhibition of CXCR4/CXCL12 biological axis reduces magnitude of pulmonary fibrosis in animal model
	Atherosclerosis	[108]	CXCL12 is highly expressed in smooth muscle cells, endothelial cells, and macrophages in human atherosclerotic plaques but not in normal vessels
	Cancer Metastasis (Lung, Breast, Prostate)	[109, 110]	CXCR12 enhances cell adhesion, migration, metastasis; CXCR4 blockade reduces cancer cell metastasis
	WHIMS	[111, 112]	Mutant CXCR4 is associated with aberrant function, lymphocyte deficiencies
	Pulmonary Fibrosis	[113]	Recruitment of fibrocytes to the lung is dependent on the chemokine ligand CXCL12; inhibition CXCR4/CXCL12biological reduces the magnitude of pulmonary fibrosis
	Asthma	[114]	CXCL12 and CXCR4 may be associated with airway remodeling in mice with asthma
CCR5	Animal Allograft Rejection	[115]	Combined CXCR3 and CCR5 blockade prolongs allograft survival in a fully MHC mismatched murine model
	HIV	[116]	Virus co-receptor
	Asthma	[117, 118]	CCR5 blockade prevents asthma like symptoms in mouse model
	Juvenile Idiopathic Arthritis (JIA)	[119]	CCR5 expression level could influence migration of proinflammatory T cells into the synovium and thus susceptibility to JIA
	Atherosclerosis	[120]	CCR5Δ32 mutation associated with lower risk of disease, receptor antagonists reduce atherosclerosis in mice models
	Prostate Cancer	[121]	Inflammatory chemokines, such as CCL5, expressed by prostate cells may act directly on the growth and survival of PCa cells
	Breast Cancer	[122]	CCR5 expression influences the progression of human breast cancer in a p53-dependent manner

Chapter 2 : MATERIALS AND METHODS

2.1 Reagents

Reagents were obtained from the following sources: fetal bovine serum, Lipofectamine 2000 and Lipofectamine LTX and Plus reagents were from Invitrogen (Etobicoke, ON, Canada). Dulbecco's Modified Eagle's Medium High Glucose, RPMI 1640 Medium, monoclonal anti-FLAG antibody, protein A-Sepharose, crystal violet, forskolin and all chemicals, unless otherwise noted, were from Sigma-Aldrich (Oakville, ON, Canada). EZ-Link Sulfo-NHS-LC-Biotin and Streptavidin Agarose Resin were purchased from Thermo Scientific (Rockford, IL). Complete EDTA-free cocktail protease inhibitors tablets were purchased from Roche Applied Science (Branford, CT). CD4 shRNA plasmid (sc-29246-SH), polyclonal anti-GFP, monoclonal anti-c-myc, polyclonal anti-Rab1, monoclonal anti-VSVG, polyclonal anti-CD4 antibodies, and horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Human recombinant CCL5 and CXCL12 were from Cedarlane Labs, (Hornby, ON, Canada). Hs-Rab1A-5 (SI00301560), Hs-Rab1A-9 (SI02662716), Hs-Rab11A-5 (SI00301553) and Hs-Rab11A-7 (SI02663206) Flexitube shRNAs were purchased from Qiagen (Valencia, CA). Bovine serum albumin was obtained from Bio Basic Inc. (Markham, ON, Canada).

2.2 Constructs

CCR5 and CXCR4 receptors were purchased from the Missouri University of Science and Technology cDNA resource center and transferred into a pcDNA3.1 vector containing the N- (Venus1) or C- terminal (Venus2) fragment of Venus yellow fluorescent protein as previously described [123]. Each receptor is coupled to either an N-terminal or C-terminal fragment of a yellow fluorescent protein variant, Venus. Alone, each fragment of the Venus protein is non-functional and does not emit fluorescence or bind an anti-GFP antibody. However, upon dimerization of their associated receptors, which is not influenced by the presence of the venus 'tags', the two venus fragments are brought together in very close proximity, allowing for the reconstitution of a functional fluorescent protein, which fluoresces and is detected by our anti-GFP antibody (Figure 2.1). Wild-type and dominant-negative Rab GTPases were generated as previously described [29]. Expression levels of the receptor constructs and the different WT and dominant negative GTPase constructs were verified by Western Blotting total cell lysates.

2.3 Cell Culture and Transfection

HEK293 cells were grown in DMEM supplemented with 10% FBS. Cells were plated in 6-well plates and transfected with 1μg of each cDNA using Lipofectamine 2000 as per the manufacturer's instructions. Jurkat cell lines CRL-10915 and TIB-152 were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum. Cells were plated in 6-well plates and transfected with 6uL each cDNA using Lipofectamine LTX and Plus reagent as per the manufacturer's instructions. siRNAs were transfected using

Lipofectamine LTX and plus reagent as per manufacturer's instructions. All experiments were performed 48h after transfection.

2.4 Biotin-Streptavidin Cell Surface Assay

The biotin-streptavidin cell surface assay technique makes use of the extremely high affinity of the biotin-streptavidin interaction to isolate cell membrane proteins from a heterogeneous cell lysate. Whole cells are first treated with EZ-link Sulfo-NHS-Biotin, a long-chain amine reactive reagent that forms stable bonds with primary amines in the lysine side-chains and N-termini of proteins (Figure 2.2a). Due to a long, flexible spacer arm and the relatively small size of biotin, steric interference is minimized allowing for multiple biotin molecules to bind a single protein. As EZ-link Sulfo-NHS-Biotin is not membrane permeable it can only react with cell surface proteins. Cells are then lysed and streptavidin-agarose beads are added to the cell lysate (Figure 2.2b). The affinity of avidin for biotin is one of the strongest known non-covalent protein-ligand interactions, which allows for the biotinylated molecules within the cell lysate mixture to discretely bind the streptavidin conjugated agarose beads (Figure 2.2c). The agarose beads can then be washed several times to eliminate unbiotinylated proteins. The biotin/streptavidin interaction can then be reversed by the addition of dithiothreitol (DTT) to release biotinylated oligonucleotides, resulting in the purification of cell surface proteins (Figure 2.2d).

HEK 293 cells expressing a receptor pair and a wild-type or dominant negative Rab in the presence or absence of exogenous CD4 were harvested in PBS (1X) 48h after transfection. Jurkat CRL-10915 and TIB-152 cells expressing a venus-tagged receptor

pair and a Rab construct (and endogenous CD4 in TIB-152 cells) were harvested 48h post-transfection. Cells were washed once with cold PBS and incubated with 0.9mM EZ-link Sulfo-NHS-LC-Biotin for 30 minutes. Cells were then washed with PBS (1X) +100mM glycine twice. Samples were lysed with radioimmune precipitation assay (RIPA) buffer (50mM Tris, pH 7.5, 10mM $MgCl_2$, 150mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% SDS, complete protease inhibitors and DNase I). The supernatant was incubated overnight with Streptavidin Agarose resin. Samples were washed three times with cold RIPA buffer and incubated in SDS- sample buffer containing 2.8M DTT for 1h at room temperature to elute bound proteins. Immunoblots were probed with anti-GFP antibody or anti-CD4 antibody (1:2000) and horseradish peroxidase-conjugated secondary antibody (anti-rabbit, 1:10,000).

2.5 Cell Migration Assay

Jurkat cells transfected with chemokine receptors and Rab constructs or Rab shRNAs were serum-starved 24h post-transfection. 48h post-transfection cells were harvested and diluted to 1.5×10^6 cells/mL using sterile filtered RPMI media + 0.1% BSA [124]. Cell migration was assayed using a 24 well transwell permeable support system with polycarbonate membrane, pore size 5.0μm (Costar, Corning, NY). 1.5×10^5 cells were then seeded into to the upper chamber of the transwell apparatus, separated from a lower chamber by a polycarbonate membrane with 5.0μM diameter pores. 600μL of RPMI + 0.1%BSA, in the presence or absence of 30ng/mL CXCL12 or 10ng/mL CCL5, was then added to the bottom chamber (Figure 2.3a). Transwells were incubated for 5h at 37°C and 5% CO_2. During this time Jurkat cells are able to migrate through the

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membrane toward the chemoattractant but then remain attached to the underside of the membrane. The membranes can be fixed and stained with a dye such as Crystal Violet to allow the visualization and quantification of the number of cells that migrated toward the chemoattractant (Figure 2.3b). Membrane inserts were then washed once with PBS (1X), fixed using ice-cold methanol for 15 minutes and incubated in a 0.5% Crystal Violet solution for 5 minutes. Non-migrated cells were removed from the upper side of the membrane using a cotton tip. Membranes were washed with H_2O until runoff was clear and air-dried before mounting on a glass slide. At least 3 areas of each membrane photographed under the 10X objective of an Olympus IX81 equipped with a photometrics coolSNAP HQ2 camera using MetaMorph software. Number of cells for each field of view were computed using ImageJ software. Net migration was calculated by comparing the wells receiving chemoattractant to those without. Results from cells transfected with dominant negative Rab GTPases were normalized to those of their wild-type counterparts.
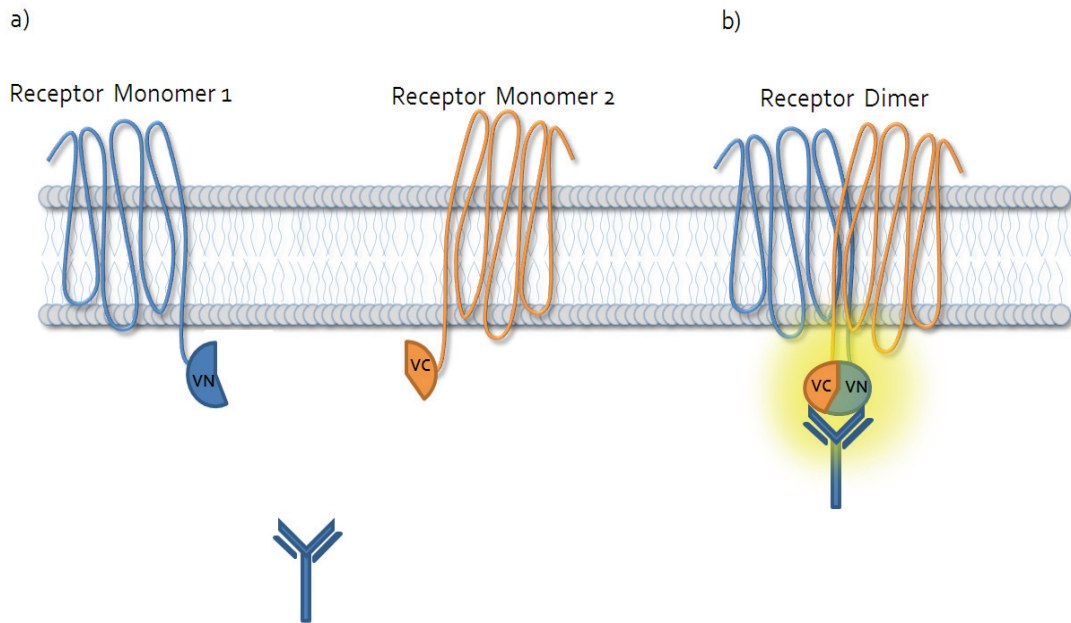
Figure 2.1 Bioluminescence Complementation
(a) Each receptor is tagged with either the N-terminal (VN) or C-terminal (VC) fragment of the GFP variant Venus, denoted Venus 1 or Venus 2 respectively. These Venus fragments are alone non-functional and do not emit fluorescence or bind our anti-GFP antibody. (b) If the two complementary Venus fragments come in very close proximity to each other through the dimerization of their attached receptors, the two fragments will covalently reconstitute into a functional, fluorescent protein and emit florescence. The reconstituted protein is detected by an anti-GTP antibody.
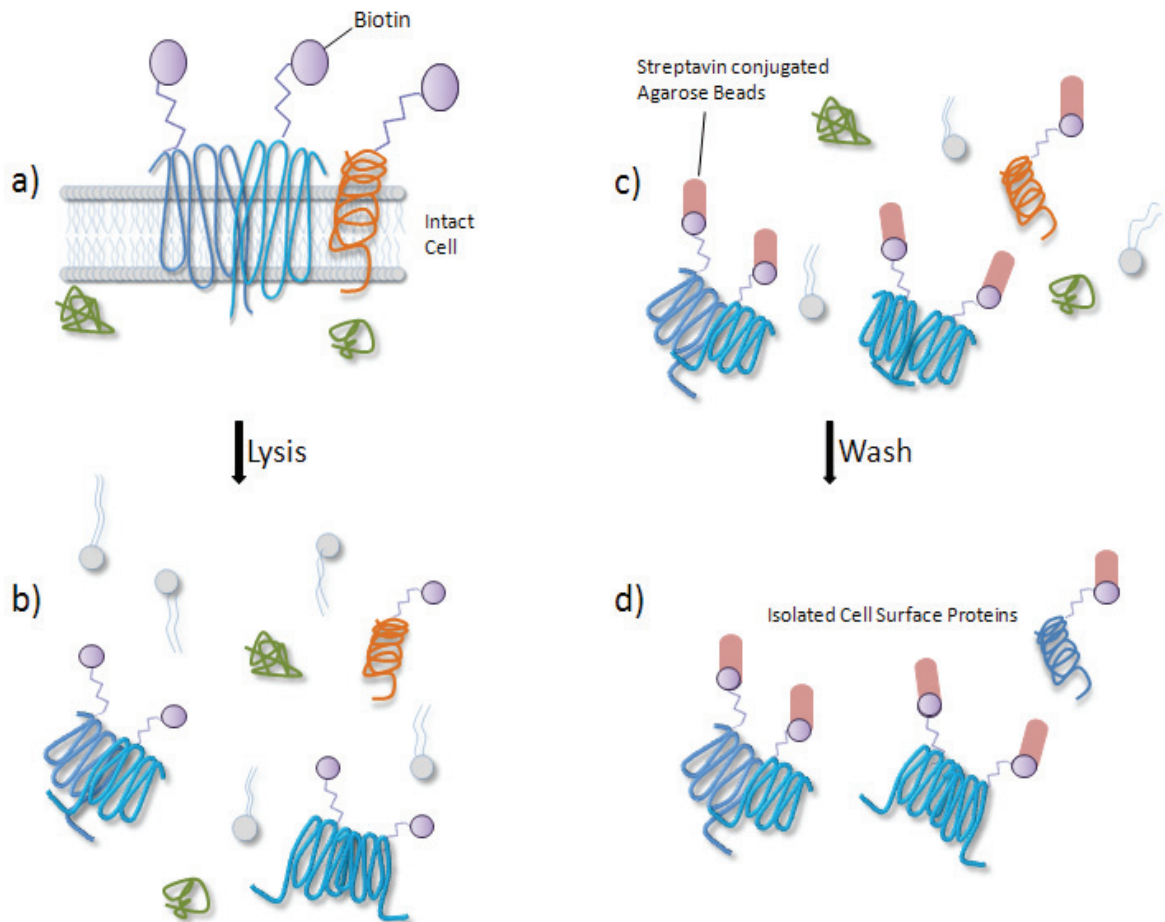
Figure 2.2 Biotin-Streptavidin Cell Surface Labeling
(a) In our biotin-streptavidin cell surface assay whole cells are first treated with EZ-link Sulfo-NHS-Biotin (purple), which reacts with primary amines on proteins to form stable bonds. EZ-link Sulfo-NHS-Biotin is not membrane permeable and only reacts with cell surface proteins (blue and orange) and does not label intracellular proteins (green). (b) Biotinylated cells are then lysed. (c) Streptavidin-conjugated agarose beads (red) are added to the cell lysate and discretely bind the biotinylated proteins. (d) The agarose beads are washed several times to eliminate unbiotinylated proteins, resulting in the purification of cell surface proteins.
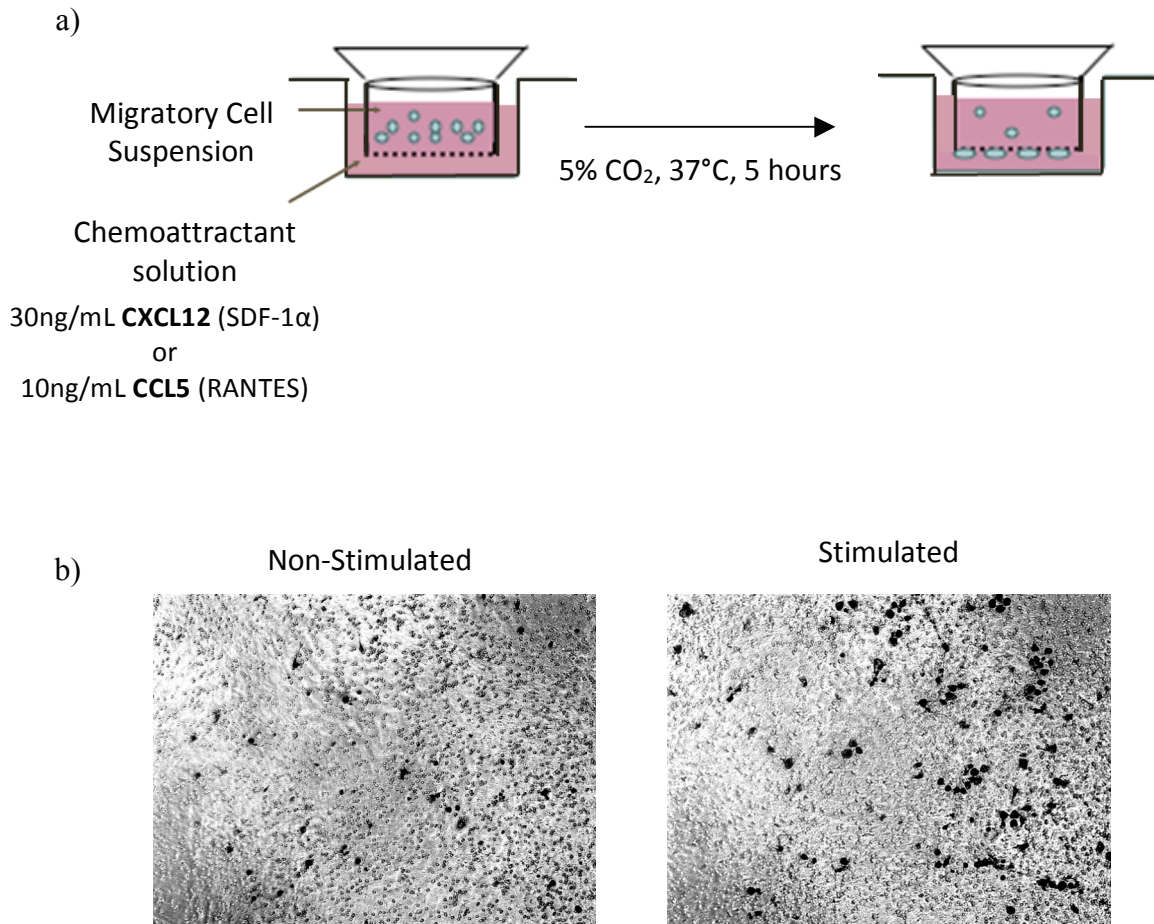
a)

