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UNIVERSITY OF GLASGOW

COLLEGE OF MEDICAL, VETERINARY AND LIFE SCIENCES
PhD in Inflammation



**IN VITRO PHARMACOLOGICAL
CHARACTERISATION OF THE ROLE
OF ACKR3 ON CXCR4-DEPENDENT
AND -INDEPENDENT FUNCTION**

A thesis submitted
in fulfilment of the requirements for the degree of
Doctor of Philosophy

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Prof.
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July 2019

Abstract

CXC-motif chemokine Receptor 4 (CXCR4) and Atypical ChemoKine Receptor 3 (ACKR3) are two 7-transmembrane domain receptors often studied together due to their common ligand CXC-motif chemokine Ligand 12 (CXCL12) and the implication of said ligand in several pathophysiological processes, cancer growth and metastasis formation among them.

This thesis project started with the aim of clarifying the interaction between these receptors and their second messengers, namely the different G proteins and arrestins, upon stimulation with different ligands, establishing a relationship between them in order to adjust the model of biased agonism to this system. Chapter 3 indeed gathers the efforts made to design and test molecular sensors that might have helped to understand whether the two receptors, alone or combined, give different types of signal in relation to differences in their expression or to different ligands. Even though several approaches were followed, this goal was not reached in full. The products generated from this first big molecular part had been propedeutical to the characterization of a new class of nanobodies against CXCR4 and ACKR3 synthesized by a project partner company.

Further, the focus narrowed on ACKR3, whose role on CXCR4-dependent cell migration was investigated *in vitro* through CRISPR. Indeed the first part of Chapter 4 describes that the lack of human ACKR3 in Jurkat cells showed a significant reduction in the amount of cells migrated towards CXCL12, indicating that ACKR3 has an indirect role in CXCR4-dependent cell migration. In the second part tumor growth dependent of ACKR3 alone was investigated: the knock-out of murine ACKR3 in LLC cells demonstrated that ACKR3 has a positive role in tumor growth regardless the expression of CXCR4.

Finally, the project headed towards the characterization of a very discussed interaction, namely that between human ACKR3 and human Adrenomedullin. Chapter 5 was dedicated to this topic, and several approaches led to the conclusions that this interaction exists, is slightly less potent than that with canonical chemokine ligands, but has a therapeutic potential still undiscovered. The results and the models build a strong body of evidence that converge on the aforementioned conclusions, and constitute a cue to consider this interaction worthy of further consideration.

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Author's Declaration

I declare that, except where explicit reference is made to the contribution of others, that this thesis is the result of my own work and has not previously been submitted for a degree or diploma at the University of Glasgow or at any other institution.

Name: -----Davide Capoferri-----

Date: -12th July 2019

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List of abbreviations

Aminoacid letter codes:

A: Alanine

R: Arginine

N: Asparagine

D: Aspartic acid

C: Cysteine

E: Glutamic acid

Q: Glutamine

G: Glycine

H: Histidine

I: Isoleucine

L: Leucine

K: Lysine

M: Methionine

F: Phenylalanine

P: Proline

S: Serine

T: Threonine

W: Tryptophan

Y: Tyrosine

V: Valine

2D-BD: 2 Dimensional Brownian Dynamics
5-HT_#: Serotonin receptor #
7-TMD: seven TransMembrane Domains
A#: Adenosine # receptor
ACKR#: Atypical ChemoKine Receptor #
aGPCR: adhesion GPCR
Akt: Ak mouse strain thymoma
AP-2: Adaptor Protein-2
 α -SNAP: Soluble NSF Attachment Protein α
AT1R: Angiotensin 1 Receptor
ATP: Adenosine TriPhosphate
 β_2 -AR: β_2 Adrenergic Receptor
BCA: BiCinchonic Acid
bFGF: basic Fibroblast Growth Factor
BLT-1: B LeukoTriene receptor 1
BN-PAGE: Blue Native PolyAcrylamide Gel Electrophoresis
BRET: Bioluminescence Resonance Energy Transfer
BSA: Bovine Serum Albumin
BTK: Bruton Tyrosine Kinase
cAMP: cyclic Adenosine MonoPhosphate
Cas#: CRISPR associated gene #
CaSR: Calcium-Sensing Receptor
CB₁: Cannabinoid receptor type 1
CC(R/L): CC motif chemokine (receptor/ligand)
CD4: Cluster of Differentiation 4
CGMD: Coarse-Grained Molecular Dynamics
Co-IP: Co-ImmunoPrecipitation
CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats
CRISPRi: CRISPR interference
crRNA: CRISPR RNA
CRS#: Chemokine Recognition Sequence #
CT: Computer Tomography
C-terminal: Carboxy-terminal

CTF: Carboxy-Terminal Fragment (of adhesion GPCRs)
CX₃C(R/L): CX₃C motif chemokine (receptor/ligand)
CXC(R/L): CXC motif chemokine (receptor/ligand)
D#: Dopamine receptor #
DAG: DiAcyl-Glycerol
DNA: DeoxyriboNucleic Acid
DNA-PKcs: DNA-Protein Kinase catalytic subunit
dNTP: deoxyNucleotide TriPhosphate
DOR: Delta Opiate Receptor
DRY motif: Aspartate-Arginine-Tyrosine motif
DRYLAIIV: Aspartate-Arginine-Tyrosine-Leucine-Alanine-Isoleucine-Valine motif
DSB: Double Strand Break
dsDNA: double strand DNA
ECL#: ExtraCellular Loop #
EDTA: EthyleneDiaminoTetracetic Acid
EGF: Epithelial Growth Factor
ELR motif: Glutamate-Leucine-Arginine motif
ERK: Extracellular signal-Regulated Kinase
ET: Evolutionary Trace
EXO1: Exonuclease-1
eYFP: enhanced Yellow Fluorescent Protein
F(B/C)S: Fetal (Bovine/Calf) Serum
Fc: fragment crystallizable region (of an antibody)
FLAG: tag peptide with sequence DYKDDDK
FRAP: Fluorescence Recovery After Photobleaching
FRET: Förster Resonance Energy Transfer
FZD#: Frizzled #
G protein: Guanosine nucleotide-binding protein
GABA: γ -AminoButyric Acid
GAG: GlycosAminoGlycan
GAIN: GPCR Autoproteolysis-INducing
Gap1: General Amino acid Permease 1
GDP: Guanosine DiPhosphate

GI tract: GastroIntestinal tract
GPCR: G Protein-Coupled Receptor
gp120: glycoprotein 120 kDa (HIV envelope)
gp41: glycoprotein 41 kDa (HIV envelope)
GPS: GPCR Proteolysis Site
GRAFS: Glutamate Rhodopsin Adhesion Frizzled Secretin (GPCR classification system)
GRK: GPCR Kinase
GTP: Guanosine TriPhosphate
H#: Helix # or Histamine # receptor
Hbegf: Heparin-Binding EGF-like growth factor
HDR: Homology-Driven Recombination
HEK: Human Embryonic Kidney
HEPES: 4-(2-HydroxyEthyl)-1-PiperazineEthaneSulfonic acid
HIF-1 α : Hypoxia-Induced Factor 1 α
hiPSC: human induced Pluripotent Stem Cell
HIV: Human Immunodeficiency Virus
HUS: Hemolytic-Uremic Syndrome
ICL#: IntraCellular Loop #
IgM: Class M Immunoglobulin
IL-1: InterLeukin 1
IP3: Inositol 1,4,5-trisPhosphate
IUPHAR: International Union of Basic and Clinical Pharmacology
JAK: Janus Kinase
KI: Knock-In
KO: Knock-Out
KOR: Kappa Opiate Receptor
KRAB: Krüppel Associated Box
LB: Luria-Bertani
LH-R: Luteinizing Hormone Receptor
Lig4: DNA ligase IV
LLC: Lewis Lung Carcinoma
M#: Muscarinic receptor #
MAPK: Mitogen Activate Protein Kinase

mCitrine: monomeric Citrine
MD: Molecular Dynamics
mGluR#: metabotropic Glutamate Receptor #
MOR: Mu Opiate Receptor
MRE11: Meiotic Recombination 11 Homolog 1
MRN: MRE11/RAD50/NBS1 complex
mRNA: messenger RNA
MT-#: Melatonin receptor #
MYD88: Myeloid Differentiation primary response 88
NBS1: Nijmegen Breakage Syndrome 1
NC-IUPHAR: International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification
NF- κ B: Nuclear Factor kappa B
NHEJ: Non-Homologous End Joining
NRF-1: Nuclear Respiratory Factor-1
N-terminal: Amino-terminal (domain)
NTF: Amino-Terminal Fragment (of adhesion GPCRs)
OX₁: Orexin-1 receptor
p115RhoGEF: 115 kDa Rho Guanine Nucleotide Exchange Factor
P2Y#: Purinergic receptor Y #
PAM: Protospacer Adjacent Motif
PAMP: Pathogen-Associated Molecular Pattern
PAXX: PAralog of XRCC4 and XLF
PBS: Phosphate Buffered Saline
PCA: Protein-fragment Complementation Assay
PCR: Polymerase Chain Reaction
PDZRhoGEF: PDZ domain containing Rho Guanine Nucleotide Exchange Factor
PEB: PBS EDTA BSA buffer
PEI: Polyethylenimine
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-Kinase
PKA: Protein Kinase A
PLC- β : Phospholipase C β
Pol: DNA Polymerase

PVDF: PolyVinylDenFluoride
R5 virus: CCR5-tropic HIV virus
R5X4 virus: CXCR4 and CCR5 tropic HIV virus
Rac: Ras-related C3 botulinum toxin substrate
RAD50: DNA repair protein RAD50
RAD51: DNA repair protein RAD51 Homolog 1
RasGAP: Ras GTPase activating protein
RDC-1: Receptor Dog cDNA-1
RET: Resonance Energy Transfer
RhoGEF: Rho Guanosine Exchange Factor
RIPA buffer: RadioImmunoPrecipitation Assay buffer
RLuc6: Renilla Luciferase 6
RNA: RiboNucleic Acid
rNTP: riboNucleotide TriPhosphate
RPA: Replication Protein A
SAG: S-Antigen
SCD: Spatial Cluster Detection
SCM: Subtractive Correlated Mutation
SCTR: rat Secretin Receptor
SDM: Site-Directed Mutagenesis
SDS: Sodium Dodecyl Sulphate
SDS-PAGE: Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
(s)gRNA: (single) guide RNA
SMO: Smoothened
SpIDA: Spatial Intensity Distribution Analysis
Src: v-src avian sarcoma (Schmidt-Ruppin-A-2) viral oncogene homolog
SRS2: ATP-dependent DNA helicase SRS2
ssDNA: single stranded DNA
SSTR#: Somatostatin receptor #
STAT: Signal Transducer and Activator of Transcription
 T_h : T helper cells
T2R: Taste 2 Receptor
TAS1: Taste receptor 1

TE buffer: Tris-EDTA buffer
TIRFM: Total Internal Reflection Fluorescence Microscopy
TM#: TransMembrane (domain) #
TNF- α : Tumor Necrosis Factor α
TR-FRET: Time Resolved FRET
tracrRNA: transactivating crRNA
Tris: Trishydroxymethylaminomethane
TSH-R: Thyrotropin Receptor
UV: UltraViolet
V#: Vasopressin # receptor
VEGF: Vascular Endothelium Growth Factor
VFD: Venus Flytrap Domain
WB: Western Blotting
WGS: Whole Genome Sequencing
WHIM: Warts, Hypogammaglobulinemia, Immunodeficiency, Myelokathexis syndrome
WM: Waldenström Macrogammaglobulinemia
X4 virus: CXCR4-tropic HIV-1 strain
XC(R/L): XC motif chemokine (receptor/ligand)
XLF: XRCC4-Like Factor
XRCC4: X-ray repair cross-complementing 4

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Chapter 1

Introduction

1.1 G protein-coupled receptors (GPCRs)

GPCR is a fundamental acronym in pharmacology. G protein-coupled receptors constitute the most frequently targeted superfamily of cell-membrane receptor proteins as they play a significant role in human pathophysiology: even though they constitute 12% of human druggable targets, 33% of all the small-molecule drugs on the market target them (Santos et al., 2016).

The core structure of a GPCR is that of a protein which spans the plasma membrane with 7 α -helical domains, linked one to another by alternating intra- or extracellular loop domains, an extracellular N-terminal, and an intracellular C-terminal. Because of this simplified structure, GPCRs are often referred to as 7-transmembrane domain receptors (7-TMD), serpentine receptors, or heptahelical receptors.

A wide plethora of cellular and physiological processes involve the action of GPCRs, as they can mediate responses to hormones, neurotransmitters, metabolites and pathogens, and are involved in vision, olfaction and taste (Rosenbaum et al., 2009).

1.1.1 Structure of a GPCR

Studying the structure of GPCRs allows us to better understand the mechanisms involved in signal transduction and helps in the design of smarter drugs. Since the first structure reported, bovine rhodopsin (Palczewski et al., 2000), great steps have been made in terms the technology of crystallography, generating more stable crystals and

higher resolution structures: all of these improvements have resulted in the availability today of over 100 GPCR structures (Rosenbaum et al., 2007), (Tate and Schertler, 2009). Together with the core structure of a GPCR, we are now able to describe with great precision, for some of the receptors, the exact topological site where ligands bind, what kind of conformational change the receptor undergoes, which cytosolic domains interact with various intracellular proteins and predict the ability of a molecule to target a receptor (Zhang et al., 2015).

GPCRs have evolutionarily conserved features that are common to many of them. A recurrent feature is the length of ECL2 (ExtraCellular Loop 2), which is the longest extracellular loop in almost all GPCRs (Kmiecik et al., 2014). Furthermore, ECL2 is involved in disulphide bridges with residues in TM3 (TransMembrane domain 3), whose function is to contribute to receptor stability (Venkatakrisnan et al., 2013). The transmembrane region represents the core of a receptor, and functions as a “communication wire” between the ligand-binding pocket and the intracellular proteins downstream of the receptor itself (Katritch et al., 2013). The occurrence of an eighth helix (H8) parallel to the plasma membrane and separated from TM7 by a short linker is reported in most GPCRs: this together with the ICLs (IntraCellular Loops) is involved in receptor activation and interaction with signalling effectors (Wess et al., 2008).

1.1.2 Classification of the GPCR superfamily

GPCRs differ from each other in several ways, such as the structure of their non-transmembrane domains, the nature of ligands with which they can interact, their expression pattern, the signalling pathway(s) activated upon receptor stimulation, and their pathophysiological role. Several attempts have been made to understand in depth which common traits could be the *fil rouge* that can be used to roughly group the members of this superfamily, and so far there are two commonly used systems of classification: the ABCDEF system (Attwood and Findlay, 1994) and the GRAFS system. The ABCDEF system has the advantage of being comprehensive, in that it includes all the GPCRs present in vertebrates and invertebrates, however some families do not have representatives in humans, such as the family of fungal pheromone receptors (family D) and the cAMP receptors (family E). The GRAFS classification (Figure 1.1) was instead developed as the result of a comprehensive bioinformatic approach applied to human data, and

this allowed all the human receptors to be sorted into 5 families: Glutamate, Rhodopsin, Adhesion, Frizzled/Tas2 and Secretin receptors, the first letter of which family names form the acronym GRAFS itself (Fredriksson et al., 2003).

metabotropic-Glutamate-like receptors

This family of receptors includes all the 8 metabotropic glutamate receptors, the GABA_B1/2 receptors, the extracellular calcium-sensing receptor CaSR, the four taste receptors (TAS1) (Fredriksson et al., 2003), and corresponds to Class C of the ABCDEF classification. Those receptors are functional only when they form dimers: one monomer binds the ligand, and the conformational change makes the other monomer trigger signalling (Brauner-Osborne et al., 2007). The family is characterised by a large amino-terminal domain, typically made of 280-580 amino acids, which forms two lobes and it is usually referred to as Venus Flytrap Domain (VFD): when the orthosteric ligand binds between the two lobes, they move towards one another (Cao et al., 2009).