Migratory Cell Suspension

Chemoattractant solution

30ng/mL **CXCL12** (SDF-1α)
or
10ng/mL **CCL5** (RANTES)

5% CO_2, 37°C, 5 hours

b)

Non-Stimulated

Stimulated

Figure 2.3 Transwell Migration Assay Apparatus
(a) In the transwell migration assay, cells are seeded in the upper chamber of an apparatus that is separated from a lower chamber containing media with or without a chemoattractant by a polycarbonate membrane with 5.0μM diameter pores. Cells migrate through the membrane toward the chemoattractant but then remain attached to the underside of the membrane. The membrane can be fixed, stained with crystal violet and photographed to allow the quantification of the number of migrated cells. (b) Representative pictures of two membranes. For each membrane, both non-stimulated and stimulated, at least 3 areas were photographed under the 10X objective of an Olympus IX81 equipped with a photometrics coolSNAP HQ2 camera and MetaMorph software. Number of cells in each field of view were computed using ImageJ software. Results from cells transfected with dominant negative Rab GTPases were normalized to those of their wild-type counterparts.

Chapter 3: RESULTS

3.1 Expression of Constructs in HEK 293 and Jurkat cells

In various cell lines, including HEK293 and cardiac myocytes, mutated forms of Rab GTPases can modify the anterograde trafficking pathway of several GPCRs [29, 35, 125]. Very little is known about the anterograde trafficking of chemokine receptors, including CCR5 and CXCR4, two major HIV co-receptors along with CD4. Some mutants of chemokine receptors, such as the CCR5Δ32 mutant, were shown to be ER retained, and provided some resistance against HIV-1 infection. Our interest was to determine whether we could modulate the plasma membrane expression of CXCR4 and CCR5 by affecting receptor trafficking from synthesis to the plasma membrane.

As it is not clear whether the effectors involved in the trafficking of CXCR4 and CCR5 are cell type specific or general, we chose to study trafficking in two cell types. The first was human embryonic kidney cells (HEK293A), a commonly used, easily transfected cell type. The second cell type examined was a more physiologically representative line of T-lymphocytes, Jurkat cells, which endogenously express CXCR4 and low levels of CCR5 [126-128]. We wanted first to verify that we could express our receptor constructs in both HEK293 and Jurkat cells. HEK293 cells were transfected with either each receptor construct, CXCR4-venus1, CXCR4-venus2, CCR5-venus1 or CCR5-venus2, alone or in complementary pairs (Figure 3.1a). Jurkat TIB-152 cells were transfected with the receptor construct pairs (Figure 3.1b). CXCR4-venus1 and CXCR4-venus2 were co-transfected to form the CXCR4 homodimeric receptor pair, CCR5-

venus1 and CCR5-venus2 to form the CCR5 homodimeric receptor, or CXCR4-venus1 and CCR5-venus2 were co-expressed to form the CXCR4-CCR5 heterodimer. The CCR5-venus1 and CXCR4-venus2 pair was not used in this study since, as previously shown [123] the pairing of those constructs did not generate significant fluorescence reconstitution. Given that in a single cell expressing both a CXCR4-v1 and a CCR5-v2 construct would have both homo and heterodimeric complexes (CXCR4 v1/CXCR4-v1, CCR5-v2/CCR5-v2, CXCR4-v1/CCR5-v2), it was necessary to able to specifically isolate one receptor complex from the others. In order to do so, an anti-GFP antibody capable of recognizing only the functional reconstituted venus was identified. In absence of receptor interaction, such as when cells are transfected with only one of the venus fragments or when non-complementary receptors interact, no fluorescence can be observed, which allows for the specific study of the receptor pairing of interest. All of the receptors could be well expressed in both HEK293 and Jurkat cell lines.

We then expressed several Rab GTPase isoforms, which were previously shown to affect the trafficking between the ER and plasma membrane of some GPCRs, in both HEK293 cells and Jurkat cells. HEK293 or Jurkat TIB-152 cells were transfected myc-tagged Rab1WT or Rab1 S25N, Rab2 WT or Rab2 S20N, Rab6 WT or Rab6 T27N, Rab11 WT or Rab11 S25N, or flag-tagged Rab 8WT or Rab8 T22N for 48 hours, then cells were lysed with RIPA buffer and loaded on an 8% SDS-PAGE gel. The levels of expression of both WT and mutant constructs were similar for each Rab GTPase we expressed in both HEK293 (Figure 3.1c) and Jurkat TIB-152 cells (Figure 3.1d).

3.2 Effect of Rab GTPases Rab1, Rab 2 and Rab 6 on the ER to Golgi Trafficking of Chemokine Receptor Dimers in HEK293 Cells, With or Without Co-Transfection of CD4

It was recently shown that a CD4-CCR5 interaction within intracellular compartments contributes to CCR5 expression at PM in CHO cells, and that silencing of CD4 negatively modulated CCR5 surface expression in human THP-1 and primary T-cells [93]. Interestingly, in that study CD4 was unable to affect CXCR4 levels at the PM. Here, HEK cells either co-transfected with CD4 or not were used to measure the involvement of several Rab GTPases on the trafficking of CCR5 and CXCR4 receptors, when expressed as homo or heterodimers (CXCR4 homodimer, CCR5 homodimer and CXCR4-CCR5 heterodimer). The results found using the HEK293 cell line have been separated into two sections. The first examines receptor traffic between the ER and Golgi and TGN, in which Rabs 1, 2 and 6 are known to function, and the second examines traffic between the Golgi and the PM, in which Rabs 8 and 11 have been implicated.

Figure 3.2 shows the plasma membrane expression levels of the CXCR4, CCR5 and CXCR4-CCR5 heterodimer (right column) after co-transfection with either WT or dominant negative Rab GTPases. For the CCR5 homodimer and CXCR4-CCR5 heterodimer the expression levels were examined when CD4 was not transfected (CD4-condition) and when it was co-transfected (CD4+ condition) so the effect of CD4 expression on the trafficking on each receptor pair could be investigated. WT expression of Rab GTPases were normalized to 100% in each graph (black bars), while the center (dominant negative Rab GTPase, no CD4) and right (dominant negative Rab GTPase

plus CD4) bars represent the percentage of cell surface expression in comparison to their WT counterpart. CXCR4 homodimer trafficking is significantly affected by the co-expression of the dominant Rab1 S25N, 54± 13% (Figure 3.2a, grey bar) when compared to transfection with the wild-type Rab1 (Figure 3.2a, black bar). Contrary to the results observed with the CXCR4 homodimer, the plasma membrane expression of the CCR5 homodimer was not significantly affected by Rab1 S25N when CD4 was absent, 65±15% (Figure 3.2b, grey bar) and present 118 ±9% (Figure 3.2b, white bar), although there was a trend towards a decrease in plasma membrane expression in the CD4- condition. Cell surface expression of the CXCR4-CCR5 heterodimer (Figure 3.2c) was significantly diminished to 57±12% (Figure 3.2c, grey bar) and 45±5.5% (Figure 3.2c, white bar) by the co-expression of Rab1 S25N in both CD4- and CD4+ HEK cells respectively.

When cells were transfected with dominant negative Rab2 S20N, the plasma membrane levels of the CXCR4 homodimer (Figure 3.2d, 73±7%) and the CCR5 homodimer in both CD4 conditions (Figure 3.2e, 67±8% no CD4 and 74±10% with CD4) decreased when compared to cell surface expression when transfected with wild-type Rab2, although only the decrease seen in the CD4- condition was significant. The expression of the CXCR4-CCR5 heterodimer was not significantly decreased by transfection of Rab2 S20N both when CD4 was absent (Figure 3.2f grey bar, 159±13%) and when it was co-transfected (Figure 3.2f white bar, 114±8%). Transfection of HEK293 cells with the CXCR4 homodimer and Rab6 T27N did not decrease the cell surface expression of the receptor (Figure 3.2g, 159±34%). However, Rab6 T27N decreased cell surface expression of the CCR5 homodimer both when CD4 was not present and when CD4 was co-transfected (Figure 3.2h, 66±10% and 60±24%

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respectively), but again only the decrease in the CD4- cells was significant. In cells without CD4, Rab6 T22N significantly decreased cell surface expression of the CXCR4-CCR5 heterodimer (Figure 3.2i grey bar, 70±10%), but when CD4 was co-transfected the reliance on Rab6 for trafficking was abolished (Figure 3.2i white bar, 134±7%). These results (summarized in Figure 4.1) show that anterograde transport of the CXCR4 homodimer involves Rab1 and Rab2, while the CCR5 homodimer involves Rabs1, 2 and 6 when CD4 is absent, but only Rab2 and Rab6 when CD4 is present. The CXCR4-CCR5 heterodimer, unlike either of its constituent homodimers, involves Rab1 and Rab6 when CD4 is absent, but only Rab1 when CD4 is present.

3.3 Effect of Rab GTPases Rab8 and Rab 11 on the Golgi to Plasma Membrane Trafficking of Chemokine Receptor Dimers in HEK293 Cells, With or Without Co-Transfection of CD4

The involvement of Rab GTPases involved in trafficking receptors between the Golgi and the plasma membrane was also investigated. Once again HEK293 cells were transfected with one of the CXCR4-CXCR4, CCR5-CCR5 or CXCR4-CCR5 dimer pairs and either a wild-type or dominant negative GTPase. For the CCR5 homodimer and CXCR4-CCR5 heterodimer the expression levels were examined in the absence or presence of co-transfected CD4. WT expression of Rab GTPases were normalized to 100% (left bar), while the center (dominant negative Rab GTPase, no CD4) and right (dominant negative Rab GTPase plus CD4) bars represent the % of cell surface expression in comparison to their WT counterpart. Figure 3.3a shows that the plasma membrane expression level of the CXCR4 homodimer is nearly unchanged from WT

levels in the presence of Rab8 T22N (119±19%). The PM expression of the CCR5

homodimer was not decreased by the Rab8 dominant negative in either the CD4+ or

CD4- conditions (Figure 3.3b, 159±11% and 106±31% respectively). However, the cell

surface expression of the CXCR4-CCR5 heterodimer is significantly reduced from WT

levels by transfection with Rab8 T22N in CD4- cells (Figure 3.3c grey bar, 66±11%) but

not in CD4+ cells (Figure 3.3c white bar, 130±5%).

Transfection with Rab11 S25N shows the same pattern for all dimers. The PM

expression of CXCR4 homodimer (Figure 3.3d, 60±20%) and the CCR5 homodimer in

cells without CD4 (Figure 3.3e, 102±5%) and with CD4 (269±33%) is not significantly

decreased by the dominant negative Rab 11. Like Rab8 T22N, Rab11 S25N significantly

decreased the PM expression of the CXCR4-CCR5 heterodimer in the absence of CD4

but not when CD4 is present (Figure 3.3f, 61±6% and 145±12% respectively). These

results (also see Figure 4.1) show that the trafficking of each homo- and heterodimer

involves Rab8 and Rab11 for anterograde transport, but that these involvements differ

between the receptor dimer combinations and are affected, in the case of the CXCR4-

CCR5 heterodimer but not the CCR5 homodimer, by the presence of CD4.