Rhodopsin-like receptors

This family corresponds to the Class A GPCRs family of the ABCDEF classification and includes the majority of human GPCRs (Fredriksson et al., 2003). The shared features of the members belonging to this family lies in the primary structure of the receptors: almost all of them have a DRY motif (or D(E)-R-Y(F)) at the junction between TM3 and IL2, whilst TM7 is characterised by a NSxxNPxxY motif. A few receptors do not have these features, but they are classified within this family because of their phylogeny (Fredriksson et al., 2003).

Adhesion receptors

Adhesion GPCRs (aGPCRs) are 7 transmembrane domain proteins with a very large N terminal region (NTF, or N-Terminal Fragment), which is cleavable from the membrane spanning domain (called also CTF or C-Terminal Fragment) by proteolysis (Monk et al., 2015). The name Adhesion derives from the fact that their NTF is rich of domains that are involved in cell-to-cell or cell-to-matrix interaction (Langenhan et al., 2013). A hallmark of aGPCRs is the presence of the GPCR proteolysis site (GPS) immediately

upstream of TM1, which in almost all aGPCRs is embedded in a GPCR autoproteolysis-inducing (GAIN) domain (Liebscher et al., 2013). Adhesion GPCRs are now subject to a new nomenclature, which starts with ADGR (Adhesion GPCR) followed by a letter that indicates the subfamily, and a number (Hamann et al., 2015).

Frizzled/Tas2 receptors

This family includes ten frizzled receptors (FZD1-10), smoothed receptors (SMO) and twenty-five T2R receptors. Frizzled and Tas2 receptors have no obvious similarities, but bioinformatic analysis has identified common consensus sequences that cluster these 2 apparently diverse groups, such as motifs IFL in TM2, SFLL in TM5 and SxKTL in TM7, that are unique to this family (Lagerström and Schiöth, 2008). The nature and functions of the ligands in the two groups are different: indeed Wnt ligands are involved in embryo development (Zhang et al., 2018), while T2Rs ligands are molecules related to taste sensing (Haraguchi et al., 2018).

Secretin-like receptors

The name of this family derives from the first of these receptors discovered, the rat SCTR (Secretin Receptor) (Ishihara et al., 1991). Common features within this family are the presence of conserved cysteine residues in the first and second extracellular loops, which stabilise the structure of the receptor by bridging between them or together with less conserved cysteines in the N terminal domain (Lagerström and Schiöth, 2008), and the fact that their endogenous ligands are peptides which share high aminoacid identity (Fredriksson et al., 2003).

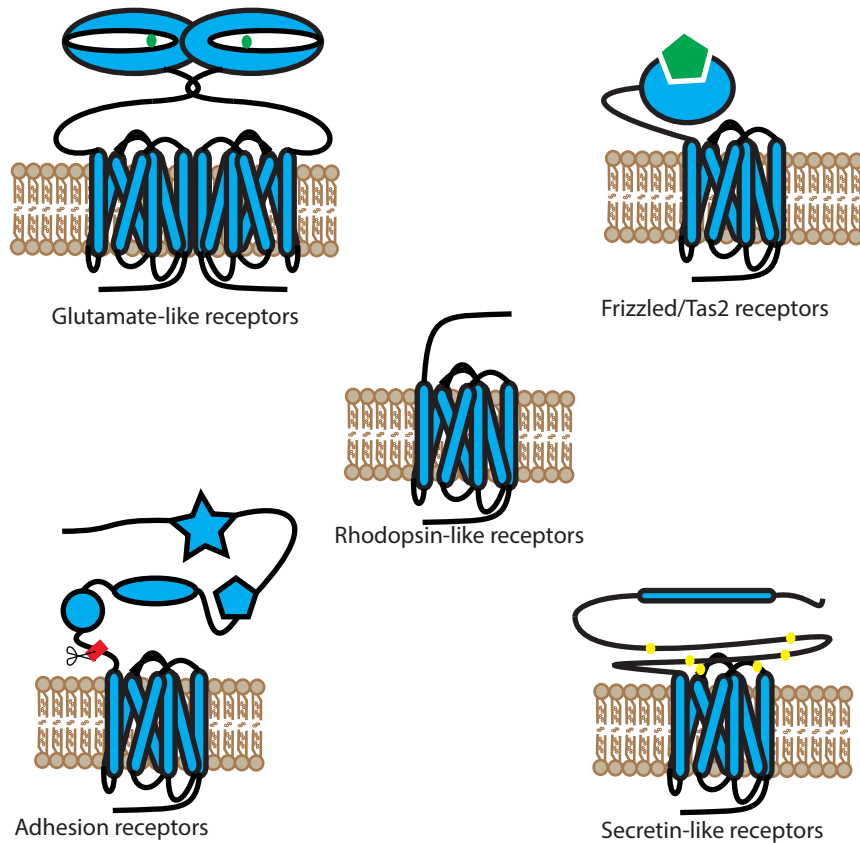


Figure 1.1: **GPCR classification.** Top left: Glutamate-like receptors are functional dimers, characterised by a long N-terminus bearing a Venus Flytrap Domain (VFD) whose role is to be the ligand binding domain. Centre: Rhodopsin-like receptors are the prototypical GPCRs, whose peculiarities are mainly in the primary structure of the receptors. Bottom left: Adhesion receptors display different cell-to-cell adhesion motifs in their long N-terminus and a proteolytic cleavage site (GPS) immediately upstream of TM1. Top right: Frizzled/Tas2 receptors have a different variety of functions and ligands, and their similarities, as per rhodopsin-like receptors, reside more in conserved motifs in their primary structures. Bottom right: Secretin-like receptors N-terminal, EL1 and EL2 domains show several conserved cysteines, responsible for a more constrained extracellular region.

Blue represents transmembrane and extracellular secondary structures. Green represents ligands. Red represents cleavage sites. Yellow represents conserved cysteines in extracellular domains.

1.1.3 Function of a GPCR: signalling

GPCRs bind a wide variety of ligands, the dimensions of which lie between the size of a photon and that of a large protein. The interaction of a ligand with the receptor leads to a series of events that have been reported as activation. During activation both extra- and intra-cellular regions play crucial roles: the extracellular domains of the GPCR, together with the non-cytosolic regions of the transmembrane domains, usually harbour the cognate ligand in a topological space called the orthosteric binding pocket, allowing it to interact with the receptor at different depths (Zhang et al., 2015). Ligand binding causes structural conformation changes in the ICLs, promoting the binding of intracellular effectors (Nygaard et al., 2009). ICL2 and ICL3 have been reported to be the G protein-interacting sites of GPCRs (Wess, 1997). According to the interacting partner recruited the signal is transduced differently (Lefkowitz, 2000).

G proteins G proteins are heterotrimeric complexes that work as binary molecular switches, with their biological activity determined by the bound nucleotide (Syrovatkina et al., 2016). These complexes directly relay the signals from activated GPCRs. G proteins are composed of α , β and γ subunits, each of which has several isoforms. The inactive state of a G protein sees the heterotrimer complete and bound by a molecule of guanosine diphosphate (GDP): upon agonist binding, the inactive G protein is recruited by the GPCR which induces an exchange of the bound GDP with GTP (guanosine triphosphate). The association of the GTP to the G protein causes the dissociation of the G_α subunit from the $G_{\beta\gamma}$ obligated heterodimer, each of which can work as a signalling effector. G_α has a low intrinsic GTPase activity that works as a negative feedback regulator for its signalling effects, and can be stimulated by GTPase-activating proteins, which accelerate the inactivation process of the G_α subunit; $G_{\beta\gamma}$ signalling instead is terminated when G_α -GDP forms and allows re-association (Figure 1.2) (Milligan and Kostenis, 2006).

G_α subunit There are different isoforms of G_α subunits, which are typically gathered in four groups: the adenylyl cyclase modulators (up- and down-regulators), phospholipase C β (PLC- β) activators and Rho-guanine nucleotide exchange factor (Rho-GEFs) activators, which are respectively named G_{α_s} , $G_{\alpha_i/0}$, $G_{\alpha_q/11}$ and $G_{\alpha_{12/13}}$. G_{α_s} stimulates adenylyl cyclase to convert ATP (adenosine triphosphate) to cAMP (cyclic

adenosine monophosphate): elevated cAMP levels result in the activation of protein kinase A (PKA) and other cAMP-regulated proteins; in contrast, $G_{\alpha_i/0}$ proteins inhibit the activity of certain isoforms of adenylyl cyclases, reducing cAMP production and decreasing the basal activity of said proteins (Wettschureck and Offermanns, 2005). The $G_{\alpha_q/11}$ family activates PLC- β , an enzyme that cleaves phosphatidylinositol 4,5 bisphosphate into inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG); the former is responsible for binding IP3 receptors on the endoplasmic reticulum causing intracellular calcium release, the latter activates protein kinase C (Goo, 2001). The $G_{\alpha_{12/13}}$ family member $G_{\alpha_{13}}$ can directly increase the activity of p115RhoGEF, PDZ-RhoGEF (PDZ domain-containing Rho guanine nucleotide exchange factor) and leukemia-associated RhoGEF by membrane recruitment and binding (Kozasa et al., 1998), $G_{\alpha_{12}}$ can interact with Gap1 (General Amino acid permease 1), rasGAP, α -SNAP and p120-catenin, while both $G_{\alpha_{12}}$ and $G_{\alpha_{13}}$ have been reported to interact with Bruton tyrosine kinases (Btk) and cadherins (Syrovatkina et al., 2016).

$G_{\beta\gamma}$ subunit The β and γ subunits of the G proteins are tightly bound to each other, and can be regarded as a single functional unit. G_{β} has five isoforms ($G_{\beta_{1-5}}$, of which G_{β_5} is the only one specific to brain cells, while the others are more widespread; G_{γ} subunit has eleven isoforms ($G_{\gamma_{1-5}}$ and $G_{\gamma_{7-12}}$) (Syrovatkina et al., 2016). Like the G_{α} subunit, the $G_{\beta\gamma}$ heterodimer can signal, regulating adenylyl cyclases, phospholipase C- β , the inwardly rectifying potassium channel and voltage-gated calcium channels (Khan et al., 2013).

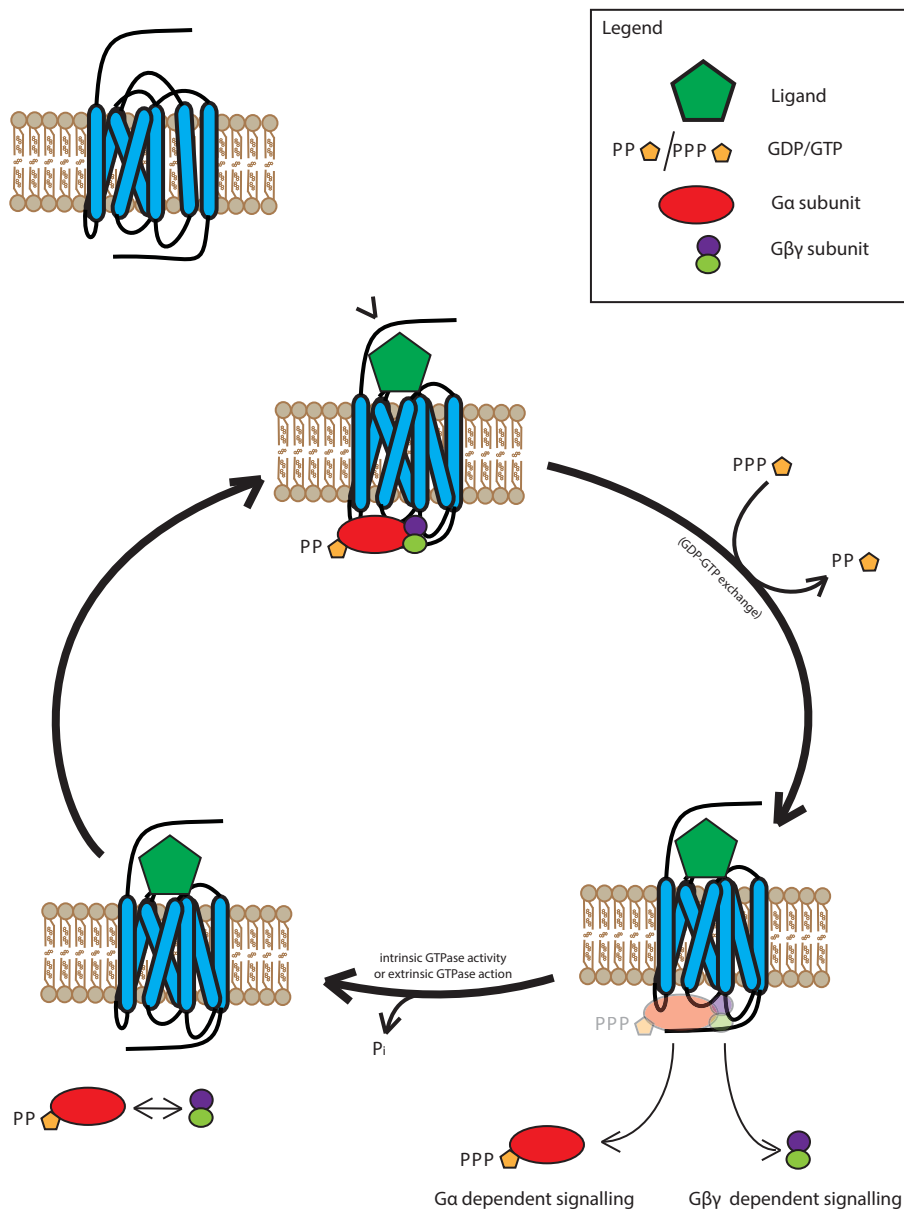


Figure 1.2: **G protein dependent signalling.** Upon ligand binding, GDP-bound G protein gets recruited to the activated GPCR. GDP-GTP exchange allows the heterotrimer to dissociate into the G α and G $\beta\gamma$ subunits, which are responsible for regulation of different signalling pathways. The GTP bound to G α gets dephosphorylated by the intrinsic GTPase activity of G α itself, and the latter is able to form a complex with G $\beta\gamma$ again and to initiate further signalling events.

Non G protein-dependent pathways On the cytosolic side of the phospholipidic bilayer GPCRs do not only interact with G proteins. The first compelling evidence that non G protein-dependent pathways are relevant in cell physiology was provided by studying the angiotensin II type 1 receptor (AT1R) in mouse heart. Since GPCR IL2 is fundamental for G protein coupling, AT1R IL2 mutant-overexpressing transgenic mice were generated and their phenotype was characterised compared to a transgenic control which overexpressed WT AT1R (Zhai et al., 2005).

In vitro studies on this AT1R-i2m (intracellular [loop] 2 mutant) already showed an impairment of G_{α_q} and G_{α_i} dependent signalling, but retained capability to activate Src kinases and ERK (Seta et al., 2002).

Mutant IL2 mice showed greater cardiac hypertrophy, cardiac dysfunction, and bradycardia, but less cardiomyocyte apoptosis and heart fibrosis than the AT1R overexpressing mice upon chronic administration of angiotensin II. This demonstrated for the first time that the impairment of G protein signalling does not result in total receptor inertia, and that two physiological phenomena can contribute to signalling pathways both G protein mediated and non mediated (Rajagopal et al., 2005).

Now we know that the principal G protein-independent signal transducers are arrestins and GRKs. GRKs will be discussed in the context of phosphorylation of GPCRs, in the next subsection.

Arrestins Arrestins are a small family of proteins which are important in the regulation of GPCR downstream signal transduction.

They were first discovered in the late 1980s as part of a conserved two-step mechanism that regulates GPCR activity in rhodopsin (Wilden et al., 1986) and in the β -adrenergic system (Lohse et al., 1990).

The most widely described role of arrestins is as a component in GPCR desensitisation: after GRKs (G protein coupled Receptor Kinases) phosphorylate the C terminus of GPCRs, arrestins can bind the receptors and mediate their removal from the plasma membrane and the uncoupling from G proteins.