3.4 The CD4 Trafficking Pathway in Jurkat Cells

Jurkat cells are a human T-lymphocyte cell line which normally expresses

endogenous CD4, CXCR4 and low levels of CCR5. As these cells are a much more

relevant physiologically system to study our receptors of interest in, the trafficking

experiments performed in HEK293 cells were replicated in Jurkat cells. As previously

mentioned, CD4 has been shown to contribute to CCR5 expression at plasma membrane

[93]. In order to study the effect of CD4 in Jurkat cells two cell lines were used, one that express CD4 (TIB-152) and one that does not (CRL-10915). First, we chose to examine the trafficking pathway of endogenous CD4 so it could be compared to the trafficking pathways of the CCR5 containing receptor dimers. TIB-152 (CD4$^+$) cells were transfected with WT or dominant negative isoforms of Rab GTPases for 48h. Cell surface expression of CD4 was evaluated using the previously described biotin labeling cell surface assay. A western blot analysis using an anti-CD4 antibody allowed us to probe the levels of expression of CD4 at the PM, depending on the expression of the different Rab isoforms. Rab1 S25N (Figure 3.4a) and Rab11 S25N (Figure 3.4e) were able to affect PM localization of CD4 compared to their WT isoforms (36±12% and 25±10% reduction respectively). Rab2 S20N (Figure 3.4b), Rab6 T27N (Figure 3.4c) and Rab8 T22N (Figure 3.4d) did not affect PM localization in comparison with their WT equivalents. We validated these findings using shRNA knockdown of the Rab GTPases believed to be involved in CD4 trafficking. TIB-152 cells were transfected with Rab1A or Rab11A shRNAs to knockdown endogenous expression of Rab1 or Rab 11 (Figure 3.4h and 3.4i). 48h post-transfection surface expression of CD4 was evaluated using the biotin-streptavidin labeling procedure. A western blot analysis using an anti-CD4 antibody allowed us to probe the levels of expression of endogenous CD4 at the plasma membrane. In both cases, knockdown of Rab1 (Figure 3.4f, right bar) and Rab11 (Figure 3.4g, right bar) using 2μL of 10μM shRNA resulted in significantly decreased cell surface expression when compared to WT levels (Figure 3.4f and g; left bars), and reproduced the effects observed when using dominant-negative knockdown of these

GTPases. Our results suggest that CD4 uses Rab1 and Rab11 to traffic from ER to plasma membrane, but not Rab2, Rab6 or Rab8.

3.5 Effect of Rab1 on the Trafficking and Migratory Ability of Chemokine Receptor Dimers in CD4+ and CD4- Jurkat Cell Lines

The involvement of the previously mentioned dominant-negative Rabs on the trafficking of CCR5 and CXCR4 receptors, when expressed as homo or heterodimers (CXCR4 homodimer, CCR5 homodimer and CXCR4-CCR5 heterodimer) was then investigated. TIB-152 or CRL-10915 cells were transfected with the same CXCR4 homodimeric, CCR5 homodimeric or CXCR4-CCR5 heterodimeric receptor construct pairs as were used in the HEK293 cell experiments.

Figure 3.5 a, b, and c show the PM expression levels of the CXCR4 homodimer, CCR5 homodimer and CXCR4-CCR5 heterodimer, respectively, after co-transfection with either Rab1 WT or dominant-negative Rab1 S25N. Each graph shows a comparison of the expression levels in both Jurkat cells lines, CD4- cells (CRL-10915, grey bars) and CD4+ (TIB-152, white bars), so the effect of CD4 expression on the trafficking of each receptor pair can be investigated. WT expression of Rab GTPases was normalized to 100% (left bar), while the center (CRL-10915 cells expressing a dominant negative Rab GTPase) and right (TIB-152 cells expressing a dominant negative Rab GTPase) bars represent the percent of cell surface expression in comparison to their WT counterpart. CXCR4 trafficking does not appear to be significantly affected by the co-expression of Rab1 S25N (Figure 3.5a). Both cell lines showed similar levels of expression at the PM, and were not significantly different from the expression of their WT counterpart. These results agree with the findings of Achour et al. where CD4 expression was not able to

modify the level of CXCR4 expression at plasma membrane. Contrary to the results observed with the CXCR4 homodimer, the plasma membrane expression of both the CCR5 homodimer (Figure 3.5b) and CXCR4-CCR5 heterodimer (Figure 3.5c) was significantly affected by the co-expression of Rab1 S25N in both CD4+ and CD4- cells. The CCR5 homodimer levels of plasma membrane expression were of 45±13% and 68±10% of wild-type expression in CRL-10915 cells and TIB-152 cells, respectively. The expression of CXCR4-CCR5 homodimeric receptors at the PM were 64±16% in CRL-10915, and 50±26% in TIB-152 cells.

We also wanted to examine the effect that altering the trafficking of the homo- and heterodimers of CXCR4 and CCR5 would have on a signalling pathway. As CXCR4 and CCR5 are both primarily expressed on immune cells, including T-lymphocytes, and play a crucial role in enabling the chemotaxis of these cells, we chose to examine migratory ability using a Transwell Cell Migration assay. Figure 3.5 d, e, f and g show the effect of Rab1 S25N on the migration of Jurkat cells towards 30ng/mL CXCL12 for the CXCR4 homodimer (Figure 3.5d) and CXCR4-CCR5 heterodimer (Figure 3.5f) or 10ng/mL CCL5 for CCR5 homodimer (Figure 3.5e) and CXCR4-CCR5 heterodimer (Figure 3.5g). These results demonstrate the same trend previously observed for the trafficking of each receptor dimer; when the anterograde receptor trafficking is affected, chemotaxis towards a respective ligand is affected as well.

3.6 Effect of Rab2 on the Trafficking and Migratory Ability of Chemokine Receptor Dimers in CD4+ and CD4- Jurkat Cell Lines

Figure 3.6a, b, and c show the PM expression levels as measured by cell surface assays of the CXCR4 homodimer, CCR5 homodimer and CXCR4-CCR5 heterodimer, respectively. Contrary to the results with Rab1 S25N, CXCR4 trafficking to the PM is affected by the dominant negative isoform of Rab2 (S20N). The levels of expression of the receptor at plasma membrane are diminished to 65±1% and 42±12%, in CRL-10915 and TIB-152 cells, respectively (Figure 3.6a). Mirroring the trafficking results, migration in both cell lines is also affected by Rab2 S20N (Figure 3.6d); migration was reduced by approximately 50% in both cell lines (49±22% for CRL-10915 and 48±16% for TIB-152 cells). Unlike the results observed with the CXCR4 homodimer, CCR5 homodimer trafficking (Figure 3.6b) and migration (Figure 3.6e) are not significantly affected by expression of Rab2 S20N in both cell lines. PM expression of CXCR4-CCR5 heterodimers (Figure 3.6c) in cells expressing Rab2 S20N was of 58±12% in CRL-10915, and 85±17% in TIB-152 cells, which suggests that the trafficking is significantly affected in CD4- cells bearing the heterodimer, but not in cells that express CD4. The same trend is observed for the migration towards CXCL12 or CCL5 in Figure 3.6f and g, where migration is affected only in CRL-10915 cells expressing Rab2 S20N and not significantly affected in TIB-152 cells.

3.7 Effect of Rab6 on the Trafficking and Migratory Ability of Chemokine Receptor Dimers in CD4+ and CD4- Cell Lines

The effect of Rab6 on chemokine receptor trafficking and migration (Figure 3.7), are the complete reverse of what was observed with the Rab1 S25N isoform. As observed for Rab2 S20N, CXCR4 trafficking toward the PM is affected by the dominant negative isoform of Rab6 (T27N) (Figure 3.7a). The levels of expression of the receptor at the PM are diminished to $60\pm15\%$ and $54\pm12\%$, in CRL-10915 and TIB-152 cells, respectively. Similar to the trafficking results, migration in both cell lines is also affected by Rab6 T27N (Figure 3.7d), shown by reduction in migration in both cell lines ($21\pm6\%$ for CRL-10915 and $65\pm22\%$ for TIB-152 cells). Contrary to the results observed with the CXCR4 homodimer, the CCR5 homodimer and CXCR4-CCR5 heterodimer trafficking (Figure 3.7 b and c respectively) and migration (Figure 3.7e for CCR5 homodimer and 3.7f and g for CXCR4-CCR5 heterodimer) are not significantly affected by expression of Rab6 T27N, in both cell lines.

3.8 Effect of Rab8 on the Trafficking and Migratory Ability of Chemokine Receptor Dimers in CD4+ and CD4- Cell Lines

Figure 3.8 shows the results on trafficking and migration in Jurkat cells expressing either Rab8 WT or T22N isoforms. As observed for Rab2 S20N and Rab6 T27N, CXCR4 trafficking towards PM is inhibited here by the dominant negative isoform of Rab8 (T22N). Figure 3.8a shows that the levels of expression of the CXCR4 homodimer at plasma membrane are diminished to $78\pm7\%$ and $62\pm2\%$, in CRL-10915 and TIB-152 cells, respectively. Similar to the trafficking results, migration in both cell

lines is also affected by Rab8 T22N, as shown in Figure 3.8d. The migration was reduced in both cell lines (18±3% for CRL-10915 and 38±9% for TIB-152 cells). Unlike what was observed with the CXCR4 homodimer, CCR5 homodimer trafficking and migration (Figure 3.8b and e, grey bars) and CXCR4-CCR5 heterodimer trafficking (Figure 3.8b center bar; and Figure 3.8c) and migration (Figure 3.8f center bar; and Figure 3.8g) in CRL-10915 (CD4-) cells are not significantly affected by expression of Rab8 T22N. However, the trafficking and migration of CCR5 homodimers was affected in Jurkat cells expressing CD4 (Figure 3.8e and 3.8f, white bars). Surprisingly, CD4 trafficking in the same cell line didn't show any change when expressed alone with Rab8 T22N, which leads to the conclusion that the combination of CD4-CCR5 homodimers react differently than when each receptor is expressed individually.

3.9 Effect of Rab11 on the Trafficking and Migratory Ability of Chemokine Receptor Dimers in CD4+ and CD4- Cell Lines

As observed for Rab1 S25N, CXCR4 trafficking towards PM (Figure 3.9a, 121±24% for CRL-10915 and 122±20% for TIB-152) and chemotactic migration (Figure 3.9d) are not significantly decreased by the dominant negative isoform of Rab11. The trafficking of the CCR5 homodimer (67±14%) and CXCR4-CCR5 heterodimer (34±15%) in the CD4- CRL-10915 cell line were significantly affected by the expression of Rab11 S25N (Figure 3.9b and c, grey bars). Similarly, the migration of cells expressing these dimers was also significantly decreased by the co-expression of Rab11 S25N (Figure 3.9e, f and g; grey bars). The trafficking (168±0.3% for CCR5 homodimer and 74±22% the CXCR4-CCR5 heterodimer) and migration of the CD4+ TIB-152 cell

line were not significantly decreased from the levels observed with their WT counterpart (Figure 3.9 b and c; white bars and Figure 3.9 e, f and g; white bars).

3.10 Effect of Manipulating CD4 Expression on Trafficking and Migration Phenotype

Because the presence of CD4 was seen to affect the trafficking of CCR5 containing dimers we wanted to examine whether addition of CD4 to CRL-10915 (CD4-) cells or knockdown of endogenous CD4 in TIB-152 (CD4+) cells using 5uL shRNA (Figure 3.6e) would reverse the cell trafficking phenotype. CRL-10915 (CD4-) cells were transfected with the CXCR4v1-CCR5v2 receptor pair, CD4 and either WT or S20N Rab2. When CRL-10915 cells lack CD4 plasma membrane expression of the CXCR4-CCR5 heterodimer is affected by dominant-negative Rab2 (Figure 3.10a grey bar; also shown in Figure 3.6c,). When CD4 pcDNA is expressed in these CD4- cells trafficking no longer involves Rab2 (Figure 3.10a; checkered bar) and the cells adopt a TIB-152 like plasma membrane expression pattern (Figure 3.10a; white bar). These effects are mirrored in a similar assessment of migratory ability. CRL-10915 cells in the presence of Rab2 S20N show significantly decreased migration towards 10ng/mL CCL5 (Figure 3.10b; grey bar), however, co-transfection with both CD4 and Rab2S20N (Figure 3.10b; checkered bar) allows for these cells to migrate in a similar manner to the TIB-152 cell type (Figure 3.10b; white bar). Similar experiments, in which endogenous CD4 was knocked down in the CD4+ TIB-152 cell line, were also performed. Proper PM expression of the CCR5v1-CCR5v2 homodimer in TIB-152 cells normally involves Rab8 (both Figure 3.8b and Figure 3.10c; white bars) but when CD4 expression is knocked down in TIB-152 cells using shRNA against CD4 (using 5μL of a 0.2μM shRNA

solution), Rab8 was no longer involved Rab8 for trafficking of the homodimer (Figure 3.10c; checkered bar), mimicking the Rab8 dependence of CRL-10915 cells (Figure 3.10c; grey bar). Once again, migration experiments mirrored these results; TIB-152 cells normally show decreased migratory ability toward CCL5 in the presence of Rab8 T22N (Figure 3.10d; white bar), but upon knockdown of CD4 in these cells migratory ability is restored to wild-type levels (Figure 3.10d; checkered bar) and closely resembles the migration of CRL-10915 cells (Figure 3.10d; grey bar).
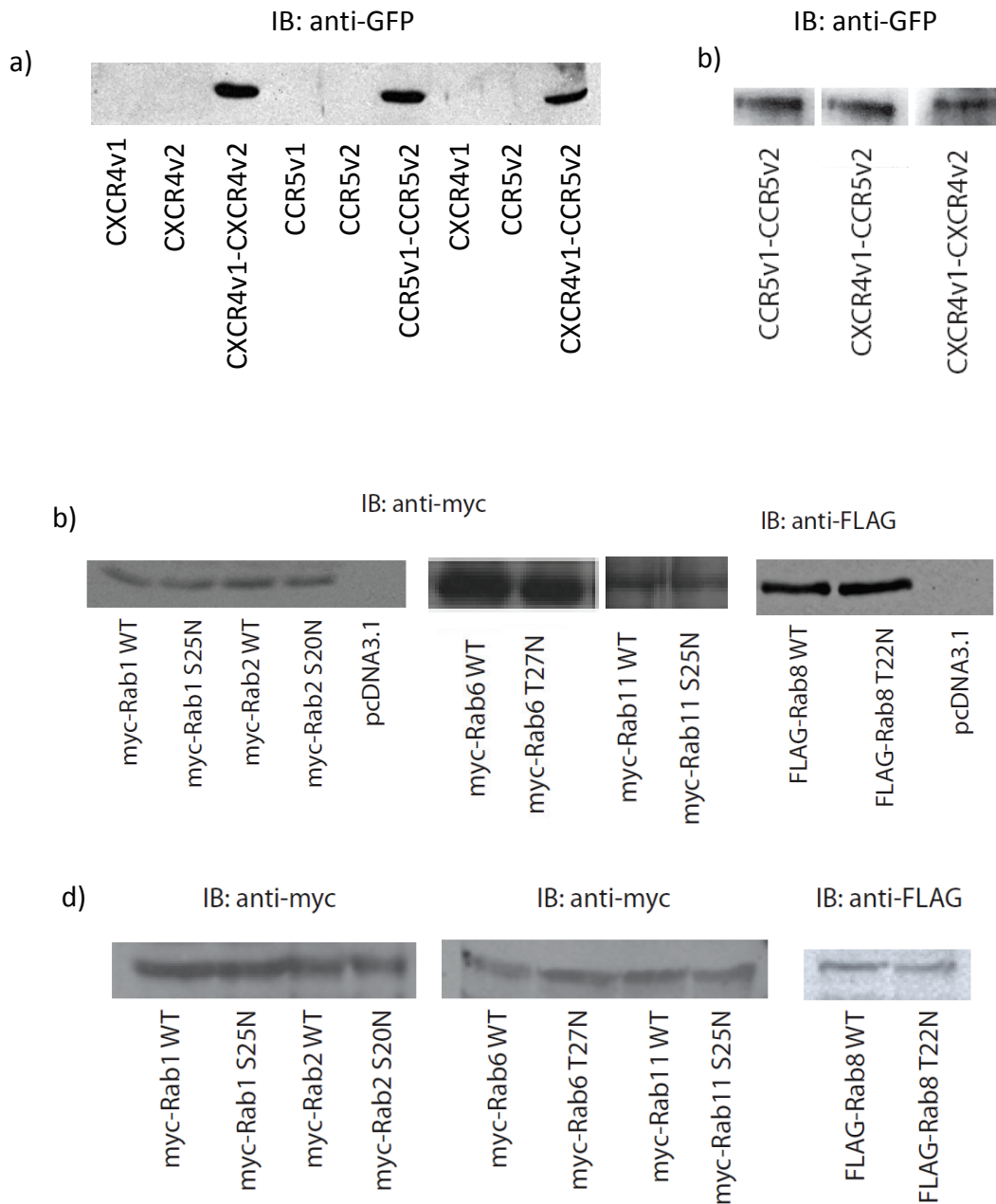
Figure 3.1: Expression of CXCR4, CCR5 and Rab GTPase Constructs in HEK293 and Jurkat Cells

HEK293 cells or TIB-152 Jurkat cells were transfected with either CXCR4 or CCR5 venus constructs or WT and dominant negative Rab constructs. 48h after transfection, cells were harvested, washed and lysed with RIPA buffer. Samples were run on a 10% SDS-PAGE gel and membranes were probed with anti-GFP, anti-myc or anti-Flag antibodies (1:2000). Results are representative of 3 experiments. (a) Receptor construct expression in HEK293 cells. (b) Receptor construct expression in Jurkat TIB-152 cells. (c) Rab construct expression in HEK293 cells. (d) Rab construct expression in Jurkat TIB-152 cells.

CXCR4-CXCR4 Homodimer

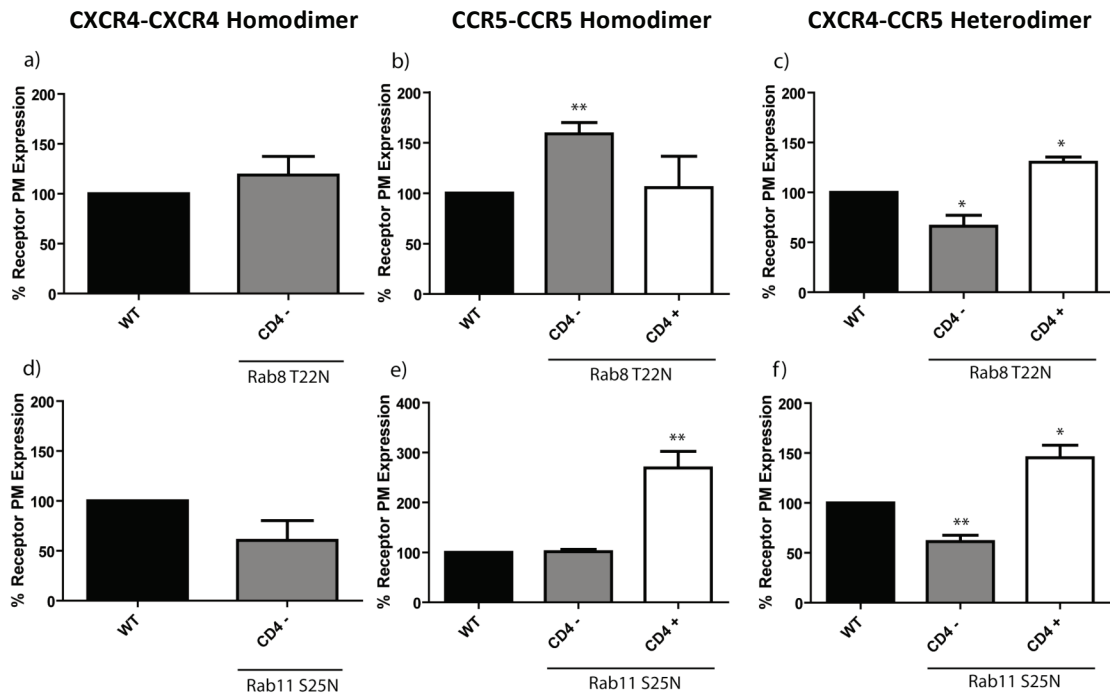**CCR5-CCR5 Homodimer**

CXCR4-CCR5 Heterodimer
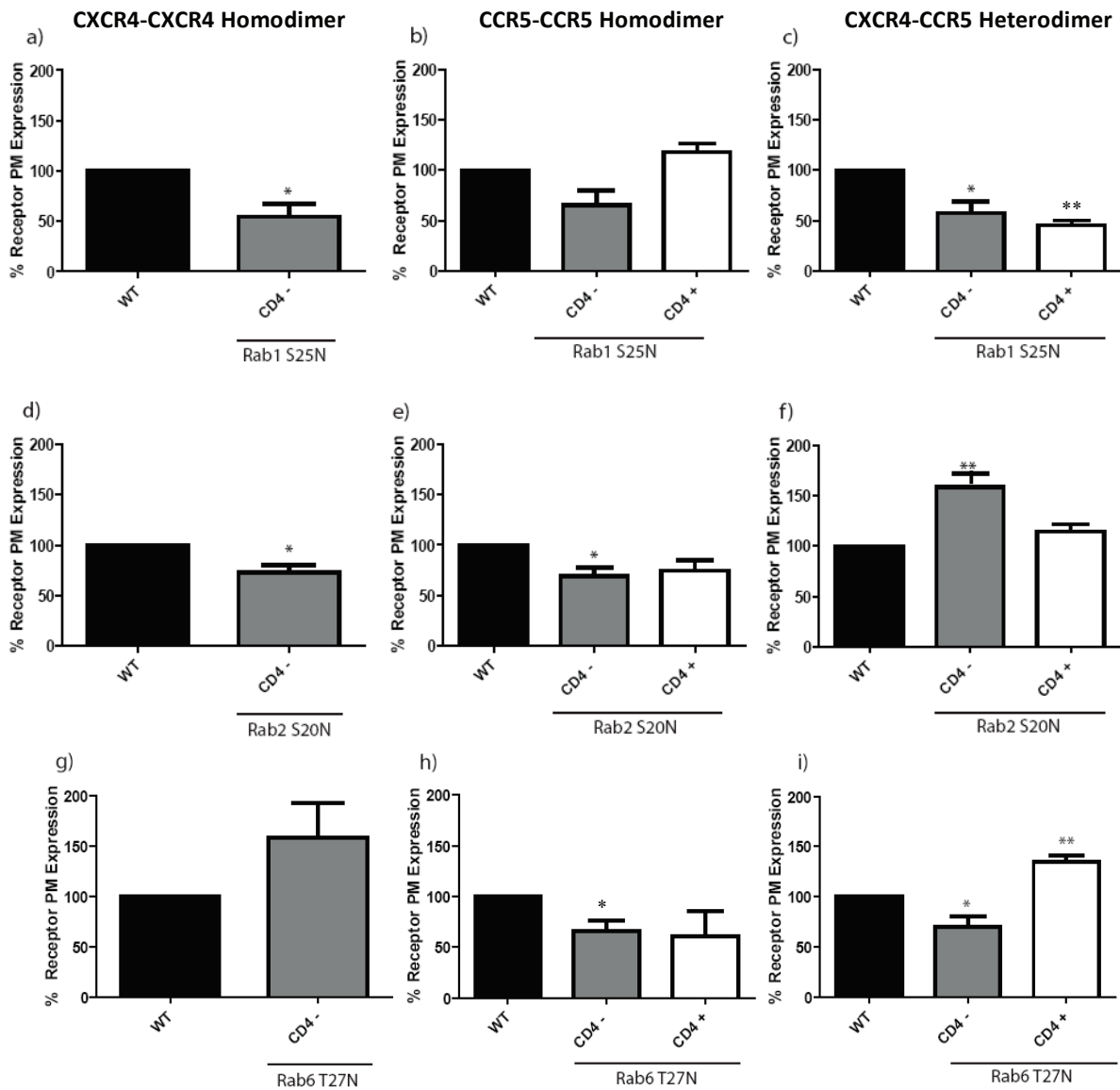
Figure 3.2: Effects of the ER-Golgi Rab GTPase Dominant Negatives Rab1 S25N, Rab2 S20N and Rab6T27N on the Trafficking of CXCR4 and CCR5 Homo- and Heterodimers to the Plasma Membrane of HEK293 Cells With or Without Co-Expression of CD4
Trafficking experiments were performed 48h post-transfection with receptor dimer and either Rab WT or Rab1 S25N, Rab2 WT or Rab2 S20N or Rab6 WT or Rab6 T27N constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized the specified receptor dimers. a) Effect of Rab1 S25N on PM expression of CXCR4 homodimer. b) Effect of Rab1 S25N on PM expression of CCR5 homodimer. c) Effect of Rab1 S25N on PM expression of CXCR4-CCR5 heterodimer. d) Effect of Rab2 S20N on PM expression of CXCR4-CXCR4 homodimer. e) Effect of Rab2 S20N on PM expression of CCR5 homodimer. f) Effect of Rab2 S20N on CXCR4-CCR5 heterodimer. g) Effect of Rab6 T27N on PM expression of CXCR4 homodimer. h) Effect of Rab6 T22N on PM expression of CCR5 homodimer. i) Effect of Rab6 T27N on PM expression of CXCR4-CCR5 heterodimer. Results representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$ using a two-tailed paired student's t-test.

CXCR4-CXCR4 Homodimer **CCR5-CCR5 Homodimer** **CXCR4-CCR5 Heterodimer**

Figure 3.3: Effects of the Golgi-PM Rab GTPase Dominant Negatives Rab8 T22N and Rab11 S25N on the Trafficking of CXCR4 and CCR5 Homo- and Heterodimers to the Plasma Membrane of HEK293 Cells With or Without Co-Expression of CD4
Trafficking experiments were performed 48h post-transfection with receptor dimer and either Rab8 WT or Rab8 S25N, or Rab11 WT or Rab11 S25N constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized the specified receptor dimers. a) Effect of Rab8 T27N on PM expression of CXCR4 homodimer. b) Effect of Rab8 T22N on PM expression of CCR5 homodimer. c) Effect of Rab8 T22N on PM expression of CXCR4-CCR5 heterodimer. d) Effect of Rab11 S25N on PM expression of CXCR4 homodimer. e) Effect of Rab11 S25N on PM expression of CCR5 homodimer. f) Effect of Rab11 S25N on PM expression of CXCR4-CCR5 heterodimer. Results representative of at least 3 independent experiments. *$p<0.05$, **$p<0.01$ using a two-tailed paired student's t-test.
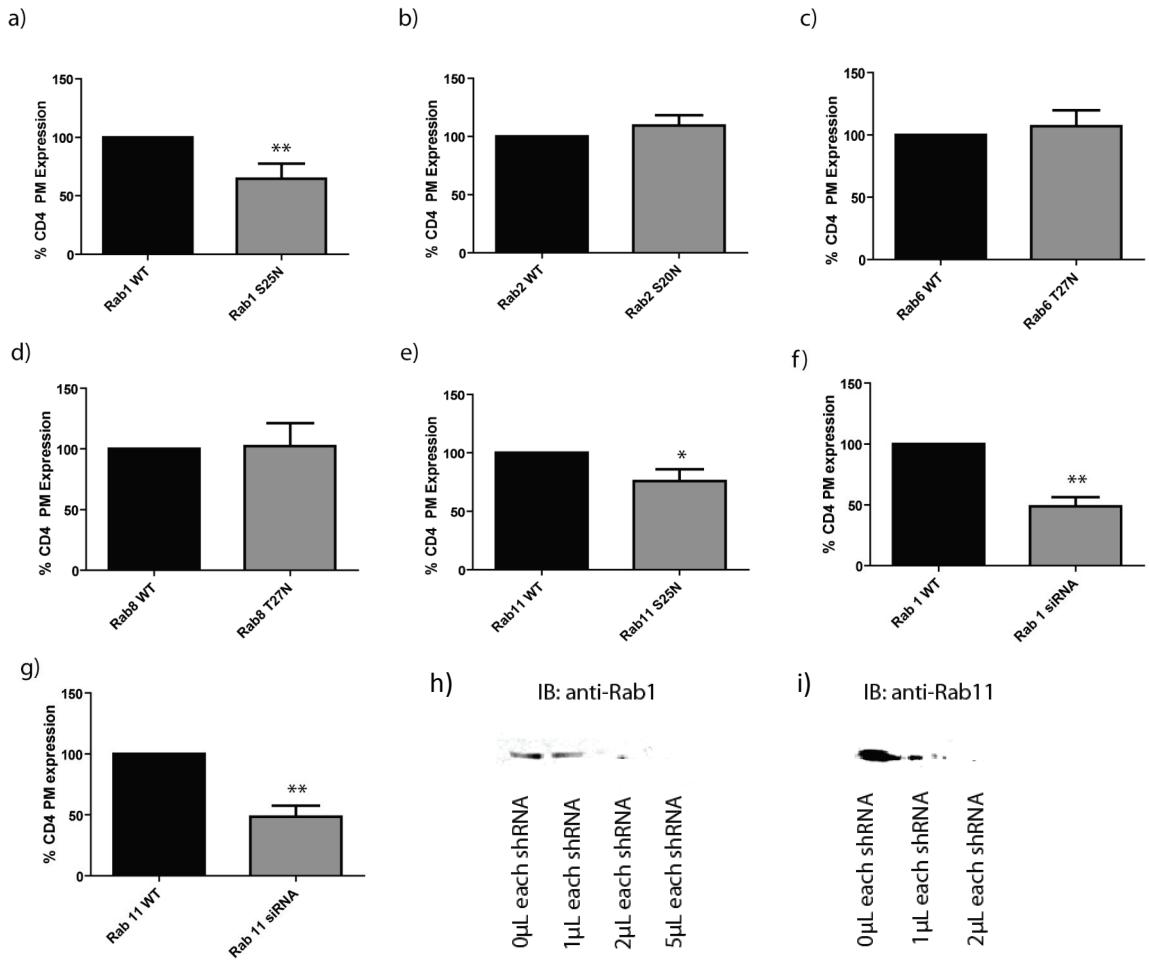
Figure 3.4: Effect of Rab GTPases on Trafficking of Endogenous CD4 to the Plasma Membrane in TIB-152 Cells