This interaction between arrestins and GPCRs can activate different signalling cascades, such as ERK (Extracellular signal-Regulated Kinase) phosphorylation (Kumari et al., 2017), Akt (Ak mouse strain thymoma) ser/thr-kinase, Src (v-src avian sarcoma viral oncogene homolog) tyrosine-kinase, NF- κ B (Nuclear Factor κ B) and PI3K (Phos-

phatidylinositol 3 Kinase) (Smith and Rajagopal, 2016a). The role of arrestins in these pathways is mainly as an adaptor protein or scaffold (Eichel et al., 2016).

There are four different types of arrestins:

- Arrestin-1, also known as the S-Antigen (SAG) or visual arrestin, it is expressed at high levels in both retinal rods and cones
- Arrestin-2, the first non-visual arrestin cloned, this was named β -arrestin as between rhodopsin and β_2 -adrenergic receptors, it showed preference for the latter
- Arrestin-3, due to its cloning chronology, was named β -arrestin2 as it was the second non-visual arrestin discovered
- Arrestin-4, called also X-arrestin or cone arrestin, as it is a visual arrestin expressed predominantly by cone photoreceptor cells

Every mammalian cell expresses one or more arrestins: arrestin-1 and -4 are confined to photoreceptor cells in the retina, while arrestin-2 and -3 are ubiquitous.

1.1.4 Regulation of GPCRs

Since GPCR signalling is fundamental to many important cellular functions, a negative feedback regulation is essential as agonist stimulation can be persistent. GPCRs have two ways to regulate their signalling: desensitisation and internalisation.

Desensitisation prevents potentially detrimental effects that chronic overstimulation of GPCR signalling can have on cells and tissues, while internalisation is a physical removal of both ligand-bound and unbound GPCRs that usually results in a lower amount of receptor.

Desensitisation

Desensitisation, or tachyphylaxis, is defined from the International Union of Basic and Clinical Pharmacology Committee on Receptor Nomenclature and Drug Classification (NC-IUPHAR) as the

spontaneous decline in the response to a continuous application of an agonist, or to repeated applications or doses (Neubig et al., 2003).

Tachyphylaxis is commonly used to describe the loss of response *in vivo* or in patients, while desensitisation refers to *in vitro* experiments.

Almost every GPCR studied so far undergoes desensitisation. Studying desensitisation is therefore useful for clinical purposes: if it occurs, the therapeutic effectiveness of drugs may decrease over time. Overcoming it would improve sensitively the treatment of patients, especially those who suffer of chronic diseases, as the receptor targeting and hence the whole therapy would not lose its effectiveness (Rosethorne et al., 2015).

Desensitisation occurs when two families of proteins act coordinately, GPCR Kinases (GRKs) and arrestins (Drake et al., 2006), supporting the action of proteins required for endocytosis and ubiquitination (Rajagopal and Shenoy, 2018). Indeed GRK-mediated phosphorylation alone is not sufficient to uncouple GPCR and G protein, there is need for β -arrestin recruitment: the arrestin acts to sterically hinder the receptor, preventing further interactions with the G protein.

Desensitisation is finely tuned by the different isoforms of GRKs and arrestins, and by the modulation of these proteins by further accessory proteins.

This phenomenon can be dissected into individual processes: receptor phosphorylation, β -arrestin recruitment, receptor internalisation, receptor recycling and resensitisation or receptor down-regulation. All of these steps contribute to an overall loss of the cells ability to respond to agonists, though recent studies showed that, for example, internalised receptors can still signal from the endosome (Feinstein et al., 2013); furthermore, β -arrestin itself can mediate a series of non-G protein-mediated signalling pathways (Smith and Rajagopal, 2016b).

Phosphorylation and GRKs GRKs are a group of seven different proteins whose main function is to phosphorylate the C-terminal of GPCRs to promote desensitisation. GRK1 and GRK7 are expressed respectively in retinal rods and cones, while the expression of GRK4 is limited to cerebellar, testicular and kidney tissues. GRK 2, 3, 5 and 6 are ubiquitously expressed (Pitcher et al., 1998). GRK5 and GRK6 are constitutively located to the plasma membrane, while GRK2 and GRK3 localise to the membrane due to their binding to the $G_{\beta\gamma}$ subunit of G proteins. GRK6 is associated with the plasma membrane as a result of its palmitoylation (Drake et al., 2006).

GPCR phosphorylation does not depend on GRKs only: PKA and PKC (Protein Kinase C) may play a role in this process too, even though their action might originate from

a different receptor activation, determining what is called heterologous desensitisation (Rajagopal and Shenoy, 2018).

GPCRs have been described as being phosphorylated on the C terminal and third intracellular loop (Alfonzo-Méndez et al., 2016). The residues that can undergo this functional modification are serine, threonine and tyrosine, which can differentially get phosphorylated according to the ligand bound to the receptor and to the expression system. The effects of a specific phosphorylation event however is determined by the kinase that carries out the phosphorylation and the location of the phosphorylated residue (Kohout and Lefkowitz, 2003). These patterns are called “phosphorylation barcodes” and they might be useful in defining signalling outcomes from receptor activation (Butcher et al., 2011).

Down-regulation GPCR down-regulation is the part of the desensitisation process where the receptors eventually undergo proteolysis, the outcome of which is a decrease in the number of receptors available for signalling. During down-regulation receptors are dismantled in lysosomes and additionally their mRNA level often decreases. Ubiquitin, a 76 amino acid protein, attaches to a GPCR promoting its degradation through progress from early to late endosomes or maturing vesicles (Kennedy and Marchese, 2015). Before the actual degradation process takes place, ubiquitin-specific proteinases remove the ubiquitin moieties from the receptor: this process occurs also during receptor recycling to the cell membrane (Alfonzo-Méndez et al., 2016).

Recycling and resensitisation When GPCRs are incorporated into the membrane of an endosome, most of them are dephosphorylated and return to the plasma membrane as resensitised receptors, able to undergo another round of stimulation and signalling; this process requires both dephosphorylation and dissociation of the β -arrestin (Krupnick and Benovic, 1998).

The dephosphorylation of the receptors occurs in acidified vesicle compartments, and the need to be internalised before resensitisation has been shown using mutants defective of the ability to undergo endocytosis (Luttrell and Lefkowitz, 2002).

Internalisation

Internalisation of a GPCR results in a drop in the total number of receptors present on the plasma membrane, often causing a reduction of the cellular response upon agonist

treatment: it is one of the key processes of GPCR regulation (Ferguson, 2001). However internalisation and desensitisation should not be viewed as in a causative relationship, as the loss of receptor due to internalisation might be irrelevant to alter the overall function of the receptor. Indeed, there are cases in which those two phenomena occur independently (Koenig and Edwardson, 1997). Moreover, relatively recent studies reported that some internalised GPCRs remain active and can sustain signalling (Feinstein et al., 2013), (Thomsen et al., 2016).

Many internalisation pathways have been reported in different cell types, or in the same cell type under different conditions. A significant number of GPCRs are characterised by the presence of different internalisation motifs in their sequence, and likely other trafficking-related motifs are yet to be discovered. Arrestins and trafficking proteins compete for these sites, but there is no consensus relationship between said motifs and specific internalisation pathway (Gurevich and Gurevich, 2006).

Agonist-dependent It is widely documented that upon agonist stimulation GPCRs undergo internalisation within minutes. Indeed, the activated GPCR undergoes phosphorylation and β -arrestins are recruited. This interaction increases the accessibility of the adaptor protein complex 2 (AP-2) to the C tail of the GPCR, which is involved in the formation of clathrin-coated pits as it constitutes the adaptor between the receptor and the clathrin monomers (Kohout and Lefkowitz, 2003).

Clathrin-mediated internalisation is not the only mechanism involved in agonist-dependent internalisation, indeed GPCRs can be internalised through the formation of non-coated membrane invaginations named caveolae (Nichols and Lippincott-Schwartz, 2001). Caveolae are associated with a family of cholesterol-binding proteins named caveolins.

Agonist-independent Agonist-independent internalisation has been observed for many GPCRs, including angiotensin 1A (AT1A) receptor, cannabinoid type 1 (CB1) receptor, α 1-adrenergic receptor, CXCR4, mGlu1R and mGlu5R, M2 muscarinic receptor and thyrotropin receptor (Xu et al., 2007). It is referred to also as constitutive internalisation, and it works more slowly than the agonist-dependent form (Drake et al., 2006).

Constitutive internalisation can occur both clathrin-dependently and -independently (Fourgeaud et al., 2003).

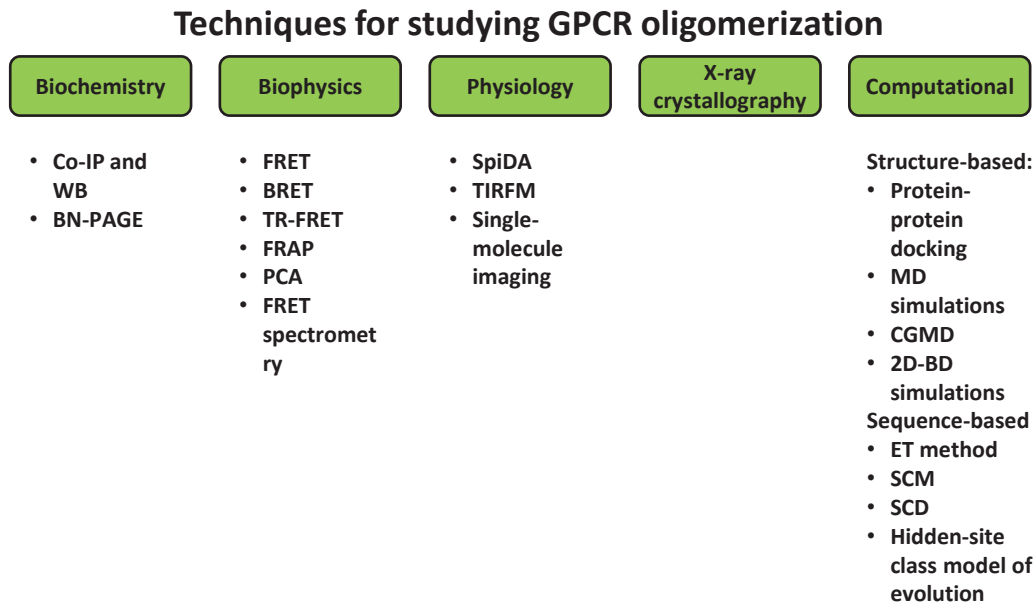
1.1.5 GPCR quaternary structures

Classical models of GPCR interaction with either ligands or intracellular early messengers have generally depicted the receptors as monomeric units. Now, however, we know that GPCRs can form homo- and hetero-oligomers, a phenomenon which has been described for a large number of GPCRs (Milligan, 2007).

The first characterisation of GPCR oligomerisation was functional, and dates back to the discovery that Gamma Amino Butyric Acid class B receptor ($GABA_B$) was actually an obligate heterodimer between the two isoforms $GABA_{BR-1}$ and $GABA_{BR-2}$ (Jones et al., 1998), (Kaupmann et al., 1998). Since then the potential of GPCR oligomerisation has been recognised, and possible interactions investigated. The current models of GPCR oligomerisation suggest the involvement of elements of TM1 for the interaction between rows of dimers, and of TM4 as well as TM5 for key interactions between monomers (Fotiadis et al., 2004).

Different techniques have been used to study homo- and hetero-merisation. Figure 1.3 summarises the techniques and the receptors by which oligomerisation has been reported. The first proposed role for GPCR oligomerisation has been linked to G protein binding. Indeed interaction models of rhodopsin with its G protein transducin demonstrated that a dimer of receptors would be necessary to accommodate the G protein, as the monomeric cytoplasmic surface of rhodopsin is too small to dock the α and the $\beta\gamma$ subunits (Fotiadis et al., 2006). A similar conclusion has been reached for the leukotriene B4 receptor BLT1, whose model showed that the signalling unit was made of two receptors and the G protein heterotrimer (Baneres et al., 2003).

Although the scientific debate regarding the authenticity of GPCR oligomerisation is still ongoing for a number of these interactions, various research groups have assigned both structural and functional roles to this phenomenon. The former includes proper receptor folding, maturation and physiological delivery to the plasma membrane (Milligan, 2007). Functional consequences of the formation of quaternary structures instead involve the modulation of signalling, for instance, the orexin-1 (OX_1) receptor co-expression with the cannabinoid receptor CB_1 has been shown to enhance the potency of orexin-A 100 fold in ERK phosphorylation compared to the treatment of OX_1 -only transfected cells; furthermore, the inhibition of CB_1 with its antagonist rimonabant reduced the synergistic effect (Ellis et al., 2006), (Ward et al., 2011).



Examples of homo-oligomer forming GPCRs

Glutamate-like:	Rhodopsin-like:
• mGluR1	• A1
• mGluR5	• β 2-AR
• CaSR	• AT ₁ R
• GABA _B 1	• B2
• GABA _B 2	• CCR2
	• CCR5
	• CXCR4
	• D1
	• D2
	• D3
	• H2
	• H4
	• LH-R
	• MT1
	• MT2
	• M2
	• M3
	• MOR
	• DOR
	• KOR
	• 5-HT ₁ B
	• 5-HT ₁ D
	• SSTR1A
	• SSTR1B
	• SSTR1C
	• SSTR2A
	• TSH-R
	• V ₂

Examples of hetero-oligomer forming GPCRs

• 5-HT ₁ B–5-HT ₁ D	• MT1–MT2
• A1–D1	• SSTR2A–SSTR1B
• A1–mGluR1	• SSTR1A–MOR
• A1–P2Y ₁	• SSTR1A–SSTR1C
• A2–D2	• SSTR1B–D2
• AT ₁ R–AT ₂ R	• T1R1–T1R3
• AT ₁ R–B2	• T1R2–T1R3
• CCR2–CCR5	• DOR–KOR
• D2–D3	• MOR–DOR
• GABA _B 1–GABA _B 2	• DOR– β 2-AR
• M2–M3	• KOR– β 2-AR

Figure 1.3: List of techniques (top) used to study GPCR oligomers (Guo et al., 2017), and list of GPCRs that are described forming homo- and hetero-oligomers (George et al., 2002). For abbreviations: see “List of abbreviations”

1.2 Chemokines and chemokine receptors

The word “chemokine” was created as a portmanteau between the words “chemotactic” and “cytokine”, and describes a class of mostly secreted proteins of size 8-12 kDa. The etymology of the word suggests that these ligands belong to the family of the cytokines, and that they are involved in cell migration. Indeed their action is implicated physiologically in phenomena such as leukocyte migration, organogenesis, hematopoiesis and immunomodulation; pathologically they play a crucial role in inflammation, allergy, autoimmune disease, cancer and in all the processes that start from innate immunity, such as leukocyte recruitment to a full delivery of antigen-presenting cells to the effector cells of the adaptive immune system, including the maturation of lymphocyte themselves(Chen et al., 2018).

They are secreted by stromal cells and leukocytes, but their heparin-binding properties allow them to be displayed to leukocytes in the capillary lumen by glycosaminoglycan(GAG)-coated endothelial cells.

Chemokine ligands are classified by their primary structure into four subfamilies, which are known as CC, CXC, XC and CX₃C. The first cysteine residue encountered starting from the N terminal of these molecules is involved in a disulphide bridge that is very important for their tertiary structure and can be followed by a second cysteine residue directly (CC), spaced by a random amino acid (CXC), spaced by three amino acids (CX₃C), or can lack a proximal second cysteine (XC). Furthermore they can be roughly classified into homeostatic or inflammatory chemokines, depending on whether they play a role in physiological cell trafficking or they are secreted on demand in response to an inflammatory cue(Ransohoff, 2009).

Chemokine receptors are GPCRs belonging to the family of rhodopsin-like receptors, γ subgroup, chemokine receptor cluster, according to the GRAFS classification. Cytogenetically these receptors are located in clusters on different chromosomes, suggesting a common ancestral origin: this might be due to several local gene duplications combined with genome duplications (Fredriksson et al., 2003). Chemokine receptors are divided into “typical” and “atypical” chemokine receptors, according to their ability to elicit a G protein-mediated response. Furthermore, typical chemokine receptors are discriminated by the class of chemokine they bind: CCR bind CC chemokines, CXCR bind CXC chemokines, XCR bind XC chemokines and CX₃CR bind CX₃C chemokines (Figure 1.4).