TIB-152 cells were transfected with either WT or dominant negative Rab constructs or shRNAs against Rab1 or Rab11. Cell surface expression of endogenous CD4 was analyzed by performing a Biotin-Streptavidin Cell Surface Assay followed by an SDS-PAGE and Western analysis using anti-CD4 antibody (1:2000). a) Effect of Rab1 S25N on plasma membrane expression of CD4. b) Effect of Rab2 S20N on PM expression of CD4. c) Effect of Rab6 T27N on PM expression of CD4. d) Effect of Rab8 T22N on PM expression of CD4. e) Effect of Rab11 S25N on PM expression of CD4. f) Effect of shRNA knockdown of Rab1 on plasma membrane expression of CD4. g) Effect of shRNA knockdown of Rab11 on plasma membrane expression of CD4. h) Rab1 knockdown in TIB-152 cells using several volumes of 10μM solutions of Hs-Rab1A-5 (SI00301560) and Hs-Rab1A-9 (SI02662716) Flexitube shRNAs from Qiagen (Valencia, CA). i) Rab 11 knockdown in TIB-152 cells using several volumes of 10μM solutions of Hs-Rab11A-5 (SI00301553) and Hs-Rab11A-7 (SI02663206)Flexitube shRNAs from Qiagen (Valencia, CA). Results representative of at least 3 independent experiments. $*p<0.05$, $**p<0.01$ using a two-tailed paired student's t-test.
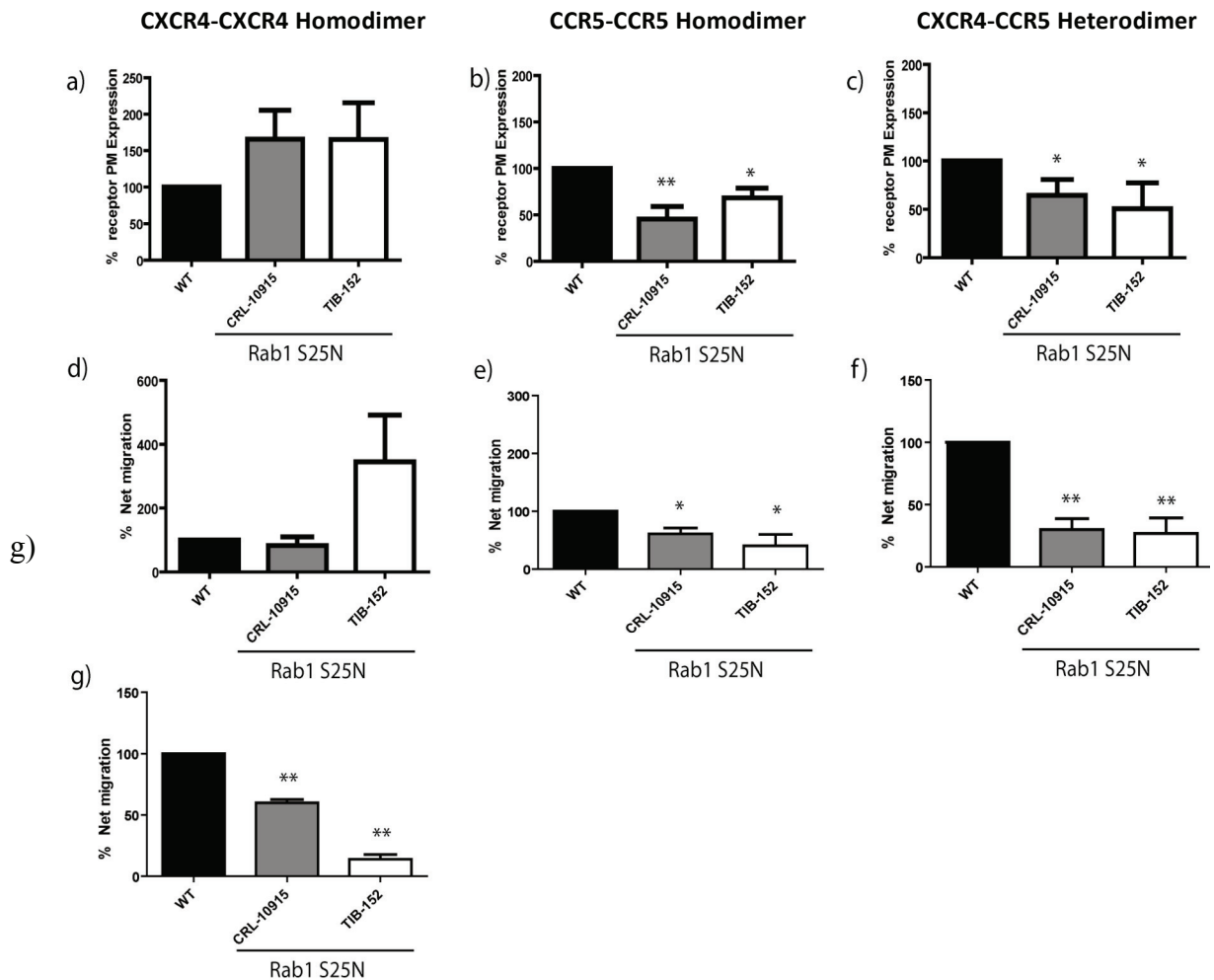
Figure 3.5: Effect of Rab1 S25N on the Trafficking of CXCR4 and CCR5 Homo- and Heterodimers to the Plasma Membrane and Migratory Ability in CRL-10915 and TIB-152 Cells

Trafficking experiments were performed 48h post-transfection with receptor dimer and either WT or S25N Rab1 constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized prescribed receptor dimers. For migration experiments, 48h after transfection with a receptor pair and either WT or S25N Rab1 constructs, cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Trafficking of the CXCR4v1-CXCR4v2 homodimer to the PM in the presence of WT or S25N Rab1. b) Trafficking of the CCR5v1-CCR5v2 homodimer to the PM in the presence of WT or S25N Rab1. c) Trafficking of the CXCR4v1-CCR5v2 heterodimer to the PM in the presence of WT or S25N Rab1. d) Migration of Jurkat TIB-152 and CRL-10915 cells expressing the CXCR4v1-CXCR4v2 homodimer toward CXCL12 in presence of WT or S25N Rab1. e) Migration of Jurkat cells expressing the CCR5v1-CCR5v2 homodimer toward CCL5 in presence of WT or S25N Rab1. f) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CXCL12 in presence of WT or S25N Rab1. g) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CCL5 in presence of WT or S25N Rab1. Results the means ±SEM. *$p<0.05$; **$p<0.01$ compared with Rab1 WT controls, using two-tailed Student's *t* test.
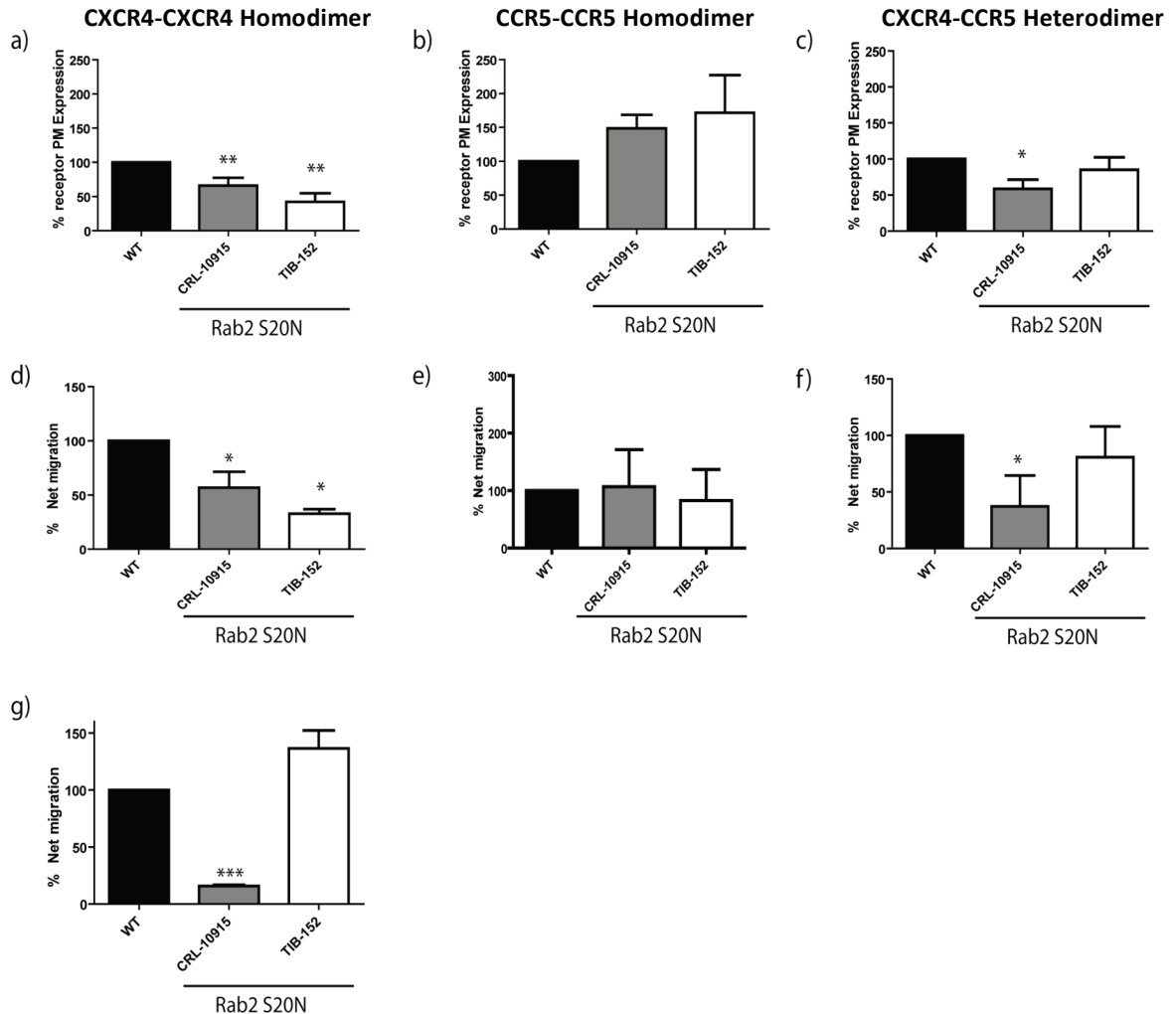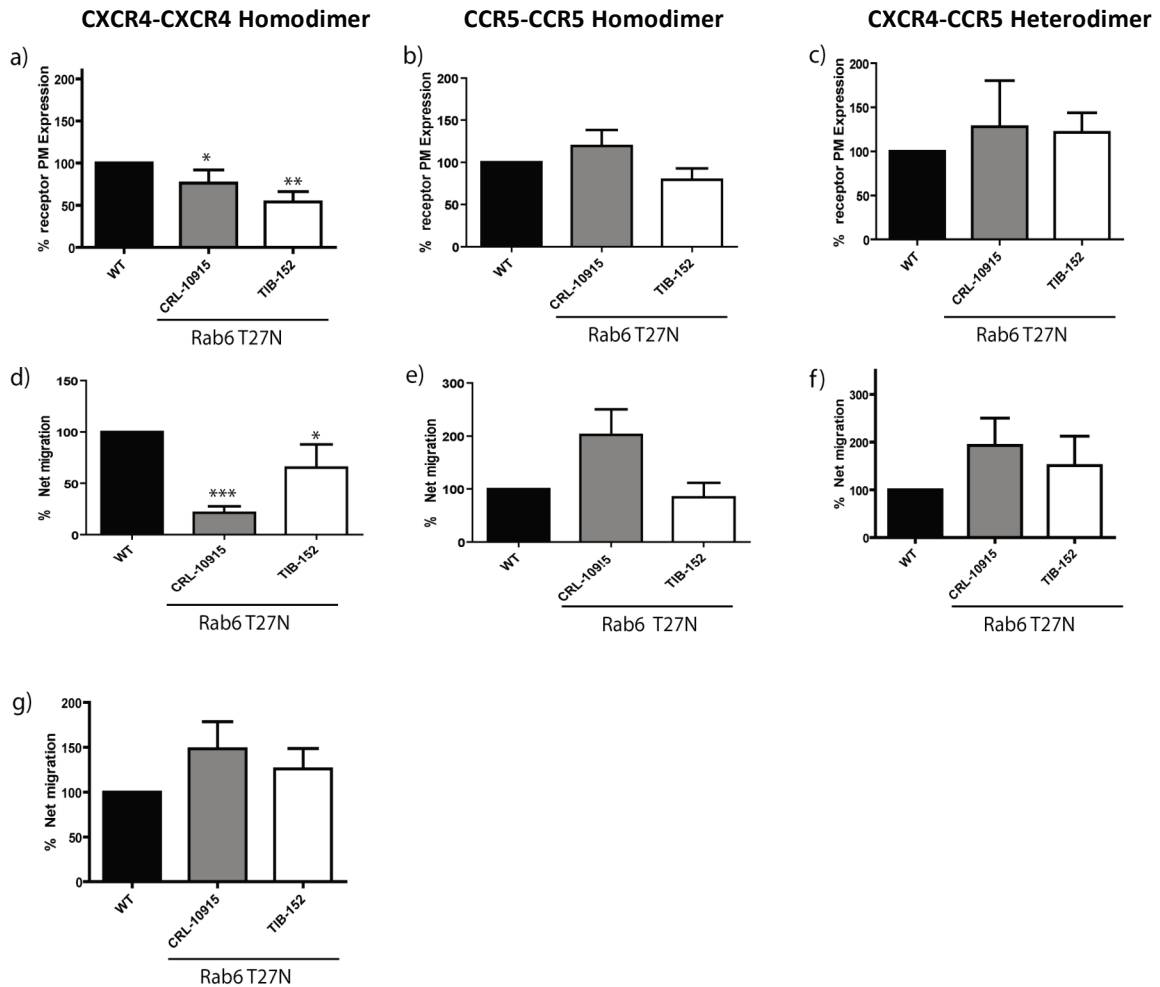
Figure 3.6: Effect of Rab2 S20N on the Trafficking of Homo- and Heterodimers of CXCR4 and CCR5 to the Plasma Membrane and Migratory Ability in CRL-10915 and TIB-152 Cells

Trafficking experiments were performed 48h post-transfection with receptor dimer and either WT or S20N Rab2 constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized our prescribed receptor dimers. For migration experiments, 48h after transfection with a receptor pair and either WT or S20N Rab2 constructs, cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Trafficking of the CXCR4v1-CXCR4v2 homodimer to the plasma membrane in the presence of WT or S20N Rab2. b) Trafficking of the CCR5v1-CCR5v2 homodimer to the PM in the presence of WT or S20N Rab2. c) Trafficking of the CXCR4v1-CCR5v2 heterodimer to the PM in the presence of WT or S20N Rab2. d) Migration of Jurkat TIB-152 and CRL-10915 cells expressing the CXCR4v1-CXCR4v2 homodimer toward CXCL12 in presence of WT or S20N Rab2. e) Migration of Jurkat cells expressing the CCR5v1-CCR5v2 homodimer toward CCL5 in presence of WT or S20N Rab2. f) Migration Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CXCL12 in presence of WT or S20N Rab2. g) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CCL5 in presence of WT or S20N Rab2. Results are the means ±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with Rab2 WT controls, using two-tailed Student's t test.
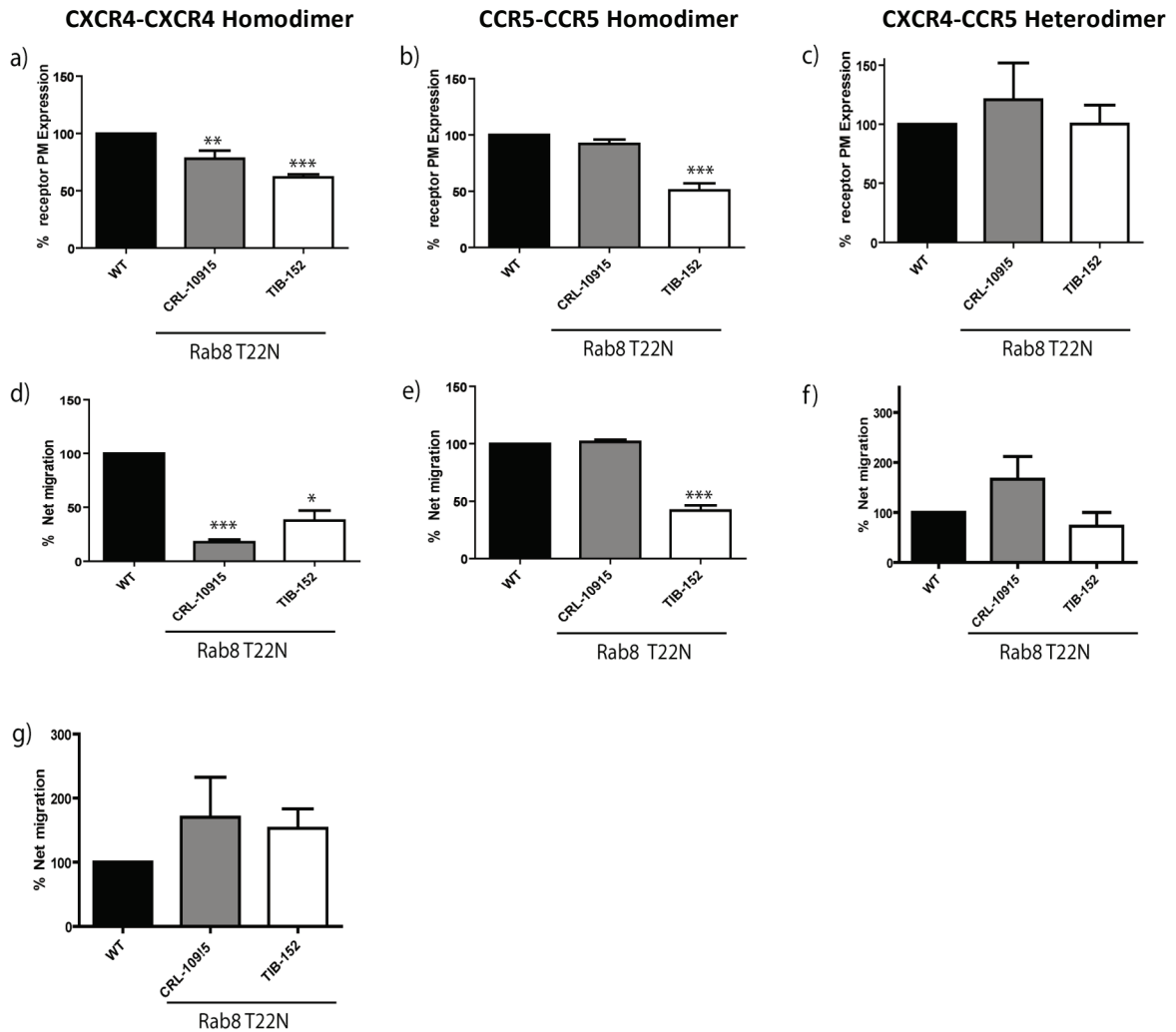
Figure 3.7: Effect of Rab6 T27N on the Trafficking of Homo- and Heterodimers of CXCR4 and CCR5 to the Plasma Membrane and Migratory Ability in CRL-10915 and TIB-152 Cells

Trafficking experiments were performed 48h post-transfection with receptor dimer and either WT or T27N Rab6 constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized prescribed receptor dimers. For migration experiments, 48h after transfection with a receptor pair and either WT or T27N Rab6 constructs, cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Trafficking of the CXCR4v1-CXCR4v2 homodimer to the plasma membrane in the presence of WT or T27N Rab6. b) Trafficking of the CCR5v1-CCR5v2 homodimer to the PM in the presence of WT or T27N Rab6. c) Trafficking of the CXCR4v1-CCR5v2 heterodimer to the PM in the presence of WT or T27N Rab6. d) Migration of Jurkat TIB-152 and CRL-10915 cells expressing the CXCR4v1-CXCR4v2 homodimer toward CXCL12 in presence of WT or T27N Rab6. e) Migration of Jurkat cells expressing the CCR5v1-CCR5v2 homodimer toward CCL5 in presence of WT or T27N Rab6. f) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CXCL12 in presence of WT or T27N Rab6. g) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CCL5 in presence of WT or T27N Rab6. Results the means ±SEM. $*p<0.05$; $**p<0.01$; $***p<0.001$ compared with Rab6 WT controls, using two-tailed Student's t test.

Figure 3.8: Effect of Rab8 T22N on the Trafficking of Homo- and Heterodimers of CXCR4 and CCR5 to the Plasma Membrane and Migratory Ability in CRL-10915 and TIB-152 Cells

Trafficking experiments were performed 48h post-transfection with receptor dimer and either WT or T22N Rab8 constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized prescribed receptor dimers. For migration experiments, 48h after transfection with a receptor pair and either WT or T22N Rab8 constructs, cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Trafficking of the CXCR4v1-CXCR4v2 homodimer to the plasma membrane in the presence of WT or T22N Rab8. b) Trafficking of the CCR5v1-CCR5v2 homodimer to the PM in the presence of WT or T22N Rab8. c) Trafficking of the CXCR4v1-CCR5v2 heterodimer to the PM in the presence of WT or T22N Rab8. d) Migration of Jurkat TIB-152 and CRL-10915 cells expressing the CXCR4v1-CXCR4v2 homodimer toward CXCL12 in presence of WT or T22N Rab8. e) Migration of Jurkat cells expressing the CCR5v1-CCR5v2 homodimer toward CCL5 in presence of WT or T22N Rab8. f) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CXCL12 in presence of WT or T22N Rab8. g) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CCL5 in presence of WT or T22N Rab8. Results the means ±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with Rab8 WT controls, using two-tailed Student's t test.

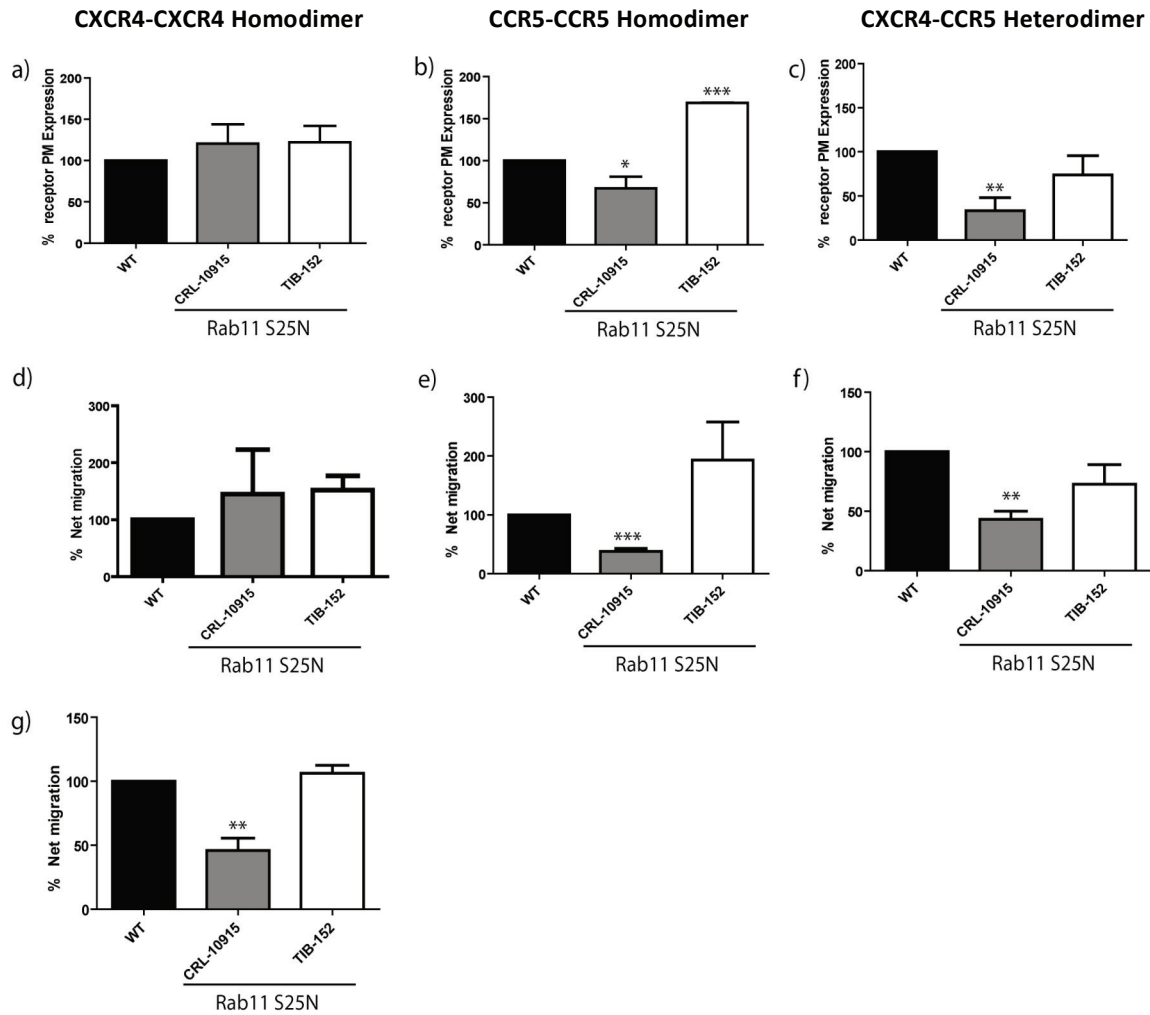**CXCR4-CXCR4 Homodimer** **CCR5-CCR5 Homodimer** **CXCR4-CCR5 Heterodimer**

Figure 3.9: Effect of Rab11 S25N on the Trafficking of CXCR4 and CCR5 Homo- and Heterodimers to the Plasma Membrane and Migratory Ability in CRL-10915 and TIB-152 Cells

Trafficking experiments were performed 48h post-transfection with receptor dimer and either WT or S25N Rab11 constructs. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized prescribed receptor dimers. For migration experiments, 48h after transfection with a receptor pair and either WT or S25N Rab11 constructs, cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Trafficking of the CXCR4v1-CXCR4v2 homodimer to the plasma membrane in the presence of WT or S25N Rab11. b) Trafficking of the CCR5v1-CCR5v2 homodimer to the PM in the presence of WT or S25N Rab11. c) Trafficking of the CXCR4v1-CCR5v2 heterodimer to the PM in the presence of WT or S25N Rab11. d) Migration of Jurkat TIB-152 and CRL-10915 cells expressing the CXCR4v1-CXCR4v2 homodimer toward CXCL12 in presence of WT or S25N Rab11. e) Migration of Jurkat cells expressing the CCR5v1-CCR5v2 homodimer toward CCL12 in presence of WT or S25N Rab11. f) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CXCL12 in presence of WT or S25N Rab11. g) Migration of Jurkat cells expressing the CXCR4v1-CCR5v2 heterodimer toward CCL5 in presence of WT or S25N Rab11. Results are the means ±SEM. *$p<0.05$; **$p<0.01$; ***$p<0.001$ compared with Rab11 WT controls, using two-tailed Student's *t* test.
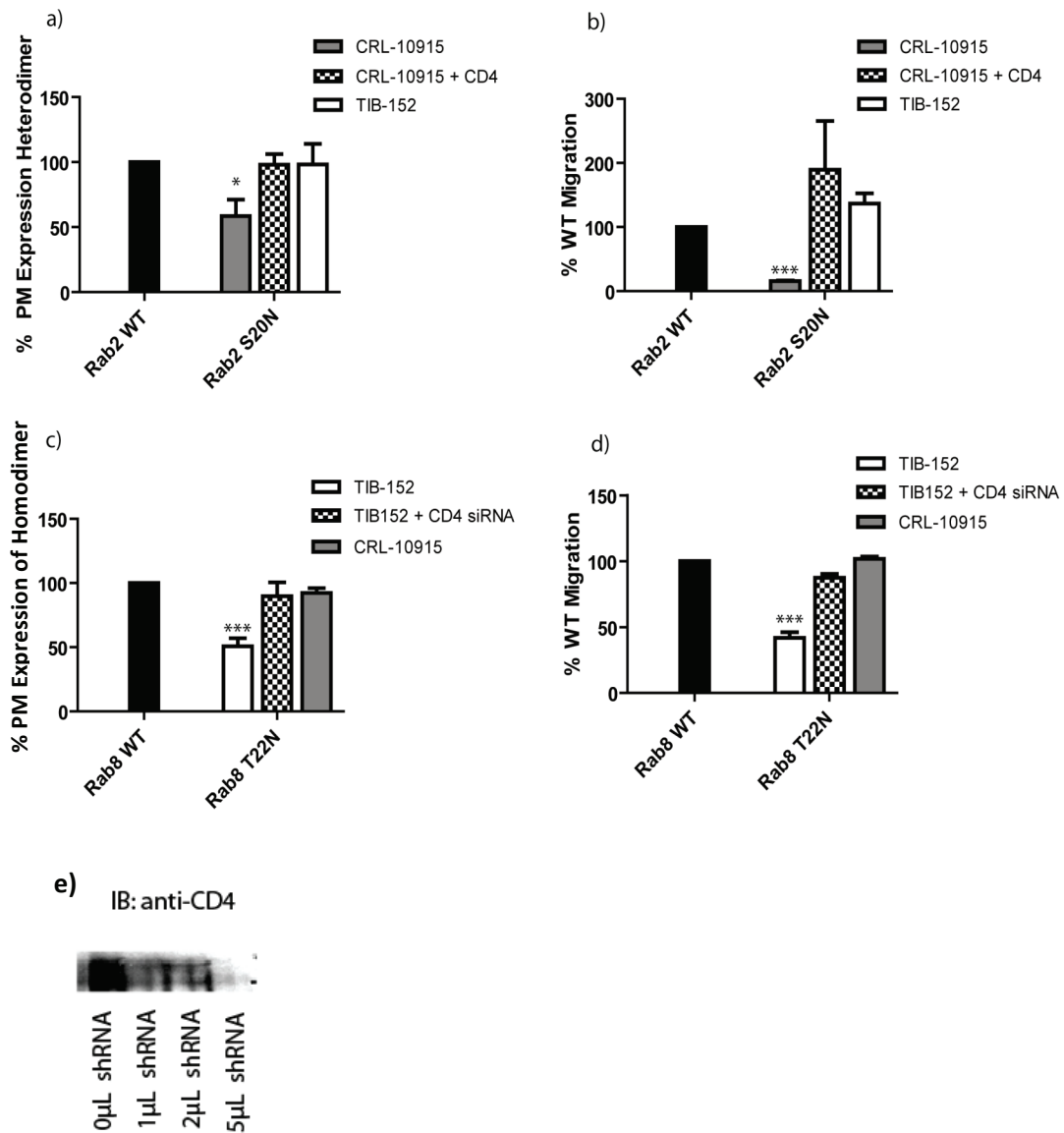
Figure 3.10: Effect of Manipulating CD4 Expression on the Reliance Upon Rab GTPases for Receptor Plasma Membrane Expression and Migration Toward CCL5 in TIB-152 and CRL-10915 Cells