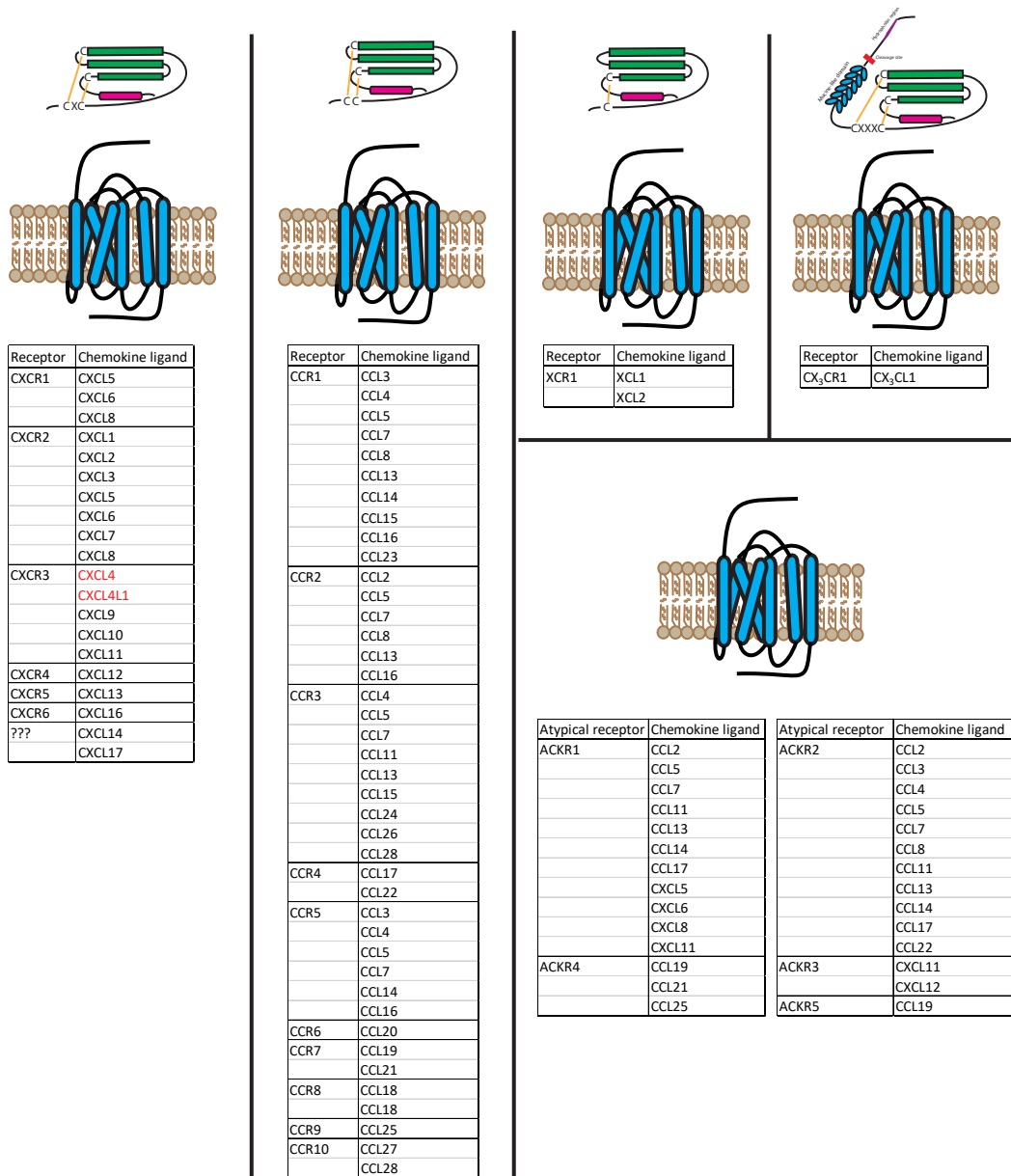


Figure 1.4: Chemokine receptors and their cognate chemokine ligands (Bachelierie et al., 2014).

1.2.1 Chemokine ligands

Currently 45 human chemokine ligands are recognised by IUPHAR (Bachelierie et al., 2014). CC and CXC chemokines generally have a short N-terminal domain, usually 6-10 amino acids long, that precedes the first structural cysteine, followed by a long loop (N-loop), a 3_{10} helix, three β strands and a C-terminal α -helix 20-30 amino acids long with a high degree of structural disorder (Rajaratnam et al., 1995), (Allen et al., 2007). This structural arrangement is conserved within the subfamilies, even though the sequence identity is highly divergent. The XC chemokine group contains only two members, lymphotactin- α and - β (XCL-1 and XCL2), while the only CX₃C chemokine, fractalkine (CX₃CL1), is membrane bound.

CXC chemokines are further divided into ELR⁺ and ELR⁻ subgroups, due to the presence and absence respectively of a three amino acid glutamate-leucine-arginine motif in their N-terminal region before the first conserved cysteine. ELR⁺ CXC chemokines are grouped on chromosome 4 in humans, and they are chemoattractants of neutrophils and have been shown to contribute to wound repair: their function is to attract neutrophils and induce degranulation and respiratory burst. Production and secretion of ELR⁺ CXC chemokines is promoted by pro-inflammatory cytokines such as IL-1, TNF- α or microbial PAMPs (Baggiolini, 2001).

Both ELR⁺ and ELR⁻ CXC chemokines have been linked to wound repair processes. Absence of CXCR4 or its ligand CXCL12 impairs the vascularisation of the gastrointestinal (GI) tract in mice and CXCR2 is expressed by endothelial cells during wound healing (Romagnani et al., 2004).

CC chemokines number at least 28 (CCL1-28) and signal through 10 known CC chemokine receptors (CCR1-10). They are known to have a key role in the recruitment of monocytes and macrophages, which are involved both in acute and chronic inflammation, atherosclerosis, rheumatoid arthritis and adipose inflammation (Charo and Ransohoff, 2006).

Due to their only conserved cysteine, XC chemokines exist in two different conformations that are in equilibrium with one another. The monomeric state resembles the conventional chemokine fold, while the other conformation is a four-stranded β -sheet which forms when the usually extended N terminal regions pairs with the third β -strand in the sheet (Sonay Kuloglu et al., 2002).

CX₃CL1 is a mucin-like membrane protein with an extracellular chemokine domain that usually works as a tight adhesion molecule, the chemokine domain can detach from the membrane anchor upon protease action and behave like a secreted chemokine (Wojdasiewicz et al., 2014).

1.2.2 Chemokine receptors

Chemokines exert their effect upon interaction with 7 transmembrane domain receptors, some of which are G protein-coupled. Six G protein-coupled receptors have been identified as being able to bind CXC motif chemokines, named CXCR1-6, while CC motif chemokine-binding receptors comprise ten known members, CCR1-10. One receptor binds the two lymphotactin proteins, XCR1, and CX₃CR1 has been described to bind CX₃CL1 (Bachelier et al., 2014).

Along with G protein-coupled chemokine receptors, a small subfamily of atypical chemokine receptors (ACKRs) has been identified. The main anomaly resides in their inability to elicit a G protein-dependent response to chemokine binding, hence they have been postulated since their discovery to have scavenging or decoy roles upon chemokine ligand binding (Ulvmar et al., 2011).

Within the family, there is 25-80% amino acid sequence homology, high enough to constitute an independent family, and diverse enough to demonstrate how wide said family is. Chemokine receptors are generally 340-370 amino acids long; they are characterised by a relatively short N terminus, usually presenting N-linked glycosylation sites and might display sulphation of tyrosine residues. The core of these receptors are the seven α -helical trans membrane domains, linked by three intracellular and three extracellular loops. There is a highly conserved amino acid motif localised between TM3 and IL2, which is often referred to as DRYLAIV motif, whilst the third intracellular loop is characterised by its short length and by the prevalence of basic residues. EL1 and EL2 are linked by a disulphide bridge, formed between two highly conserved cysteine residues. The C terminus is characterised by a short α -helix directly downstream of TM7, and the primary structure of the C terminus itself contains serine and threonine residues that function as phosphorylation sites, which are important for receptor regulation (Allen et al., 2007).

Chemokine ligands interact with their receptors through their N terminal domain and the proximal rigid loop of the ligand backbone formed by the disulphide bridge be-

tween their second and third cysteines. Furthermore, alterations to the N terminus of the chemokines whose structure, when in complex with their receptor docks within the transmembrane binding pocket, have been characterised as crucial in the conversion of a chemokine ligand from a receptor agonist to an antagonist. Due to these features, the N terminal region of a chemokine is often referred to as CRS2 (Chemokine Recognition Site 2). Chemokine Recognition Site 1 (CRS1) refers instead to the globular core of the chemokine ligand, which interacts with the N terminal region of the chemokine receptor. The nomenclature CRS1 and CRS2 is the result of several studies which led to the theory of a two-step binding mechanism. Indeed, the interaction of monomeric chemokine ligands with their receptor starts from the recognition of their globular core (CRS1) from the N terminal domain of the receptor, which upon binding docks the N terminal part of the ligand (CRS2) into the transmembrane domain binding pocket, triggering the signalling (Kufareva et al., 2015). This model, represented in Figure 1.5, can explain why alterations of the N terminus of the chemokine can transform an agonist into an antagonist.

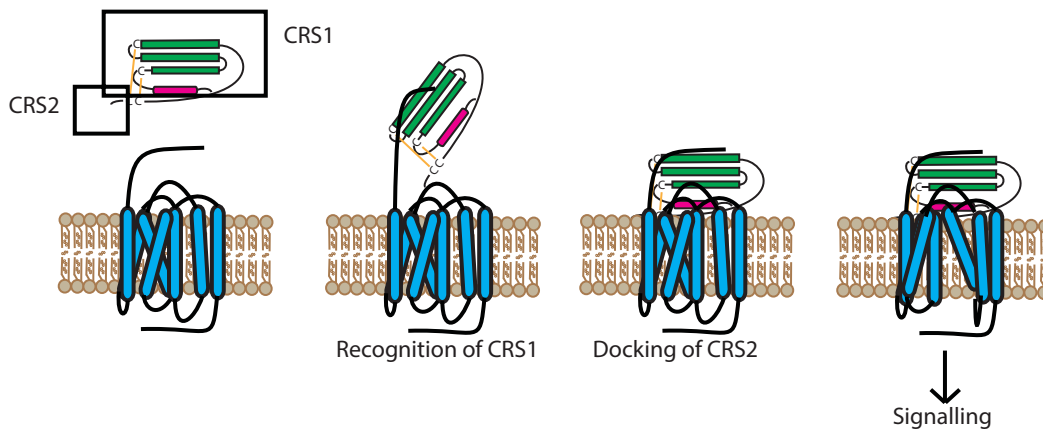


Figure 1.5: Two-step recognition binding model. The aminoterminal domain of the chemokine receptor with the CRS1 of the chemokine ligand allows the docking of the CRS2 into the chemokine receptor, allowing the latter to transmit the signal intracellularly.

1.2.3 CXCR4

CXC-motif chemokine Receptor 4 (CXCR4) is the most studied chemokine receptor, as it is involved in fundamental biological processes such as embryonic development, haematopoietic cell trafficking, mature leukocyte homing and angiogenesis (Wang and Knaut, 2014). It is involved in different immunopathologies, such as the congenital immune deficiency WHIM, acts as a co-receptor for the T-tropic HIV-1 protein gp120 (Balabanian et al., 2004), and furthermore its expression is upregulated in different neoplastic conditions (Vela et al., 2015), (Balkwill, 2004b), including Waldenström macroglobulinaemia.

CXCR4 is able to signal through different types of G proteins, predominantly the ones belonging to the $G_{\alpha_{i/0}}$ family, but it has been described as also able to recruit $G_{\alpha_{13}}$ (Tan et al., 2006) (Yagi et al., 2011) (Kumar et al., 2011), G_{α_q} (Soede et al., 2001) and β -arrestins. Upon activation of the receptor, inhibitory G_{α} proteins lead to the inhibition of the adenylyl cyclase, reducing the amount of intracellular cAMP and the activity of cAMP-dependent protein kinases. $G_{\alpha_{13}}$ protein signalling has been identified during Jurkat T cell migration, where together with Rac, Rho GTPase was activated (Tan et al., 2006), as well as in CXCR4-induced metastatic basal-like breast cancer cells (Yagi et al., 2011). Activation of PLC- β (thus G_{α_q} activation) was observed downstream of CXCR4 stimulation only in dendritic cells and granulocytes (Soede et al., 2001), suggesting that the cellular context can have an impact on the signalling of a GPCR.

Non G protein-dependent signalling via CXCR4 is mediated by β -arrestins, which influence its internalisation, G protein signalling and chemotaxis. β -arrestin1 and 2 are recruited once GRKs phosphorylate the intracellular side of the receptor, initiating the formation of clathrin-coated pits. The C terminal region of CXCR4 is clearly involved in the binding of β -arrestin, as shown by the congenital disease named WHIM (Warts, Hypogammaglobulinaemia, Immunodeficiency, Myelokathexis) syndrome, whose pathogenesis resides in a truncation of the CXCR4 C tail. WHIM patients' cells are unable to down-regulate CXCR4 expression on the membrane, resulting in a gain-of-function phenotype that prevents mature granulocytes from leaving the bone marrow niche and this leads to a chronic noncyclic neutropenia. However, the CXCR4 C tail is not the only docking site for β -arrestin recruitment. Indeed residues on internal loop 3 (IL3) of CXCR4 were also described as being sites of arrestin recruitment (Cheng et al., 2000),

(Gustavsson et al., 2017).

The first whole genome sequencing (WGS) study of Waldenström macroglobulinemia (WM) patients highlighted that nonsense mutations 20 amino acids upstream of the physiological stop codon of CXCR4 and frameshift mutations of a region up to 40 amino acid upstream of the same were almost equally frequent in patients. The driving force for this disease is a gain-of-function mutation in MYD88, present in 90% of the patients, but CXCR4 C tail mutations accounted for almost 30% of the cases, 98% of which were concurrent with MYD88 (Myeloid Differentiation primary response 88) mutations. This was the first report of WHIM-like mutations of CXCR4 in cancer (Treon et al., 2014). WM indeed is a type of B cell proliferative disease (lymphoplasmacytoid tumour) half way between non-Hodgkin lymphoma (lymphoid tumour) and multiple myeloma (plasmacytoid tumour), in which cancerous WM cells grow in the bone marrow and can crowd out the hemoatopoietic stem cells from their niche. This can lead to a drop in the level of red blood cells (anemia), leukocytes (immune deficiency), platelets (thrombocytopenia) and usually results in weak and fatigued patients with high susceptibility to bruising, to excess bleeding and with problems in fighting infections. These cells produce large amounts of IgM antibodies (macroglobulins), which thicken the blood and give symptoms such as alteration in vision, headaches, vertigo and changes in the mental status, however blood tests for IgM level evaluation are pathognomonic and differential diagnosis can be made upon CT or X-ray scans of bones or soft tissue(Treon et al., 2014).

CXCR4 has been intensively studied because of its role in T-tropic HIV-1 infection of T cells, even before its identity as a chemokine receptor was known. HIV-1 gp120 is an envelope protein that binds with high affinity to CD4, a 55 kDa membrane protein found predominantly on a subset of T cells named T helper (T_h cells), but expressed also on the surface of monocytes/macrophages and dendritic/Langerhans cells(Zaitseva et al., 1997). As gp120 binds to CD4, gp120 itself undergoes a conformational change that promotes its binding to one of the two major co-receptors (which are both chemokine receptors), CCR5 if the HIV-1 strain is M-tropic (monocyte/macrophage tropic, named also R5 virus), or CXCR4 if it is T-tropic (named also X4 virus). Many strains though are dual tropic, as they can utilise both the CXCR4 and CCR5 receptors (named R5X4 viruses) (Bleul et al., 1997). Binding to the chemokine receptor leads to a process called fusion, due to the exposure of another envelope protein named gp41 which anchors and penetrates the target cell membrane and brings the virion together with the cell. CXCR4

is crucial for the fusion process, and so because of that one of its alternative names has been “fusin” (Endres et al., 1996).