Trafficking experiments were performed 48h post-transfection. A biotin-streptavidin cell surface assay was performed to isolate plasma membrane proteins. Immunoblots were probed with an anti-GFP antibody that recognized our receptor dimers. For migration experiments, 48h after transfection cells were placed in a Transwell chamber and migration towards ligands was assessed. a) Plasma membrane expression of CXCR4v1-CCR5v2 heterodimer in CRL-10915 cells in presence or absence of CD4 and WT or S20N Rab2. b) Migration of CXCR4v1-CCR5v2 expressing CRL-10915 cells toward 10ng/mL CCL5 in presence or absence of CD4 and WT or S20N Rab2. c) Plasma membrane expression of CCR5v1-CCR5v2 homodimer in TIB-152 cells in presence or absence of CD4 and WT or T22N Rab8. d) Migration of CCR5v1-CCR5v2 expressing TIB-152 cells toward 10ng/mL CCL5 in presence or absence of CD4 and WT or T22N Rab8. e) shRNA knockdown of CD4 expression in TIB-152 cells using several volumes of 0.2M Santa Cruz CD4 shRNA plasmid solution (sc-29246-SH). Results are the means ±SEM. $*p<0.05$; $**p<0.01$; $***p<0.001$ compared with WT controls, using two-tailed Student's t test.

Chapter 4: DISCUSSION

The physiological functions of CXCR4 and CCR5 are mediated by their presence and activation at PM. Several therapeutic avenues, including those for cancer and HIV infections, have used a variety of different approaches to reduce the levels of receptor expression at cell surface, and therefore, reduce their signalling potential. Although there have been advances in surgery, chemotherapy and radiotherapy over the last several decades, the death rate from lung cancer has been almost unchanged, largely due to the high metastasic potential of the disease [129]. There is a clear, urgent need for new strategies to treat highly metastatic cancers. In recent years CXCR4 and CCR5 have emerged as a novel, attractive targets. CXCR4 antagonists such as Plerixafor (AMD3100) and T140 analogues disrupt CXCR4-mediated tumour cell adhesion to stromal cells and sensitize lung cancer cells to cytotoxic drugs [129]. Therapeutics that block the interaction between the chemokine and its receptor or inhibit aspects of the receptor-associated downstream signalling pathways are also being investigated. Candidates for this blockade include small molecule inhibitors, siRNAs and blocking antibodies. In addition, a role for chemokines in interrupting the supply of nutrients and oxygen to tumours is also being investigated. Several CCR5 antagonists also show anti-tumour activity in gene-transfer experiments and alternate methods for ligand delivery to sites of tumour growth are currently being investigated [130]. These studies highlight chemokines and their receptors as potentially multifaceted tools for disrupting not only migration of malignant cells and the formation of secondary tumours, but also promising therapeutic targets to attack the original tumour itself.

Similarly, despite successes in decreasing viral load and increasing the length and quality of life for people with HIV/AIDs, current HIV therapies, including CCR5 antagonists have not been proven sufficient to cure the disease. CCR5 receptor antagonists are used to antagonize HIV-1 binding to CCR5 *in vitro* [131]; however, only one of the several CCR5 antagonists evaluated in clinical trials for HIV, Maraviroc, has been approved by the US Food and Drug Administration (FDA) for treatment of HIV-infected patients resistant to other classes of anti-retroviral drugs [132]. New anti-HIV agents that target relatively invariant host determinants, such as co-receptor-based strategies, over agents that are directed against components of the rapidly mutating virus population would be a great benefit in targeting drug-resistant forms of HIV-1. While developing antagonists and de novo-designed synthetic ligands for the co-receptors has been the predominant research area it is also possible that disruption of initial cell surface delivery of CXCR4 or CCR5 rather than blockade or promotion of receptor removal from the plasma membrane may block HIV-1 infection.

It was previously shown that affecting one partner of a signalling complex mistraffics all the components assembled with it [29], suggesting that new targets along the exocytic pathway might be useful to regulate receptor expression at plasma membrane. It is important to understand the effectors involved in the exocytosis of these important chemokine receptors to determine possible targets for new pharmacological intervention. The GTPases involved in the trafficking of CXCR4 and CCR5 receptors, assembled as homo- and heterodimers, are key effectors along the transport pathway between the ER and the PM for each receptor. Understanding whether heterodimerization of CXCR4 and CCR5 confers changes in the trafficking itinerary used

by those receptors to reach PM is important because dimer-specific therapies may be therapeutically superior, especially for the treatment of dimer-specific diseases.

Several studies have examined the involvement of Rab effector proteins in GPCR trafficking by using selective siRNAs or dominant-negative overexpression to knockdown endogenous Rab proteins. Previously it was found that overexpression of a dominant-negative Rab1a mutant disrupted trafficking of both the AT1 receptor and the β_2AR [125]. Interestingly, the closely related α_{2B}AR was not affected by knockdown of Rab1a, so the effects appear to be receptor-specific. Similarly knockdown of Rab2 inhibited cell surface expression of both the β_2AR and the α_{2B}AR [133]. This work extends these studies into a different subset of class A GPCRs, chemokine receptors, by examining the effects of different Rab GTPases on the trafficking of CXCR4 and CCR5. We examined novel aspects of dimerization on the trafficking of these receptors, and the influence of CD4, the primary receptor during HIV infection, onthe trafficking of CXCR4 and CCR5 dimers.

Results from two different cell types suggest that each receptor complex, whether homodimeric or heterodimeric, acts individually from the other receptor complexes and uses a unique trafficking itinerary. Figure 4.1 shows a summary of the results gathered in HEK293 cells, showing the Rab GTPases involved in receptor complex transit from the ER to plasma membrane, both in the presence and absence of CD4. The CXCR4 homodimer utilizes Rab1 and Rab2 and possibly Rab11 (though not significant) for exocytic transport whereas the CCR5 homodimer uses a different subset of Rabs, Rab 1, 2 and 6 in the absence of CD4 and Rabs 2 and 6 (not significant) in the presence of CD4. Here co-expression with CD4 confers minor changes in the trafficking of the CCR5

homodimer. Interestingly, the CXCR4-CCR5 heterodimer does not traffic like either the CXCR4 or CCR5 homodimers. The heterodimer requires Rabs 1, 6 and 11 for cell surface expression when CD4 is absent, but only Rab 1 when CD4 is present. This data could indicate that the different receptor dimer complexes may be differentially localized within the cell, which could permit them to mediate different receptor functions. Like the CCR5 homodimer, CD4 slightly altered the trafficking of the heterodimer.

Figure 4.2 shows the results gathered using the same experimental protocol in a second cell type, T-lymphocytes both in the presence of endogenous CD4 and without CD4. It was found that both CCR5 and CD4 use Rab1 and Rab11 for their trafficking to plasma membrane, whether they are expressed in the same cells or not. It appears that our results concur those of the Achour study, where those two receptors could travel together to reach the plasma membrane [93]. Our finding that CXCR4 traffics via a completely different set of GTPases than CCR5-containing dimers contributes to the understanding that CD4 was not found to contribute to the expression of CXCR4 to PM. In the cases of the CCR5 homodimer and CXCR4-CCR5 heterodimer, it appears that there may be some variations in the trafficking pathway used by the receptor complex upon changes in CD4 expression. CCR5 homodimer trafficking appears to be regulated by Rab8 in CD4+ cells, despite the fact that neither CCR5 nor CD4 trafficking were influenced by Rab8 when expressed individually. The trafficking itinerary for the CXCR4 and CCR5 homodimers in Jurkat cells is very different than that in HEK293 cells, indicating that dimers of CCR4 and CCR5 may traffic differently in different tissues. Interestingly, both the TIB-152 (CD4+) Jurkat cells and the CD4+ HEK cells required only Rab1 for trafficking of the CXCR4-CCR5 heterodimer, while CRL-10915 (CD4-) Jurkat cells and the CD4- HEK

cells both required Rab 1 and Rab11 for heterodimer transport, and differed in only one Rab GTPase requirement. These results indicate that while there are major differences in the trafficking of the homodimers, the heterodimer traffics quite similarly in the two cell types. These results are intriguing, and at this point, cannot be compared the trafficking of other GPCR dimers since no other studies are available in this regard. Our study evaluating the differences between homo and heterodimers of known GPCR partners is currently the only one of its kind.

It is possible that specific structural determinants important for receptor trafficking could be modified upon interaction between CD4 and CCR5, leading to the formation of a signalling complex capable of interacting with a subset of intracellular proteins distinct from those formed when each receptor is individually expressed. Given the large diversity of signals GPCRs can mediate, this would not be extremely surprising. In fact, a recent study from our group showed that dimers of CCR5 interact with and are regulated by the scaffolding protein NHERF1, while CXCR4 homodimers and CXCR4-CCR5 heterodimers do not [123]. This study, along with others that showed heterodimeric receptor specificity [123, 134-136], demonstrates the importance of not considering heterodimers as simple derivatives of their constituent receptors, but rather as distinct signalling entities which must be carefully and individually characterized.

Some heterodimers have been found to be tissue-specific or disease-specific. Our results in HEK293 and Jurkat cells suggest cell-type specific differences in the trafficking of CXCR4 and CCR5. These findings warrant greater study in more cell lines, as well as in pathological tissues. If exclusive receptor interacting partners can be identified for a tissue or disease it may be possible to develop pharmacological treatments that

specifically disrupt receptor expression in those cells, while leaving other cell types or non-diseased tissues unaffected. This could possibly prevent some of the undesired side effects associated with current treatments for some diseases like cancer, or HIV and lead to the development of more therapeutically desirable drugs.

Intracellular retention of CCR5 has been suggested as a mechanism for the delayed onset of AIDS in HIV-infected patients that harbour a CCR5/CCR5Δ32 genotype [101, 137]. As previously mentioned, the CCR5Δ32 truncation mutant prevents ER export of CCR5, resulting in the intracellular retention of the receptor. Homozygotes for the mutation are virtually immune to HIV-1 infection while, heterozygotes have a delayed progression of the disease and onset of AIDS, presumably due to heterodimerization of the mutant receptor with the wild-type receptor. These findings, in light of the unperturbed health of those with the CCR5Δ32 mutation, highlight a potential benefit for the intracellular retention of CCR5 in conferring protection against HIV. Unfortunately, though CCR5 antagonists are currently investigated for treating HIV, as well as a number of chemokine receptor associated diseases, incomplete antagonism or a lack of specificity hinder the wide and effective application of these treatments and contribute to a high attrition rate for these compounds. For these reasons it is important to identify other points along the lifecycle of these chemokine receptors which could be targeted for pharmacological interventions aimed at disrupting the plasma membrane expression of these receptors.

It has previously been found that truncated mutants of GPCRs can impede the cell surface expression of their wild-type counterparts, possibly due to an association between the wild-type and mutant receptors in the ER and the prevention of the proper

dimerization of the wild-type receptors. The co-expression of CCR2 with a loss of function YSK tagged CCR2b mutant receptor, results in the formation of dimers but cannot trigger signalling pathways. CCR2b acts as a dominant negative mutant, forming non-productive complexes and blocking the chemokine responses of complexes containing the functional tyrosine domains [138]. The CCR5Δ32 receptor mutant produces a truncated protein lacking the cytoplasmic tail and ER export sequences and was able to significantly reduce the cell surface expression of the wild-type CCR5 receptor in both HeLA and Jurkat cells [137]. Truncated mutants of the Vasopressin V2R receptor [139], D3 Dopamine receptor [140] and gonadotropin-releasing hormone (GnRH) receptor [141] have also been found to behave like dominant-negatives of their respective wild-type receptors, preventing their efficient cell surface expression or inhibiting their function. These findings suggest promise for therapeutics that aim to interrupt GPCR function by exploiting GPCR dimerization. Unfortunately, therapies that aim to alter receptor dimerization by delivering mutant receptors are therapeutically difficult to implement. It may be more feasible to identify small, molecular effectors that interrupt their export and or dimerization of GPCRs, which may perhaps elicit the same overall effects on receptor cell surface expression.

GPCR transport is a very dynamic and highly regulated process. There are numerous other small protein effectors of membrane proteins that are essential for proper cell surface expression and subsequently the function of GPCRs, for example receptor-activity-modifying proteins (RAMPs), and proteins that regulate Rab GTPases (GTPase-activating proteins, Rab escort proteins, guanine nucleotide exchange factors, GDP dissociation inhibitors) [26, 33, 142]. These effectors present new targets for potential

therapies because unlike antagonists, which occupy receptors and prevent their activation by other ligands or promote receptor removal from the plasma membrane, these therapies interfere with receptor expression prior to membrane insertion.