Several cancer cell types show alterations in CXCR4 expression, in some cases correlating with an enhanced ability to invade other tissues and metastasise, therefore resulting in a more aggressive phenotype, and lower disease-free and survival rates (Liu et al., 2009), (Iwasa et al., 2009), (Liang et al., 2010). Overexpression of CXCR4 has been found in more than 23 different human cancer types, such as blood, lung, brain, kidney, ovary, pancreas and skin neoplastic diseases (Balkwill, 2004a).

Some of the upregulation mechanisms of CXCR4 have been described and are cogent to what is known about cancer biology. Hypoxia-induced factor-1 α (HIF-1 α) activation, stimulation with basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF) and epithelial growth factor (EGF), and activation of transcription factors such as nuclear respiratory factor-1 (NRF-1) are all events described both prior to CXCR4 upregulation and, even if not present all at the same time within the same neoplasia, in cancer growth (Phillips et al., 2005), (Wigerup et al., 2016), (Zhuo et al., 2012).

The induction of the aggressive phenotype upon upregulation of CXCR4, resides in the ability of the cells to hijack the CXCR4/CXCL12 signalling axis in order to facilitate distant organ metastasis. This is supported by 1) common metastasis sites, such as brain, liver, bone marrow and lung, express high levels of CXCL12, and 2) signalling impairment of the CXCR4/CXCL12 axis significantly reduced the formation of metastases in murine models of cancer (Chatterjee, Azad and Nimmagadda, 2014).

CXCR4 plays a significant role in embryo development, as CXCR4 knock-out (KO) mice show aberrant vascular formation in the intestine (Tachibana et al., 1998), cardiac ventricular malformations (Ivins et al., 2015) and abnormal migration of the cerebellar neurons (Qing et al., 1999), (Zou et al., 1998), together with a severe impairment of haemopoiesis, concerning particularly the myeloid progenitors and the B lymphoid lineage (Qing et al., 1999). As a result of these developmental phenotypes, CXCR4 KO mice die perinatally (Janssens et al., 2018).

The expression of CXCR4 in myeloid progenitors is decreased as the maturation of the cells proceed. This observation, that CXCR4 is involved in hemopoiesis as it helps to retain immature cells into the bone marrow hemopoietic niche until they reach a maturation stage in which CXCR4 expression decreases and allow their release into the

bloodstream, led to a breakthrough in clinical practice. A CXCR4 antagonist, named plerixafor, was developed, and its main use in healthy volunteers is to allow hemopoietic stem cell mobilisation from the bone marrow for transplantation to patients who received immunodepletion treatments mainly as a therapy against blood cancer.

The physiological ligand of CXCR4 is the chemokine ligand Stromal-Derived Factor-1 (SDF-1), mainly known now as CXCL12 (CXC motif chemokine ligand 12). CXCL12 is an ELR⁻ CXC chemokine, expressed ubiquitously in most tissues and cells. It binds to CXCR4, to the atypical chemokine receptor ACKR3 and to glycosaminoglycans (GAGs). Human CXCL12 exists in six different splice variants (α to ϕ), each of which has a specific tissue distribution and activity (Janssens et al., 2018). CXCL12 KO mice display similar hemopoietic, neurogenetic and cardiac phenotypes as CXCR4 KO, linking their pharmacology to physiology.

Kaposi's sarcoma Herpes virus, or Human Herpesvirus 8 (KSHV or HHV8) is a gamma-herpesvirus which, upon infection, activates the production of a CXCR4 antagonist, named v-MIP II or v-CCL2 (viral Macrophage Inflammatory protein II, or viral CC motif chemokine ligand 2). v-CCL2 works in concert with the other two viral chemokines (vMIP I/v-CCL1 and vMIP III/v-CCL3) to antagonise the T_h1-mediated antiviral response: vCCL2 in particular acts as a neutral ligand to many chemokine receptors, included CXCR4, and contributes to the immune evasion due to the blockade of chemokine-mediated responses to viral infection (Nicholas, 2010).

Synthetic ligands, such as the already mentioned plerixafor (or AMD3100) and isothioureait (IT-1t), were initially developed as T-tropic HIV-1 blockers, but they failed to show efficacy during clinical trials (Hendrix et al., 2004).

1.2.4 CXCR7/ACKR3

Atypical Chemokine Receptor 3 (ACKR3), called also Receptor Dog cDNA-1 (RDC-1) or CXC-motif chemokine receptor 7 (CXCR7), is a relatively new component in the CXCR4/CXCL12 axis, being characterised by its interaction with CXCL12 only in 2005 (Balabanian et al., 2005). Unlike CXCR4, upon CXCL12 binding ACKR3 does not elicit G protein dependent signalling, resulting only in β -arrestin recruitment. Indeed ACKR3, like other atypical chemokine receptors, shows an altered DRYLAIV motif between TM3 and IL2, presenting a DRYLSIT sequence instead; however this is not the only obsta-

cle to G protein recruitment. A chimeric version of ACKR3 with the DRYLAIV motif has been engineered and its signalling characterised. However, it also failed to show CXCL12-mediated G protein activation, intracellular calcium mobilisation, ERK phosphorylation or chemotaxis (Hoffmann et al., 2012), (Naumann et al., 2010).

G protein coupling though is not totally ruled out, as evidence has shown a BRET signal between ACKR3-eYFP and $G_{\alpha_{i2}}$ that decreased upon treatment with GTP- γ S, suggesting that ACKR3 recruits $G_{\alpha_{i/0}}$ proteins during the resting state, but fails to activate them (Levoye et al., 2009).

A large amount of literature describes ACKR3 as a decoy receptor for CXCL12, efficiently internalising the ligand and shaping the extracellular chemokine gradient necessary for CXCR4-mediated migratory responses (Boldajipour et al., 2008), (Dambly-Chaudière et al., 2007). Upon CXCL12 treatment ACKR3 can recruit β -arrestin and result in Akt and ERK activation and phosphorylation, together with JAK2/STAT3 (Janus Kinase 2/Signal Transducer and Activator of Transcription 3) activation (Décaillot et al., 2011), (Hattermann et al., 2010), (Ödemis et al., 2012), (Rajagopal et al., 2010), (Torossian et al., 2014), (Torossian et al., 2014). CXCL11 treatment induces β -arrestin recruitment as well, showing ERK phosphorylation in HEK293 cells overexpressing ACKR3; however, stimulation of untransfected rat vascular smooth muscle cells expressing endogenous amounts of ACKR3 with CXCL11, failed to show phosphorylation of ERK (Rajagopal et al., 2010), indicating the importance of the cellular context.

Unlike typical chemokine receptors, activation of ACKR3 does not actively drive cell chemotaxis, but it triggers mainly internalisation pathways which have an indirect effect on chemokine-dependent cell migration as the removal of part of the chemokine ligands present in the microenvironment helps shape the gradient.

In the resting state, ACKR3-only transfectants express the receptor mostly on the membrane of endocytic vesicles. A study showed that shortening the receptor C-terminal tail in ACKR3-GFP resulted in an increased plasma membrane localisation by up to 100% when the whole domain was missing (Ray et al., 2012). Receptor internalisation is β -arrestin-, clathrin- and dynamin-dependent, thus in the presence of a dominant negative dynamin (K44A dynamin mutant), all ACKR3 expression is localised on the cell surface. This does not alter constitutive β -arrestin recruitment, but upon CXCL12 treatment, β -arrestin recruitment significantly increased and ERK phosphorylation lasted significantly longer. Therefore, ACKR3 is able to signal when located exclusively to the plasma mem-

brane without the chance to be internalised (Ray et al., 2012).

Unlike CXCR4, whose expression decreases over time upon treatment with CXCL12, ACKR3 membrane expression undergoes a slight decrease before being restored in the presence of persistent stimulation with the chemokine ligand, after which it can resist depletion from the plasma membrane for a prolonged time. Furthermore, through radioligand internalisation studies it was demonstrated that ACKR3 brings its chemokine ligands to degradation, confirming its role as a scavenger receptor (Naumann et al., 2010).

During embryo development, the chemokine distribution in the right place at the correct time is crucial in lineage commitment, organogenesis and chemotaxis. The importance of ACKR3 in development started to be investigated after the discovery that CXCL12 could bind this receptor. One of the first studies *in vivo* used the zebrafish embryo model. The knock-down of ACKR3 through specific morpholinos resulted in an impairment of the migration of primordial germ cells (PGCs), resulting in defective gonad development (Boldajipour et al., 2008).

Almost simultaneously, ACKR3-KO mice were created and their unusual phenotype characterised. More than 95% of the $Cxcr7^{-/-}$ mice died within 24 hours after birth, showing abnormal heart valve development and an impaired expression of the angiogenic factor *Hbegf* and of adrenomedullin in cardiac valves (Sierro et al., 2007).

Adrenomedullin genome duplications ($Adm^{hi/hi}$) in mice phenocopied the cardiac defect seen in $Cxcr7^{-/-}$ mice, suggesting a link between the expression and/or the interaction of the two proteins. Genetic dosage of the two molecules obtained by crossing mice with mono- or bi-allelic adrenomedullin duplication or ACKR3 deletion reported phenotypes with variable severity, further corroborating the hypothesis of an interdependence between adrenomedullin and ACKR3. Furthermore, ACKR3-transfected HEK293 cells displayed the ability to internalise biotinyl-adrenomedullin, providing stronger evidence that adrenomedullin can be considered a ligand for ACKR3 (Klein et al., 2014).

ACKR3 has been reported to be expressed in several types of malignancies, such as lung, brain, pancreatic and prostate cancer (Liu et al., 2013), (Heinrich et al., 2012), (Kallifatidis et al., 2016).

Arteries upregulate the expression of ACKR3 following injury, and after myocardial infarction this promotes ischemia-induced angiogenesis and endothelial cell proliferation (Hao et al., 2017). Moreover, in atherosclerotic plaques ACKR3 is expressed by res-

ident macrophages and its expression is involved in their phagocytic activity through MAPK-mediated signalling (Chatterjee, Borst, Walker, Fotinos, Vogel, Seizer, Mack, Alampour-Rajabi, Rath, Geisler, Lang, Langer, Bernhagen and Gawaz, 2014).

Diabetic *db/db* circulating endothelial progenitor cells were isolated from the bone marrow and transfected to express more ACKR3 or to silence its expression. Both *in vitro* and *in vivo*, their ability to generate new vessels during angiogenesis correlated with the amount of ACKR3, revealing that this receptor is protective in hind limb ischemia models (Dai et al., 2017).

HIV-1 and -2 gp120 proteins have been described as using ACKR3 as a co-receptor (Shimizu et al., 2000) for viral envelope fusion with the plasma membrane, this has been successfully inhibited *in vitro* using monoclonal antibodies targeting ACKR3 (D'huys et al., 2018).

ACKR3 binds human chemokine ligands CXCL12 and CXCL11, the former of which has been already discussed in the previous section. Human CXCL11, previously known as Interferon-inducible T-cell alpha chemoattractant (I-TAC), is an ELR⁻ CXC chemokine whose expression is highly increased in response to interferon- β and - γ stimulation. Its cognate typical chemokine receptor is CXCR3, and its role is mainly as a chemoattractant for activated and memory T cells during the cell-mediated adaptive immune response. Many studies have investigated the physiological implications of CXCL12/ACKR3 interaction, while CXCL11/ACKR3 interaction has been characterised only pharmacologically (Quinn et al., 2018).

Non-chemokine endogenous ligands of ACKR3 include adrenomedullin, BAM22 peptide, peptide I and peptide E from the adrenal opioid proenkephalin A gene.

Adrenomedullin is a vasodilator peptide belonging to the family of calcitonin-related genes, and the study that first linked it to ACKR3 has been mentioned previously. BAM22, peptide I and peptide E can activate β -arrestin recruitment through ACKR3 and modulate the circadian glucocorticoid oscillation, revealing a role of ACKR3 also in human emotional behaviour (Ikeda et al., 2013).

Viral chemokine v-MIP II/v-CCL2, together with CXCR4, can also interact with ACKR3 (Szpakowska et al., 2016).

Attempts to design synthetic antagonist ligands against ACKR3 have been made, but most of those molecules instead also function as receptor agonists (Wijtmans et al., 2012).

1.2.5 Clinical significance of CXCR4 and ACKR3 targeting

One of the aspects of biomedical research is the impact of every discovery in a translational perspective, thus how something that occurs consistently at a lab bench can be translated to a clinical setting.

Due to the physiological roles of CXCR4 and ACKR3, their targeting to date concerns HIV gp120-mediated envelope fusion, metastasis and angiogenesis, the latter being related both to neoplastic and non-neoplastic diseases.

This thesis project was intended to investigate the oncological research field, thus greater relevance will be given to the metastatic and angiogenetic aspects of chemokine receptor targeting.

Cancer

Cancer is a very generic term that encompasses more than 270 different diseases. This occurs as cancer can develop from virtually any type of human cell, hence the wide variety, but it maintains a number of features that are universal to all the diseases, which are referred to as “the hallmarks of cancer”. Even though each cell type differs from one another, all the cells at some point undergo the same series of events during duplication, they all are ruled by the same molecular checkpoints of the cell cycle, they all live thanks to oxygen and the same classes of nutrients, they all can commit for apoptosis if they receive determined stimuli. Likewise, cancer cells have common capabilities, which according to the latest literature revision (Hanahan and Weinberg, 2011) are:

- Resist apoptosis
- Sustain mitogenic signaling
- Resist growth suppression
- Induce the formation of new vessels
- Resist anti-proliferative signals
- Activate invasion and metastasis
- Enable immune evasion

- Reprogram cellular metabolism
- Accumulate genomic mutations (genomic instability)
- Support the inflammatory responses that facilitates the evolution of the tumour

Metastasis Metastases are neoplastic entities, which can be single cells as well as masses, that have left the primary tumour site to reach different regions of the anatomy. They are the major cause of death in cancer patients.

The expression of CXCR4 on tumour cells generally correlates with a more aggressive phenotype (Zhao et al., 2014), (Zhao et al., 2015), meaning that the expression of this chemokine receptor can be a biomarker of cells with high metastatic potential. The role of ACKR3 in metastasis is not yet elucidated, as in non small cell lung carcinoma (NSCLC) models it has not been conclusively implicated in CXCL12-CXCR4 dependent behaviour (Choi et al., 2014), however, in a model of breast cancer the expression of ACKR3 mitigated the metastatic phenotype due to CXCR4 (Hernandez et al., 2011), while in another breast cancer model its activation promoted metastasis (Miao et al., 2007). Therefore, further studies will be needed to assess its role in tumour metastasis.

Angiogenesis Mammal cells require a consistent provision of oxygen and nutrients in order to survive, and this explains why blood vessels are the first organ to develop in the embryo, and why the vascular network is the most extended organ in the body (Carmeliet and Jain, 2000). Two processes regulate the initiation, extension and maintenance of the vessels: vasculogenesis and angiogenesis. Vasculogenesis occurs early during embryo development, and is defined as the *de novo* generation of primitive vessels, which will constitute the artero-venous system later on. Angiogenesis instead is the formation of capillaries originating from pre-existing vessels and can occur at any moment in a lifetime, from the extension of the primitive vasculature in the embryo to the physiological neo-formation of vessels in post-natal life (Carmeliet, 2000), including chronic inflammation- or tumour-induced vessel formation (Liekens et al., 2001).

The main components during this process are endothelial cells, soluble factors and extracellular matrix (ECM) components. The angiogenic cascade starts with the activation of endothelial cells in response to a hypoxic cue or a release of angiogenic and chemoattractant factors. The vessels in close proximity to the stimulation source increase their

permeability and lose most of their intercellular connections; also endothelial cells secrete proteases that degrade the basal membrane and the ECM, which favours cell migration. Endothelial cells proliferate and differentiate towards the area from which the angiogenic stimulus originated, matching the neo-capillary tips coming from the corresponding anastomotic vessel (arterious capillary with venous capillary). The primitive tube then matures and all the structures required for the correct function of a capillary vessel (i.e. pericytes, smooth muscle cells, basal membrane) are recruited (Risau, 1998). Angiogenesis is a finely tuned phenomenon, which has master positive and negative regulators together with minor but not less important modulators.

VEGF and angiopoietins are the most important direct positive regulators of angiogenesis, and act in concert with chemokines, cytokines and growth factors that contribute to cell proliferation and migration toward the origin of the angiogenic cue. Angiostatin, endostatin, trombospondin-1, interferon- γ , platelet factor-4, plasminogen activator-inhibitor are all soluble inhibitors of angiogenesis, whose activity is either to prevent growth factors binding to their receptor, or to inhibit ECM proteases (Carmeliet and Jain, 2000).

Tumors, like healthy cells, need oxygen and nutrients to grow, thus the need for vascularisation increases with tumour size. At the very early phases tumours can be avascular, but as soon as the mass grows it acquires an angiogenic phenotype: the ability to trigger a new vascular network around itself constitutes one of the hallmarks of cancer (Hahnan and Weinberg, 2011). Tumor vessels are architecturally different from physiological vessels, as they lack a complete and organised basal membrane, their structure is unorganised, and the vessels are collapsed and scarcely differentiated. The structure does not show any advantage concerning the provision of oxygen and food to the tumour, but it allows an easy access to the bloodstream for the neoplastic cells (Kleiner and Stetler-Stevenson, 1999).

Adrenomedullin (Ochoa-Callejero et al., 2016) and CXCL12 are both pro-angiogenic factors, and they are both able to interact with ACKR3. Thus targeting ACKR3 to prevent the interaction with adrenomedullin and CXCL12 might add to current cancer multi-therapy a further pathway to block, and may contribute to the overall control of the neoplastic disease.

Angiogenesis might also be targeted for different conditions, for example post myocardial

infarction (MI) ischemia or diabetes with microvascular complications.

In the context of myocardial infarction, activation of ACKR3 promoted post-ischemic angiogenesis, increasing the survival, heart function and remodeling in mice (Hao et al., 2017), suggesting that targeting ACKR3 as soon as possible after MI might offer cardiac protection.

Microvascular complications of diabetes are due to both abnormal neo-vascularisation (diabetic retinopathy) and to a lack of efficient neo-vascularisation (decreased response to hypoxia, peripheral ischemic events and lack of endothelial regeneration). Endothelial progenitor cells (EPCs) are the key players in post-ischemic neo-vascularisation, in that they express ACKR3, and this expression in the EPCs of patients correlates with their ability to contribute to angiogenesis and re-endothelisation (Dai et al., 2017). In this case, ACKR3 might constitute a quantitative marker to be considered for EPC transplantation in treating diabetic limb ischemia.

1.3 CRISPR/Cas9

Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) are short sequences in prokaryotic genomes that work as an adaptive immunity strategy in bacteria (Ishino et al., 1987). Indeed, as for eukaryotic adaptive immunity, it involves a genome rearrangement whose consequence is the triggering of a pathogen-specific response that impairs the biology of the pathogen itself, in an attempt to eliminate the causes of the immune response. The main biological enemies of bacteria are viruses, called bacteriophages or phages. The strategy of the phages involves attachment to the bacteria cell wall and the injection of viral DNA: viruses are not able to self-replicate, thus the aim of this attack is to hack the bacterial machinery for DNA replication and protein translation and give rise to new viral particles. However bacteria are not defenseless: indeed they have at their service the Cas (CRISPR-associated gene) enzymes system, which includes polymerases, nucleases and helicases. Cas proteins can detect exogenous dsDNA (double stranded DNA), break it into small pieces (usually around 30 bp long) and integrate it in the CRISPR Array sequence. The transcription of these sequences is then processed as short crRNAs (CRISPR RNAs), which are joined with a tracrRNA (transactivating crRNA) forming a sgRNA (single guide RNA, called also guide RNA (gRNA)), loaded on other Cas protein complexes that, through sequence homology, can

target and specifically cleave foreign dsDNA sequences upon re-exposure (Barrangou et al., 2007), generating a double strand break.

1.3.1 Rationale of DSB induction for genome editing

The potential of CRISPR was long neglected, until in 2012 it was demonstrated that this system could be programmed to target DNA cleavages *in vitro*, and in 2013 the first CRISPR genome editing protocol was described for cell cultures (Jinek et al., 2012), (Cong et al., 2013), (Mali et al., 2013). Transfected Cas9 loaded with gRNA generates a double strand break in regions highly homologous to the guide itself, which works as a template. A very important part of the guide RNA is the protospacer adjacent motif (thereafter PAM), which is a set of 3-nucleotide long species specific sequences that need to be upstream of the homology region and that is where the cut is localised. Automatic software now exists that can detect the most suitable regions of a given gene and design different guides according to the species of the Cas9 of choice.

The double strand break (DSB) generated by a loaded Cas9 can be employed in two ways, as summarised in Figure 1.6: either the generation of a knock-out (KO), or the generation of a knock-in (KI), exploiting two mechanisms of cellular DNA repair that are respectively the non-homologous end joining (NHEJ) and the homology directed repair (HDR).

The NHEJ is the prevalent pathway of DNA DSB repair, and almost all repairs happening when the cell cycle is not in phases G2 or S belong to this category (Beucher et al., 2009). The DSB is recognised and bound early by the heterodimer Ku (Ku70 and Ku80), which can recruit Artemis and DNA-dependent Protein Kinase catalytic subunit (DNA-PKcs) nucleases with greater affinity than the DSB itself (Meek et al., 2008). These nucleases are needed since the direct ligation of the DSB ends can be prevented by nucleotide matching incompatibility or chemical modification of the bases, and thus their role is mainly to prepare the ends for ligation (Pannunzio et al., 2018). When the Ku-DNA complex recruits the nucleases complex, DNA-PKcs self-phosphorylates and activates Artemis (Ma et al., 2002). Active Artemis can cut all the DNA structures that differ from a stable double strand (Chang et al., 2016). After the double strand ends are stabilised, DNA polymerases belonging to the family of Polymerases X participate in the process. DNA Pol μ and Pol λ are attracted to the Ku-DNA complex, and can

incorporate both dNTPs and rNTPs (Nick McElhinny and Ramsden, 2003) in both a template-dependent and template-independent manner (McElhinny et al., 2005) in order to create a stable ligatable joint. DNA ligase IV (Lig4), in complex with the enzyme X-ray repair cross-complementing 4 (XRCC4), constitutes a central component in NHEJ. In particular, XRCC4 stimulates the activity of Lig4 (Grawunder et al., 1997). XLF (or XRCC4-like factor) and PAXX (PARalog of XRCC4 and XLF) are other two proteins which are reported to participate in the ligation process, in particular XLF can interact and contribute to the formation of the complex XRCC4-Lig4 (Ahnesorg et al., 2006) and PAXX can interact with Ku (Ochi et al., 2015).

HDR (Moynahan and Jasin, 2010) instead is a process that takes place mostly during phases G2 and S of the cell cycle in the proximity of a replication fork, when a homologous sequence is available (usually the sister chromatid) to the site of DSB. The DSB begins processing by the complex MRN (MRE11/RAD50/NBS1), which acts as an exonuclease able to create single stranded 3' overhangs from the broken dsDNA end, together with EXO1 and other exonucleases. The overhang is then bound by RPA, which is then replaced by RAD51: at this stage these nucleoprotein filaments search and invade homologous sequences. Upon action of the helicase SRS2, RAD51 dissociates and the base-pairing of the invading and complementary donor strands takes place, together with the subsequent strand extension by DNA polymerase. The freshly repaired strand then either dissociates and anneals with the processed end of the non-invading strand on the opposite side of the DSB, or if both ends invaded the donor filament, a double Holliday junction is produced, which is resolved to crossover or non-crossover recombinants (Mimitou and Symington, 2009).

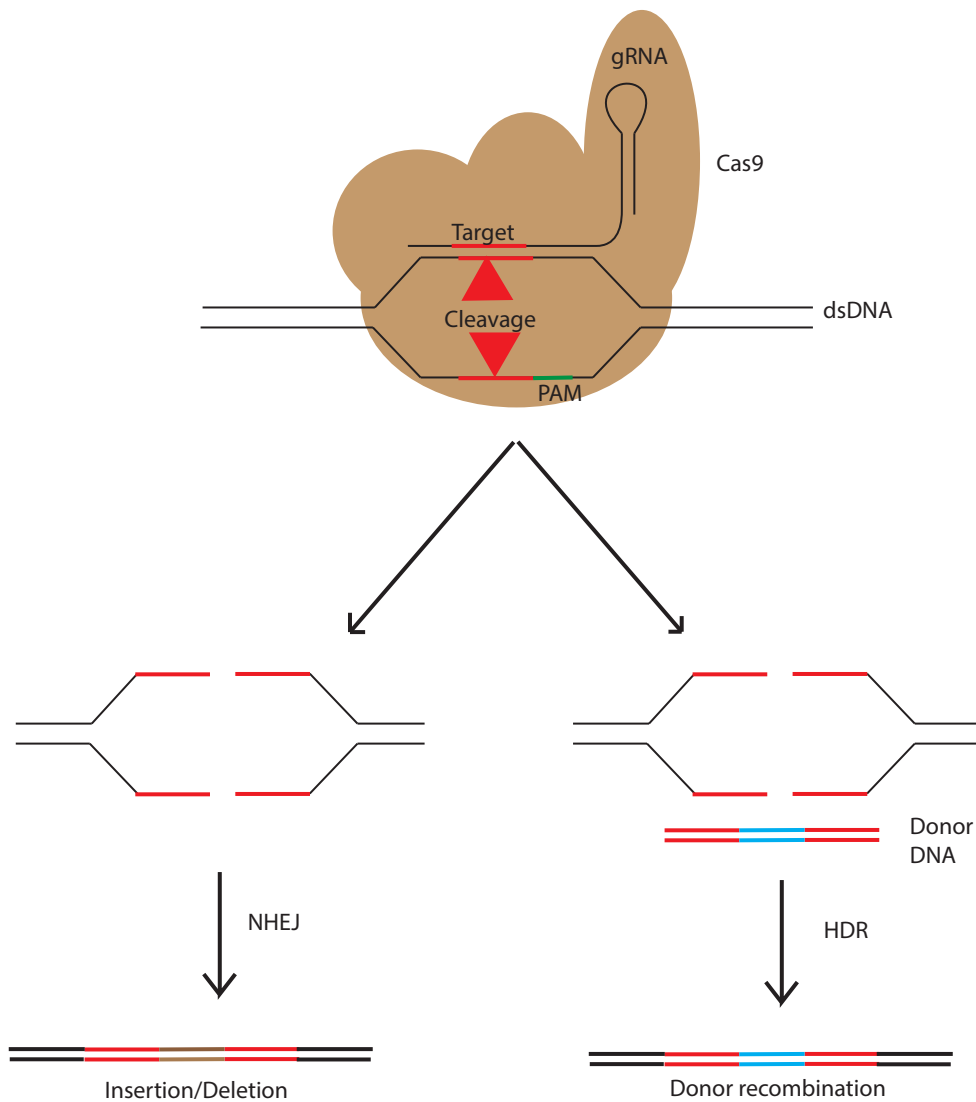


Figure 1.6: **Cas9 role in CRISPR.** Cas9 dependent double strand breaking allows the process of genome editing: when Cas9 and its gRNA are the only player in the reaction (left), the DSB undergoes the non homologous end joining pathway, leading to the formation of indels at the level of the DSB site and most likely generating a knock-out of the target sequence. When a donor DNA participates in the reaction, the DSB is more likely to undergo homology-directed recombination, which allows the generation of a knock-in in the target sequence.

1.3.2 Applications of CRISPR

The ability of Cas9 (and the other CRISPR enzymes) to generate a DSB and the knowledge of the repair mechanism provided an essential boost to the development of CRISPR technology. Indeed, by introducing a double strand break using Cas9 loaded with a guide targeting a specific gene sequence, NHEJ will produce deletion and/or random insertions in the gene, likely generating a frameshift and giving rise to a premature stop codon in the mRNA resulting from the transcription of that gene. The result of this is a gene knock out. Introducing a guide-loaded Cas9 together with a ssDNA homologous to the targeted region but carrying a mutation or an insertion, HDR will contribute to the formation of an edited version of the gene, which results in the generation of a specifically mutated or tagged protein (knock in). The advantage of CRISPR generated KI is the fact that the resulting protein is less subject to alteration of expression. This allows the study of the behaviour of the protein itself in a context which is much closer to the real physiology than a normal overexpression system (Cong et al., 2013).

The study of Cas9 mutants led to more applications, for instance dCas9 (nuclease dead Cas9) was obtained as the result of two point mutations in the active sites of the enzyme, D10A and H840A. The result is a Cas9 that is able to bind to a DNA strand complementary to its gRNA, but when it is bound to the non-coding strand it prevents the transcription of the target sequence, modestly silencing the expression of the sequence targeted (Qi et al., 2013).

Advances in CRISPRi, namely CRISPR-mediated interference, involve the fusion of dCas9 to a KRAB (Krüppel associated box) repressor domain, which works as a DNA binding-dependent transcriptional repressor when fused to a heterologous DNA-binding protein (Margolin et al., 1994). This approach is analogous to RNA interference techniques, but instead of degrading RNAs the role of dCas9 is to hamper RNA polymerase-mediated transcription. If the fusion protein of dCas9 with KRAB targets a promoter region, the repression effectiveness is increased compared to dCas9 alone. However, not all the gRNAs targeting the promoter give the same effectiveness, thus studies using this system should use multiple gRNAs (Gilbert et al., 2013).

1.4 Aim of the thesis

CXCR4 and ACKR3 represent, as described in the previous sections, potential therapeutic targets for different types of human cancers and for tackling HIV-1 infection. However, the mechanisms by which they signal remain poorly-characterised, as well as the outcome that the two receptors can promote by acting in concert. As a consequence, this thesis aimed to address the following research questions.

How do CXCR4 and ACKR3 signal upon stimulation by different ligands? Does their response influence one another? To address these questions the project started with the generation of BRET sensors: for G protein-dependent signalling the constructs were constituted by the receptor (either CXCR4 or ACKR3), a BRET couple (*Renilla* Luciferase and eYFP) and the α_5 helix of either G protein, which is the domain of said signalling molecule responsible for the interaction with GPCRs; for non-G protein-dependent signalling β -arrestin2 recruitment was chosen as readout.

Further, questions related to the behaviour that these receptors gives to the cells arose. Since chemokines are responsible for cell migration triggering classic receptors, how does the removal of the atypical receptor contribute to this phenomenon? In cancer, how does the overexpression of ACKR3 contribute to the severity of the disease? The CRISPR genome editing technique was used to generate Knock-Outs (KOs) to try to infer an answer to these questions.

In parallel, a more recently characterised and debated ligand of ACKR3, namely adrenomedullin, made its entrance in the literature with a work (Klein et al., 2014) that clearly demonstrates that ACKR3 is involved in the clearance of said ligand during development. Does adrenomedullin actually interact with ACKR3? Can this ligand be responsible for the aberrant expression of this receptor in many cancer models? To disentangle these questions a library of extracellular-domain mutants to alanine was generated and the results modelled externally.

Chapter 2

Materials and methods

The following antibodies were purchased: mouse anti-hCXCR4 clone 12G4 (unlabelled and APC-labelled) from BD Pharmingen, mouse anti-hACKR3 clone 8F11-M16 (unlabelled and PE-labelled) and rat anti-mKi67 clone 16A8 from Biolegend, goat anti-GFP clone ab5450 and rabbit anti-hCXCR4 clone ab124824 from ABCAM, and mouse anti-hACKR3 clone 11G8 from R&D Systems.