As previously mentioned Rab GTPases to be able to perform their protein shuttling functions by inter-conversion between an active-GTP bound form and inactive-GDP bound form, as well as recycling back to their membrane of origin (Figure 4.3). In the Rab GTPase life cycle, guanine nucleotide exchange factors (GEFs), accelerate the switch from a GDP-bound to a GTP-bound, active conformation, serving as positive regulators for Rab proteins [142]. This GTP-bound form associates with various downstream effectors and permits the transport of associated proteins. The Rab GTPase is then returned to its inactive form by GTP hydrolysis, which is facilitated by GTPase-activating proteins (GAPs). In its GDP-bound form there is opportunity for GDP dissociation inhibitors (GDIs) to stabilize the Rab and prevent its activation. Lastly, there are Rab escort proteins (REPs) that escort prenylated Rabs to their target membranes. While this study has focused on the expression of dominant negative Rab GTPases, the important actions of the aforementioned Rab regulatory proteins to modulate Rab function could also be utilized to disrupt protein function.

Pharmacological and chemical chaperones are ligands or chemicals that facilitate the proper folding of mutant proteins, allowing them to escape the ER quality control system and rescuing proper functionality [143]. Pharmacological or chemical agents that inhibit the folding or function of normal Rab proteins or Rab regulatory proteins could be therapeutically useful for preventing the transport of specific membrane proteins. For example, it may be possible to develop agents that could either (1) mimic GDIs and

prolong the Rab GTPases in their inactive state, (2) promote or mimic GAP activity to more rapidly convert active Rabs to an inactive form, (3) interfere with GEFs, preventing the activation of Rab GTPases, or (4) interfere with or mimic REPs to mistraffic Rab proteins and prevent Rab recycling to their membrane of origin, mimicking the effects of the dominant-negative Rabs on cell surface expression of CXCR4 and CCR5. There are regulatory proteins specific to different Rab GTPases, for example, Rabin8 is a GEF for Rab8, ICA69 is a Rab2 specific effector that recruits active Rab2 to membranes, and Sec15 appears to interact in a GTP-dependent fashion with Rab11 but not Rab4, Rab6 or Rab7 [142, 144, 145]. Disrupting the function of these Rab-specific regulatory proteins could possibly effects a small subset or even a single Rab GTPase. It may then be possible to utilize the specific Rab requirements for the efficient cell surface expression of various receptors to interfere with the trafficking of only one receptor type, or even one dimerization state of a receptor. This study suggests that understanding how the different receptor complexes traffic along the anterograde pathway to reach the PM may lead to potentially selective targets that could be exploited to reduce the expression levels of a specific receptor at the cell surface, and therefore modify the extent of the signalling events produced by those receptors.

In conclusion, it was found in this study that homodimers of CXCR4 and CCR5 traffic very differently in the two cell types studied, Jurkat T lymphocytes and human embryonic kidney cells, while trafficking of the heterodimer of these receptor is quite similar. As HEK cells do not normally express these receptors, it may not be surprising that the trafficking of CXCR4 and CCR5 in these cells is quite different that in the T cell line. An investigation into the requirements for trafficking of CXC4 and CCR5 in more cell types that endogenously express these receptors would be very beneficial to elucidate

whether cell type specific or tissue specific Rab GTPase requirements do exist. Some

interesting cell lines to study would be the a human macrophage cell line or a memory T cell

line, both of which endogenously express both receptors of interest and are important in the

pathogenesis and progression HIV-1 infection. It would also be beneficial to examine the

effectors required for CXCR4 and CCR5 expression in tissues involved in inflammatory

diseases and several cancer cell lines because, as previously mentioned in Table 1, both

receptors have been implicated in numerous inflammatory diseases and malignancies in

which interrupting of the trafficking CXCR4 or CCR5 may prove beneficial.
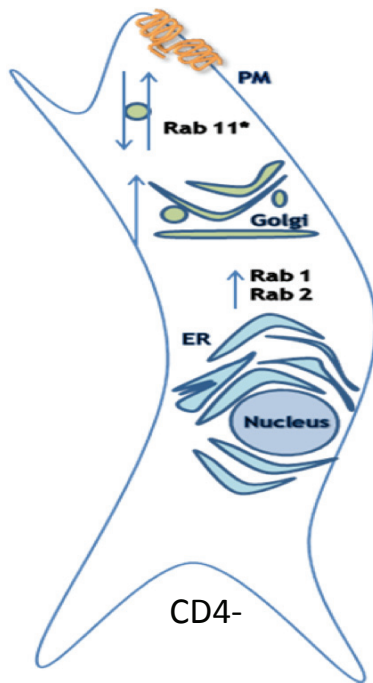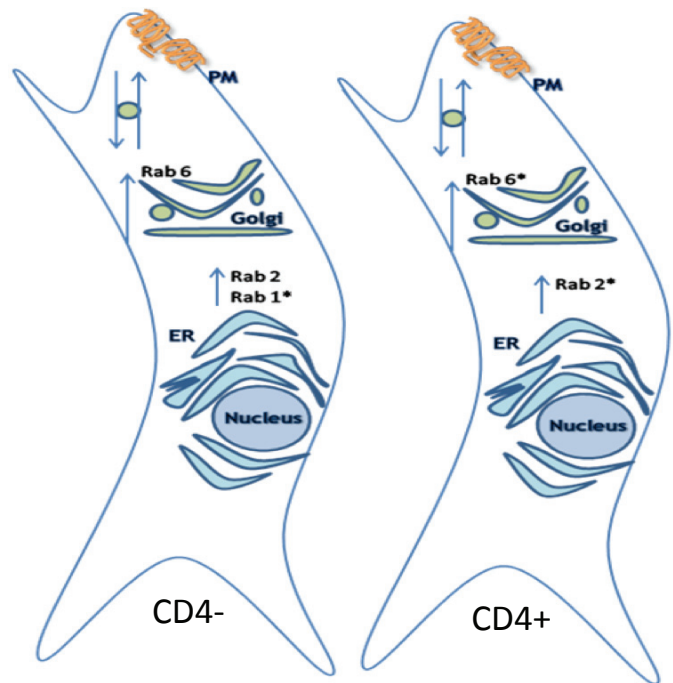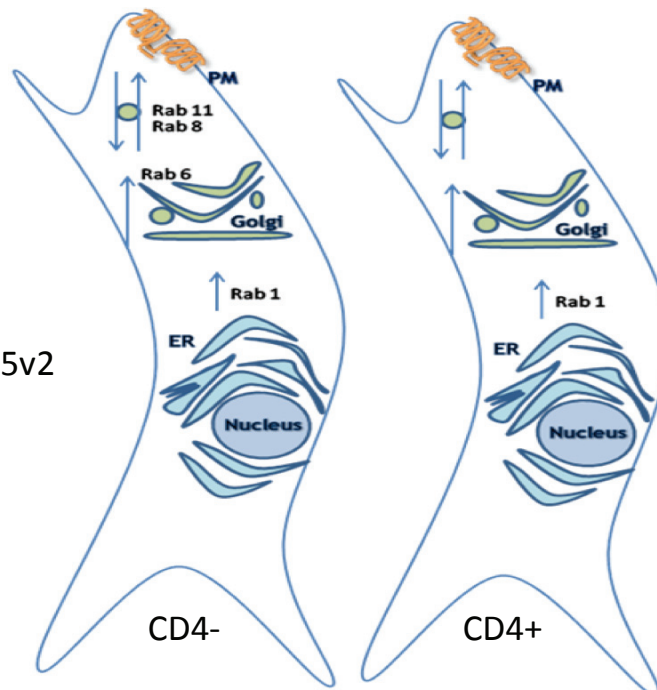
Figure 4.1 Schematic Representation of the GTPases Involved in the Trafficking of Chemokine Receptors in HEK293 cells in the Presence and Absence of CD4
Left panels represent the GTPases involved in CD4- cells, while the right column shows the GTPases involved in cells expressing CD4+. a) Represents the cells expressing CXCR4 homodimers, b) CCR5 homodimers while c) represents cells expressing the CXCR4-CCR5 heterodimers. * denotes not significant.
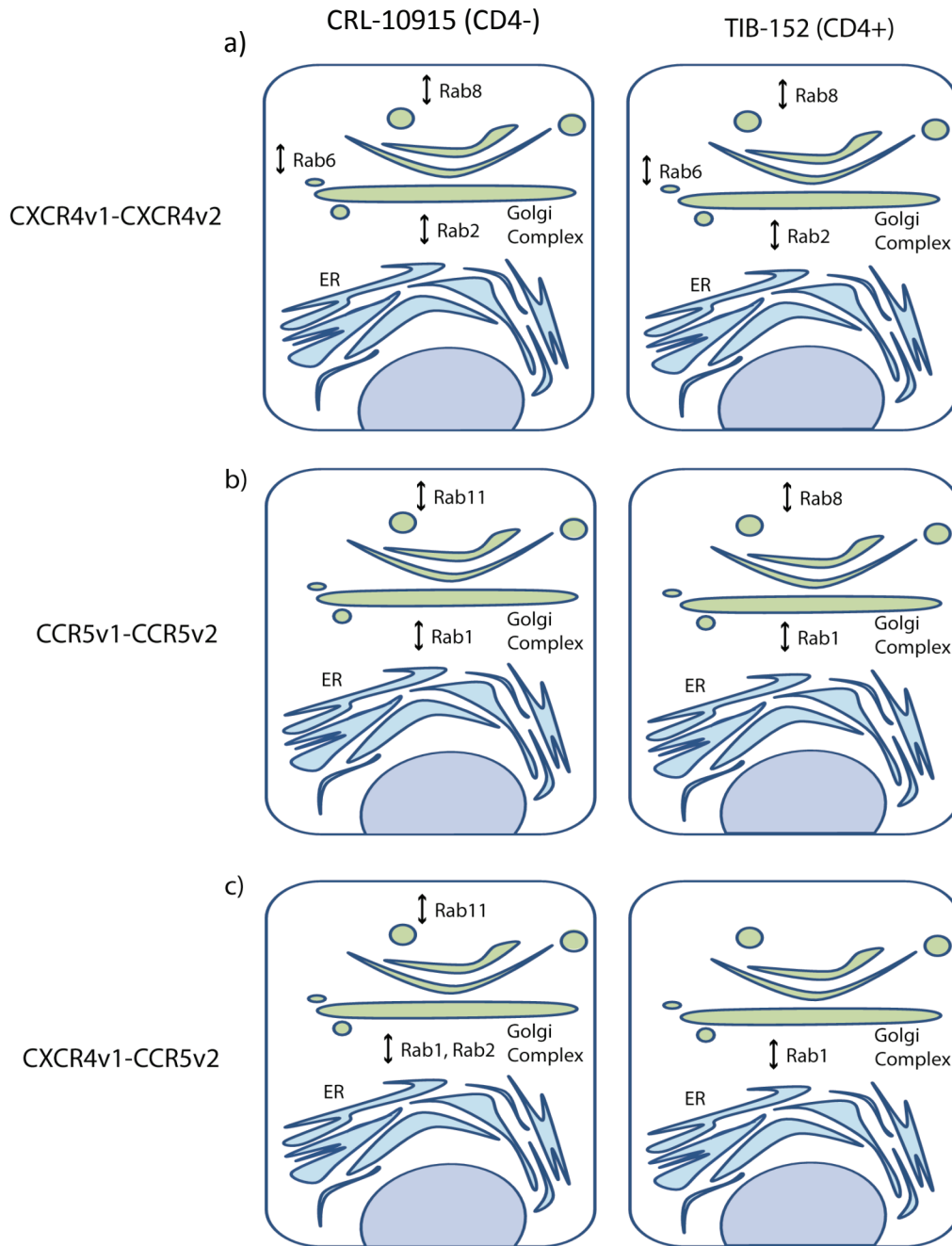
Figure 4.2 Schematic Representation of the GTPases Involved in the Trafficking of Chemokine Receptors in Jurkat Cells
Left panels represent the GTPases involved in CRL-10915 cells (CD4-), while the right column shows the GTPases involved in TIB-152 cells (CD4+). a) Represents the cells expressing CXCR4 homodimers, b) CCR5 homodimers while c) represents cells expressing the CXCR4-CCR5 heterodimers.
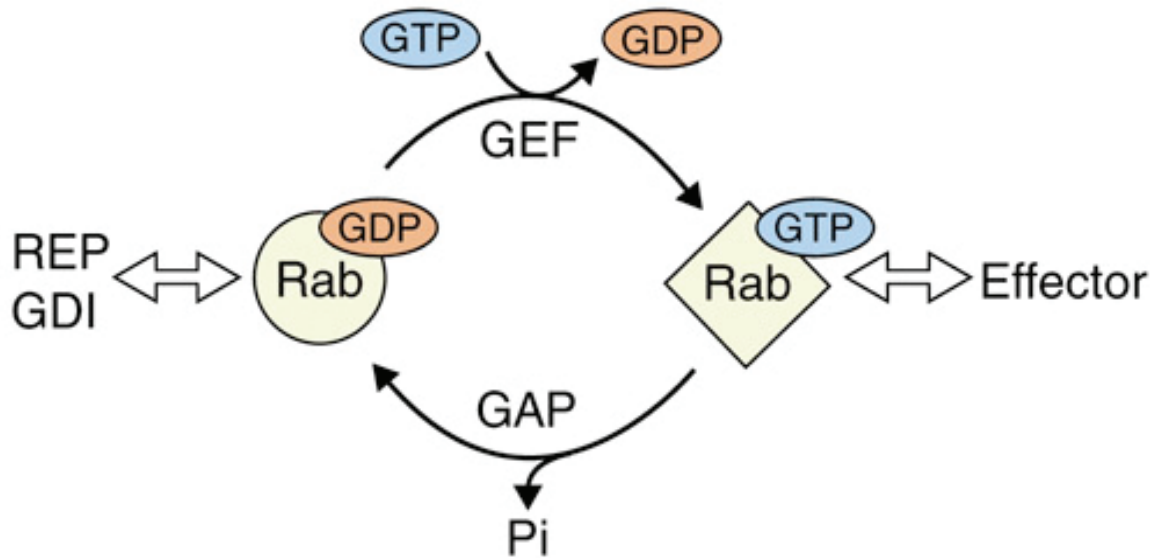
Figure 4.3 The Rab GTPase Activation Cycle
A critical component of the function of Rab GTPases is their ability to cycle between an active
GTP-bound form and an inactive GDP-bound form. The initial activation of a Rab GTPase is
initiated by guanine nucleotide exchange which is facilitated by GDP/GTP exchange factors
(GEFs). This active, GTP-bound conformation allows the Rab to interact with various
downstream effectors. GTPase-activating proteins (GAPs) facilitate the GTP hydrolysis,
returning the Rab GTPase to an inactive form. In this state GDP dissociation inhibitors can
maintain the Rab GTPase in an inactive form and Rab escort proteins can shuttle Rab GTPases to
their target membranes [adapted from Stenmark and Olkkonen, *Genome Biology* 2001,
2:reviews3007-reviews3007.7, with permission]

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