All antibodies were used according to the manufacturers' instructions.

The *E. coli* strain XL-1 blue was purchased from Agilent.

Cell lines Jurkat and LLC were purchased from ATCC, HEK293T were a kind gift from prof. Martine Smit (VU Amsterdam), Flp/In-TREx 293 were bought from Thermofisher Scientific.

The following kits were purchased: QuickExtract Solution (Lucigen), Monarch Miniprep Kit (New England Biolabs), Wizard SV plus (Promega) miniprep kit, PureLink HiPure Plasmid purification kit (InvitroGen) midiprep kit, QIAprep miniprep, midiprep and maxiprep kit and QIAquick gel extraction kit (Qiagen).

Plasmid px461 and pcDNA3 was purchased from Addgene, plasmids pcDEF3-CKR (Chemokine receptor) were a kind gift from prof. Martine Smit (VU Amsterdam), plasmids pcDNA5-FRT/TO-SPASM and pcDNA3- β Arr2-RLuc were a kind gift from Dr. Brian Hudson (University of Glasgow), plasmid pOG44 was purchased from Thermofisher Scientific.

Tissue culture plastics and 0.5 μ m transwell inserts were purchased from Corning, centrifuge tubes and pipettes were purchased from Sarstedt and 0.22 μ m filters were pur-

chased from Sartorius.

Custom primers were purchased either from ThermoFisher Scientific and IDT.

The following reagents were purchased: Alexa647-hCXCL12 from Almac, Dulbecco-Modified Eagle Medium, Foetal Bovine Serum, HEPES, L-Glutamine, Phosphate Buffered Saline, Penicillin-Streptomycin, RPMI1640 and Trypsin-EDTA (0.25% v/v) from Invitrogen, BbsI and Q5 Polymerase from New England Biosciences, hCXCL11 and hCXCL12 from Peprotech, HindIII, KpnI, Pfu Polymerase, pGEM T-Easy and Taq Polymerase from Promega, Acetic Acid, human Adrenomedullin, Agar, AMD3100, Ampicillin, Blasticidin S HCl, Calcium Chloride, Dimethylsulphoxide (DMSO), Glycerol, Hydrochloric Acid, Hygromycin B, Manganese Chloride, Sodium Chloride, Polyethylenimine (PEI), Poly-D-Lysine, Rubidium Chloride, Tryptone, Yeast Extract and TE buffer 1x (Tris 10 mM, EDTA 1 mM, pH 8.0) from Sigma-Aldrich, BCA Pierce, Coelenterazine-h, DpnI and ECL Pierce from ThermoFisher Scientific.

Sequencing analyses were purchased from Dundee DNA Sequencing and Services and Eurofins Genomics.

2.1 Molecular biology

Molecular biology techniques have been used extensively during this project. All the techniques listed have some steps in common, as the final product of each of them is a relatively large amount of the same sequence of DNA. Techniques such as PCR can amplify a fragment of DNA by using a purified enzyme (DNA-polymerase) together with two oligonucleotides that share sequence with a specific part of the target DNA (primers). Amplification of bigger polynucleotides, such as whole plasmids, can be left to competent bacteria (usually *E. coli*) and then purified using commercially available kits.

2.1.1 Microbiology

Escherichia coli is a widespread bacterium which is found in animal and human microbiota. There is a wide variety of *E. coli* strains, most of which are harmless to humans, but a few strains are pathogenic and can cause food poisoning, urinary tract infection, meningitis, gastrointestinal symptoms and hemolytic-uremic syndrome (HUS) (Kaper et al., 2004). The *E. coli* strains used in molecular biology are all harmless if handled

according to the standard safety guidelines. In particular, during this thesis work the strain XL-1 blue was used.

Reagents for use with bacteria

E. coli culture and selection are well established processes, hence all the basic techniques in microbiology were already well optimised, from the composition of growth medium to protocols for bacteria transformation and plasmid purification.

LB broth recipe Per 1 L of medium:

- 10 g Tryptone
- 5 g Yeast extract
- 10 g NaCl

Stir until the powder is completely solubilised in about 800 mL of distilled water, adjust the pH to 7.0, bring the volume to 1 L and autoclave.

Ampicillin 1000x stock recipe Dissolve ampicillin powder into distilled water (or in ethanol) to a concentration of 100 mg/mL and filter sterilise it. Working concentration will be 100 $\mu\text{g}/\text{mL}$.

LB-agar recipe Per 300 mL of gel (approx 30 Petri dishes):

- 3 g Tryptone
- 1.5 g Yeast extract
- 3 g NaCl
- 4.5 g Agar

Stir the tryptone, yeast extract and sodium chloride in distilled water until solubilised, adjust the pH to 7.0, then add the agar powder and autoclave.

LB-agar Amp recipe Prepare LB-agar according to the previous paragraph. After autoclaving, cool to 55°C and dilute 1:1000 the ampicillin 1000x stock into the LB-agar. Pour the LB-agar amp into an appropriate number of plates (approx 10 mL per dish) and let it solidify.

Solution 1 for competent cells Recipe for 100 mL.

Potassium acetate (1M)	3 mL (30 mM final)
RbCl (1M)	1 mL (10 mM final)
CaCl ₂ (1M)	1 mL (10 mM final)
MnCl ₂ (1M)	5 mL (50 mM final)
Glycerol (80% w/v)	18.75 mL (15% w/v final)

Adjust the pH to 5.8 with 100 mM acetic acid, bring up the volume to 100 mL with distilled water, filter sterilise it and store at 4°C.

Solution 2 for competent cells Recipe for 40 mL.

MOPS (100 mM, pH 6.5)	4 mL (10 mM final)
RbCl (1M)	0.4 mL (10 mM final)
CaCl ₂ (1M)	3 mL (75 mM final)
Glycerol (80% w/v)	7.5 mL (15% w/v final)

Adjust the pH to 6.5 with concentrated HCl, bring up the volume to 40 mL with distilled water, filter sterilise it and store at 4°C.

Preparation of competent bacteria

The *E. coli* strain XL-1 blue was chosen to propagate plasmid DNA.

An LB-agar plate with no antibiotics was spread with non transformed XL-1 blue cells to grow colonies overnight at 37°C. One of the colonies was picked and grown in 5 mL of LB broth with no antibiotics for the following night at 37°C. Those 5 mL were then subcultured in 100 mL of LB broth with no antibiotics and grown at 37°C until the optical density at 550 nm reached the value of 0.48.

After chilling 5 minutes on ice, cells were spun 2500 *g* for 10 min at 4°C in 50 mL sterile Falcon tubes. Each pellet was resuspended in 20 mL of solution 1 by gentle pipetting, chilled for further 5 minutes and spun as previously. Each pellet was then resuspended in

2.2 mL of solution 2, by gently pipetting up and down, chilled for a further 15 minutes, then divided into 220 μL aliquots per microcentrifuge tube, and stored at -80°C .

Transformation of competent cells with plasmids

50 μL of competent cells were kept on ice and incubated for 10 min with either 1 μL of plasmid or 5 μL of ligation/mutagenesis product in microcentrifuge tubes. The tubes were transferred to the water bath at 42°C for 90 s, then re-transferred back onto ice for further 2 min. 450 μL of LB-broth were added to the microcentrifuge tube, and the bacteria were incubated on a shaker at 37°C for 45-60 min. The tubes were spun 2 min at 2000 g in a table top minicentrifuges and 450 μL of supernatant discarded. The bacteria were resuspended in the leftover volume, spread onto LB-agar amp plates and allowed to grow overnight.

Output The output of transformation is a plate full of bacteria colonies. To proceed to DNA purification, single colonies were picked from each plate and grown in 5 mL aliquots of LB amp overnight.

Transformed bacteria were used to propagate desired DNA constructs. The purification of plasmid DNA from bacteria is usually referred to as “prep”, and according to the volumes of LB-amp in which bacteria have been seeded and to the amount of plasmid we want to obtain, we can have minipreps (5 mL bacteria, up to 20 μg DNA), midipreps (25-100 mL bacteria, up to 200 μg of DNA) and maxipreps (100-500 mL bacteria, up to 1000 μg of DNA). All the kits have common steps:

- resuspension of the bacteria
- lysis of the cells and subsequent neutralisation of the detergent
- separation (centrifugation) of the membranes (pellet) from the DNA-protein mixture (supernatant)
- elimination of the impurities from the DNA through a column
- elution of purified DNA

All the prep kits were used according to the manufacturer instructions.

Quantitation of purified DNA

Plasmid DNA purified from bacteria was quantified in a spectrophotometric system using either cuvettes, single drops (in a Nanodrop) or in a Pherastar FS purpose-built plate (BMG Tech).

Three wavelengths are usually used to quantify DNA and RNA to establish also their degree of contamination with carryovers from the lysis or purification steps: 230 nm, 260 nm and 280 nm. DNA and RNA absorb efficiently ultraviolet light at 260 nm. Most proteins, in particular the ones with aromatic residues, absorb UV light at 280 nm, thus to assess the purity from proteins the ratio $A_{260/280}$ is fundamental; likewise, guanidine and phenol, which are used for the DNA extraction, absorb UV light at 230 nm, so the ratio $A_{260/230}$ gives an indication of how efficiently the washes have been to get rid of phenol and guanidine compounds.

Rule of thumb, is that pure DNA shows ratios $A_{230:260:280}$ 1:1.8:1, while pure RNA $A_{230:260:280}$ is 1:2:1.

2.1.2 PCR

The polymerase chain reaction (PCR) is a fundamental reaction in molecular biology. Since its discovery by Kary B Mullis (Saiki et al., 1985), the ability of DNA-polymerases to synthesise complementary DNA given a template has given rise to several techniques, from molecular cloning to DNA sequencing, and provided a useful potential investigation tool.

Being enzymes, DNA polymerases can show temperature- or buffer-dependent activity impairment: to this end, the study, purification and engineering of polymerases from extremophil microorganisms gave the market more stable enzymes.

Furthermore, a desirable feature for DNA fragment synthesis in most applications is the high proofreading activity: in nature this activity helps balancing the effects of exogenous mutagenic events and from random mistakes that a DNA polymerase can make on the cell in replication phase. For laboratory work, using an efficient proofreading polymerase can facilitate cloning and give reliable results from sequencing reactions.

In the next subsection, “Molecular cloning”, there will be a thorough description of all the PCR-dependent techniques used for this project.

2.1.3 Molecular cloning

Herein molecular cloning refers to all the techniques used to generate multiple copies of a desired wild-type or engineered DNA sequence.

For this project plasmid site-directed mutagenesis and CRISPR were used.

Plasmid Site-Directed Mutagenesis

Plasmid Site-Directed Mutagenesis (SDM) is a molecular biology technique that is used to specifically edit a plasmid bourn DNA sequence by targeting that sequence with a pair of oligonucleotides carrying a specific mutation, which can be a point mutation, an insertion or a deletion.

For insertion or deletion, the primers can be designed partly by hand, taking care to put a buffer sequence at the beginning of the primer should a 5' restriction site be desired. The insertion/deletion (indel) should be flanked by the sequence of the template in correspondence of the area where the indel is desired. The same applies to the complementary strand in proximity to the final part of the mutation.

For point mutation purposes the phase of primer design can be left to specific online software packages, such as Agilent QuikChangeII (<https://www.genomics.agilent.com/primerDesignProgram.jsp>), which optimise the design according to the minimum energy cost (they seek to minimise the difference of free energy between the plasmid and the oligonucleotide) and to an annealing temperature of 65°C (Novoradovsky et al., 2005).

High-fidelity Q5 polymerase (NEB) was used for this method, in order to reduce the chance of random off-target mutation due to the lack of proofreading activity that many commercially available DNA-polymerases have.

Component	Amount
5X Q5 Reaction Buffer	5 μ L
dNTPs (10 mM)	0.5 μ L
Primer fwd (10 μ M)	1.25 μ L
Primer rev (10 μ M)	1.25 μ L
Template DNA	up to 1 μ g
Q5 High-Fidelity DNA Polymerase	0.25 μ L
Nuclease-free Water	to 25 μ L

To insert specific sequences in the receptor plasmids described below, a classic PCR

approach was used.

Step	Temperature	Time
Initial denaturation	98°C	2 min
35 cycles	98°C	10 s
	68°C	30 s
	72°C	30 s/kbase
Final extension	72°C	2 min
Hold	4°C	∞

The PCR protocol selected for the point mutagenesis was the touch-down approach, which required the following steps:

Step	Temperature	Time
Initial denaturation	98°C	2 min
25 cycles	98°C	10 s
	67°C	30 s
	72°C	30 s/kbase
15 cycles	98°C	10 s
	60°C	30 s
	72°C	30 s/kbase
Final extension	72°C	2 min
Hold	4°C	∞

After the PCR ends, the product consists of a mix of the mutated plasmids, generated from purified polymerase, and the template plasmid, which was obtained from bacteria. Plasmids purified from most bacteria, *E. coli* included, are methylated on every adenine of the sequences 5'-GATC-3' due to the activity of a bacterial enzyme called Dam methylase (Deoxyadenosine methylase); treatment of the aforementioned mix with the endonuclease *DpnI*, which recognises and cleaves only A-methylated 5'-GATC-3' sequences, allows the digestion of the bacteria-generated mutagenesis template leaving only the mutant DNA in the final product.

Component	Amount
10X FastDigest buffer	2 μ L
FastDigest Enzyme <i>DpnI</i>	1 μ L
Template DNA	2 μ L (up to 1 μ g)
Q5 High-Fidelity DNA Polymerase	0.25 μ L
Nuclease-free Water	15 μ L

After the digestion of the template at 37 °C for 5 minutes, the product was then transformed into competent cells, a few clones were picked from the plate and DNA isolated by miniprep.

To confirm that the mutagenesis product was correct, sequencing was outsourced.

Primers for CXCR4, FLAG-CXCR4, CXCR3, FLAG-ACKR3 and ACKR3 cloning into pcDNA5/FRT/TO *HindIII*-GPR35-*KpnI*-eYFP or into *HindIII*-GPR35-*KpnI*-SPASM sensors were the following (Italics: restriction site; underlined: Kozak sequence; bold: FLAG tag):

Name	Primer sequence (5' -> 3')
Fw <i>HindIII</i> -FLAG-CXCR4	CGATCGAAGCTT <u>GCCACC</u> ATG GATTACAAG-GATGACGACGATAAG GAGGGGATCAGTATA-TACTACT
Fw <i>HindIII</i> -CXCR4	CGATCGAAGCTT <u>GCCACC</u> ATG GAGGGGATCAGTATA-TACTACT
Rv <i>KpnI</i> -CXCR4 no stop	GCTAGC <i>GGTACC</i> GCTGGAGTGAAAACCTTGA
Fw <i>HindIII</i> -FLAG-ACKR3	CGATCGAAGCTT <u>GCCACC</u> ATG GATTACAAG-GATGACGACGATAAG GATCTGCATCTCTTC-GACTACTCA
Fw <i>HindIII</i> -ACKR3	CGATCGAAGCTT <u>GCCACC</u> ATG GATCTGCATCTCTTCGACTACTCA
Rv <i>KpnI</i> -ACKR3 no stop	GCTAGC <i>GGTACC</i> TTTGGTGCTCTGCTCCAAGG
Fw <i>HindIII</i> -CXCR3	CGATCGAAGCTT <u>GCCACC</u> ATG GAGTTGAGGA-AGTACGGCCCTGGA
Rv <i>KpnI</i> -CXCR3 no stop	GCTAGC <i>GGTACC</i> CAAGCCCGAGTAGGAGGC

The constructs were all fusion proteins, hence the stop codon at the end of each receptor was removed.

In order to build the first library consisting of 105 single mutations to alanine of human ACKR3, the mutagenesis primers listed in Appendix 1 have been used.

2.1.4 CRISPR and validation

For the purposes of this project, Jurkat and LLC cells have undergone genome editing processes. For all the products, after transfection and selection, cells' genomic DNA have been extracted, amplification of the gene of interest have taken place and pre-screened by their size in an agarose gel; the desired PCR products have then been TA cloned inside pGEM T-Easy vectors and sequenced.

Genomic DNA extraction

Genomic DNA was extracted using the QuickExtract Solution (Lucigen). The kit provided only a lysis buffer and did not require toxic chemicals or spin columns, only a vortex mixer and heat.

The protocol requires a small number of cells:

- Place 100 μL of sample cells in a 0.2 mL PCR tube and spin briefly to deposit them to the bottom of the tube
- Remove the supernatant and add 100 μL of QuickExtract Solution
- Vortex for 15 seconds
- Incubate at 65°C for 10 minutes
- Vortex for 15 seconds
- Incubate at 98°C for 2 minutes
- Genomic DNA is ready to use or to be stored at -5°C

For CRISPR validation, the gDNA was amplified with specific genomic primers, the product was run in a 2% agarose gel (120V for 30 minutes) and the sample of interest

was identified from the size of the band. Selected PCR products were then purified from gel according to the QIAquick gel extraction kit (Qiagen) protocol and prepared for the TA cloning.

TA cloning

Amplification products were blunt ended dsDNA oligonucleotides, and required a dATP single nucleotide 3' overhang at each strand for TA cloning. The first step was an incubation (72 °C for 25 minutes) in which only dATP was added to the dsDNA products together with Taq polymerase (New England Biolabs) and its buffer.

pGEM T-Easy (Promega) is a linearised vector with a single dTTP 5' overhang from each strand.

pGEM T-Easy, single deoxyadenosinated PCR products and T4 DNA ligase (Promega) were then incubated together according to the latter's protocol in order to obtain a closed vector with the amplification product integrated (overnight incubation at 4°C).

Bacteria were transformed with the ligation product, single colonies grown, mimiprep DNA prepared and then sequenced using a T7 primer. The T7 promoter is located upstream of the 5'T overhang in pGEM T-Easy, so consequently the insert sequence was obtained.

2.2 Tissue culture

Eucharyotic cells are perhaps the simplest model of tissue physiology. Plenty of information can be derived from cell culture, and together with the use of molecular biology, deep insights of what is happening inside the cell upon any kind of treatment or stimulation can be observed and quantified. Of course important information necessary for drug development such as most of the pharmacokinetic parameters are not obtainable from cell culture, however tissue culture is able to give information on signalling quantification, imaging and single cell physiology.

2.2.1 HEK293T cells

HEK293 cells are a cell line obtained in Leiden, The Netherlands, in 1973 in van der Eb's laboratory from the embryonic kidney of a legally aborted human foetus. The cells were

cultured and transfected with the sheared DNA of Adenovirus 5, and the 293th clone obtained was the only one showing stability as it could be cultured for several months. Since whole kidney chunks were used for transfection, primary fibroblasts, endothelial, epithelial cells or neurons could have been the ancestor of the HEK293 cell line (Graham et al., 1977). To this end, later studies on HEK293 cells were done and showed that they have a lot of functional properties in common with immature neuronal cells; moreover, their transcriptome displays similarities to adrenal cells, whose medullar region originates from the neural crest (ectoderm) (Lin et al., 2014). Given the topological proximity of the adrenal gland to the kidney, it is plausible that HEK293 cells maybe derived from it (Shaw et al., 2002). HEK293T is a variant of HEK293, developed from the latter cell line and transformed with Simian Vacuolating Virus 40 large T antigen (SV40-TAg). As most of the commercially available DNA vectors are designed including an SV40 episomal replication promoter, SV40-TAg allows the high-copy replication of the transfected DNA.

Flp/In T-REx 293

Flp/In T-REx 293 (ThermoFisher Scientific) is a HEK293 derived cell line which expresses the Tet repressor from the integrated pcDNA6/TR regulatory plasmid and contains a single stably integrated FRT (Flp Recombination Target) site. In 2.3.2 will be described more thoroughly the use of this particular engineered cell line.

Maintenance and freezing procedures

HEK293T cells were cultured in D-MEM + 10% (v/v) FCS + Pen/Strep + 2 mM L-Gln. They were passaged by incubating for 5 minutes at 37°C in trypsin-EDTA 0.5 % (v/v) when they reached 75-90% confluence.

Freezing medium was FCS + 10 % (v/v) DMSO. An 85% confluent T75 flask could be frozen in 2-4 mL of freezing medium, 1 mL per cryovial.

2.2.2 Jurkat T cells

Originally called JM cells, Jurkat T cells are an immortalised cell line derived from a T-cell Acute Lymphoblastic Leukemia (T-ALL) and isolated from the peripheral blood of a 14 year old boy in the late 1970s (Schneider et al., 1977). Commercially available Jurkat cells derive from a problem encountered in the early 1980s, when the cells from the

original batch were found to be heavily contaminated with mycoplasma, and the curing process led to the establishment of clone E6-1 as a perfectly non-infected starting culture for expansion and retail (Abraham and Weiss, 2004). This model has been extensively used to study T cell leukemia, T-Cell Receptor (TCR) components and signalling and T-tropic HIV infection (Abraham and Weiss, 2004).

Maintenance and freezing procedures

Jurkat cells were cultured in RPMI 1640 + 10% (v/v) FCS + Pen/Strep + 2 mM L-Gln. They were passaged by diluting 1:3-1:10 in fresh medium when they reached the concentration of 0.8-1.2 x10⁶ cells/mL.

Freezing medium was FCS + 10 % (v/v) DMSO. 5x10⁵ cells/mL could be frozen in freezing medium and stored 1 mL per cryovial.

2.2.3 LLC

The Lewis Lung Carcinoma cell line was first isolated from a spontaneous epidermoid carcinoma of the lung in C57BL/6 mice in 1954 by Dr. Margaret Lewis (Rashidi et al., 2000). It has been an important tumour model for cancer therapy, as it has helped studying metastasis and angiogenesis and has been involved in developing antitumoural chemotherapeutic drugs (Kellar et al., 2015).

LLCs hold an advantage that other tumour models do not have, deriving from the fact that they were isolated by a spontaneous tumour in C57BL/6 mice, thus they are immunologically compatible with this strain of mice, so they can be engrafted into immunocompetent mice and not be rejected by their immune system. This feature is called syngeneism.

Together with the lack of immune-dependent obstacles, being syngeneic allows the study of every aspect of the tumour microenvironment, including the role of the resident immune cells.

Maintenance and freezing procedures

LLCs were cultured in D-MEM + 10% (v/v) FCS + Pen/Strep + 2 mM L-Gln.

They were passaged by incubating for 5 minutes at 37°C in trypsin-EDTA 0.5 % (v/v) when they reached 80% confluence, hence every second or third day due to their extremely

efficient proliferation.

Freezing medium was FCS + 10 % (v/v) DMSO, the content of an 80% confluent T75 flask could be frozen in 2-4 mL of freezing medium, 1 mL per cryovial.

2.3 Cell biology

Cell biology experiments involve the use of live cells to get information about the status and the behaviour of the cell upon perturbation of its homeostasis.

2.3.1 Optical microscopy

Optical microscopy involves passing visible light (transmitted through the sample or reflected from it) through a system of lenses in order to give a magnified view of the sample.

Fluorescence microscopy

When cells carrying a fluorophore-labelled protein get illuminated with light of the specific wavelength for the fluorophore excitement, said light is absorbed and a longer wavelength radiation is emitted.

Fluorescent images were acquired using a Nikon TE2000-E inverted microscope (Nikon Instruments, Melville, NY) equipped with a 40x (numerical aperture 1.3) oil immersion Plan Fluor lens and a cooled digital CoolSNAPHQ charge-coupled device camera (Photometrics, Tucson AZ, USA).

This technique was used to assess the expression of SPASM sensors in stably transfected Flp/In T-REx 293 cells by detecting mCitrine signal.

2.3.2 Transfection

To transfect means to deliver exogenous nucleic acids inside eukaryotic cells.

Transfection of HEK293T cells was done using polyethylenimine (PEI) or lipofectamine 2000 (Invitrogen), the latter used for LLCs as well. Optimal transfection of Jurkat was obtained using electroporation.

PEI-transfection

Polyethylenimine (PEI) is a cationic polymer made of repetitions of an imine (NH) group spaced by two aliphatic carbons (CH₂-CH₂). PEI packs DNA into positively charged particles, which bind to the anionic residues on the cell surface and are internalised by endocytosis. Once the vesicle containing the PEI-DNA complex is inside the cell, acidification of the intravesicular environment leads to protonation of the amines, resulting in an influx of counter-ions and a progressive loss of osmotic equilibrium. The osmotic swelling then bursts the vesicle, allowing the DNA-PEI complex to diffuse into the cytoplasm. If the complex decondenses, the DNA can diffuse to the nucleus (Akinc et al., 2005).

Protocol for PET transfection of HEK293T cells:

- The day before transfection, cells were split from a confluent T75 flask 1:4 in 10 cm Petri dishes
- On the day of transfection DNA dilutions were prepared as follows: 5 µg of DNA in 250 µL of sterile 150 mM NaCl
- PEI dilution was prepared as follows: 30 µL of PEI in 250 µL of sterile 150 mM NaCl
- DNA dilutions and PEI dilutions were mixed
- PEI-DNA mix was vortexed and incubated RT for 10 minutes; in the meantime, cells medium was changed
- PEI-DNA complexes were added to the cells dispensing 500 µL per dish in a drop-wise manner.

Lipofectamine-2000 transfection

Lipofectamine (Invitrogen) is a cationic liposome formulation, which complexes with negatively charged macromolecules such as DNA or RNA. The resulting complexes can easily fuse with the negatively charged plasma membrane of living cells, allowing the delivery of the complexed nucleic acids into the cytoplasm.

To be efficiently expressed the transfected product should reach the nucleus, therefore

transfection is recommended on sub-confluent cells, which most likely undergo mitosis during transfection (Dalby et al., 2004).

Protocol for HEK293T cells in 6 well plates:

- The day before transfection, half a million cells were seeded per well
- In one microcentrifuge tube, 5 μg of DNA was diluted in 200 μL of Opti-MEM medium
- In another microcentrifuge tube, 5 μL of Lipofectamine 2000 was diluted in 200 μL of Opti-MEM medium
- The content of the two Eppendorf tubes were combined, then vortexed for 5 seconds and incubated RT for 5 minutes to allow the formation of complexes
- 400 μL of each mix was dispensed dropwise into the well

The same protocol was used to transfect LLC cells.

Electroporation

Electroporation is a transfection technique based on the sudden and short exposure to a strong electric field to increase cell membrane permeability to macromolecules which would otherwise struggle to pass through the membrane under physiological conditions. Its efficiency is calculated to be about 10 times higher than other chemical- or liposome-based transfection techniques. Modern electroporators are devices on which voltage, repetition, pulses and duration may be input, and are transferred through a docking unit to a purpose-built pipette tip that contains the cell and transfectant suspension (in 10-100 μL volumes) and acts as one of the two electrodes necessary for the exposure to the electric field.

Electroporation was chosen to transfect Jurkat cells. The electroporator, Neon Transfection System (Thermofisher Scientific), allowed for the use of 10 μL pipette tip electroporation chambers, in which 2×10^5 Jurkat cells mixed with 500 ng plasmids mixture were exposed to 3 1,325V pulses 10 ms wide.

2.3.3 Generation of stably transfectant Flp/In T-REx 293

The Flp/In T-Rex 293 cell line was purchased from ThermoFisher Scientific, and is a HEK293 derived cell line which expresses the Tet repressor from the pcDNA6/TR regulatory plasmid and contains a single stably integrated FRT (Flp Recombination Target) site. Generation of stably transfected cell lines took place when those cells were co-transfected with plasmids pOG44 and pcDNA5/FRT/TO: pOG44 encoded for a Flp recombinase, which integrated stably in the host genome the construct contained in pcDNA5/FRT/TO by recombining the DNA through FRT sites. Selection was made by adding Hygromycin B and Blasticidin S HCl to the medium since their resistance sequences were carried respectively by pcDNA5/FRT/TO and pcDNA6/TR: it took approximately 3 weeks. As the promoter upstream of the integrated construct was repressed by a Tet repressor, a 24 hours-long treatment with doxycycline was needed to express the gene of interest.

2.3.4 Genome editing using D10A Cas9

Mutant D10A Cas9 encoded in px461 vectors (Addgene) was used to CRISPR Jurkat cells and LLCs.

Mutation D10A impairs the DSB-formation ability of WT Cas9 giving it the capability of generating a single nick on the complementary strand. Therefore, in order to generate a DSB two mutant Cas9s are required, reducing greatly the likelihood of off target effects (Chiang et al., 2016). The efficiency of the genome editing is reduced if the two gRNAs are not about 20 nucleotides apart, making it more difficult to find a proper region in which the two PAMs need to be rigidly spaced, but the advantage is that the genome targeting is extremely precise.

2.3.5 β -arrestin2 recruitment assay

This assay constitutes a G protein-independent readout for receptor activation. Receptor-eYFP and β -arrestin2-RLuc6 were co-transfected transiently into HEK293T cells at a ratio of 1:5, and upon stimulation, when the tagged arrestin approaches the receptor to interact with it, the distance between RLuc6 and eYFP gets below the Förster radius (10 nm) and in presence of a substrate such as coelenterazine-h, luciferase's lumi-

nescence excites the fluorophore and a BRET signal can be detected.

The protocol took place over four days, the first of which consisted of splitting HEK293T cells 1:4 from a confluent T75 into a 10 cm Petri dish. On the second day this plate was transfected and also a white 96-well plate poly-D-lysine coated. The cells were transferred to the pre-coated 96-well plates on the third day, whilst on the fourth, after washing the cells with HBSS to get rid of the phenol red contained in the medium (which might interfere with the spectrum detected from one of the two fluorescent molecules), agonist/antagonist treatment took place together with the administration of the substrate for the luciferase (10 μ M of coelenterazine-h), and BRET was calculated as ratio of fluorescence (545 nm) over luminescence (475 nm).

2.3.6 G protein recruitment assay (SPASM sensor)

GPCR Systematic Protein Affinity Strength Modulation sensors (SPASM sensors) are chimeric proteins (Malik et al., 2017) described as being formed by a GPCR, a FRET couple separated by a flexible ER/K linker (Sivaramakrishnan and Spudich, 2011) and the $\alpha 5$ helix of the G protein of interest. The rationale behind the design of this sensor is that the $\alpha 5$ helix of the G protein has been reported as being a fundamental element for the interface between GPCRs and G proteins (Mahoney and Sunahara, 2016). Upon agonist binding, if the construct is made with the right G protein $\alpha 5$ helix, the FRET couple get close enough to allow energy transfer.

A BRET version has been generated by cloning CXCR4 and ACKR3 upstream of mCitrine, ER/K linker, NanoLuc and each of the G_{α} subunits in pcDNA5/FRT/TO vectors. NanoGlo (Promega) was used as substrate.

For a better resolution of the assay the use of stable transfectants is recommended.

The process was analogous to the protocol described for β -arrestin2 recruitment.

2.3.7 Fluorescent-ligand saturation binding assay

Fluorescent-ligand saturation binding is an assay in which different concentrations of a fluorescent ligand are used to treat the cells, the excess washed away, and the binding is measured as a function of the fluorescence detected by a flow cytometer.

The protocol was performed as follows and was carried out on ice:

- 100,000 sample cells per well were seeded into a round-bottom 96-well plate, suspended in 90 μL of PEB buffer (PBS without calcium and magnesium + 2 mM EDTA and 0.5% (w/v) BSA)
- Cells were treated by adding 10 μL of 10x concentrated fluorescent ligand to each well
- Cells were incubated 30 minutes at 4°C
- Cells were washed twice with PEB buffer (300g, 5 minutes)
- Cells were resuspend in 150-200 μL of PEB buffer, ready for flow cytometry.

2.3.8 Transwell migration assay

Transwell inserts have been developed from the concept introduced by the Boyden chemotaxis chamber. The latter is an *ad hoc* device made of two detachable plastic parts in which communicant wells are carved and separated by a porous filter (usually polyvinylpyrrolidone-coated polycarbonate). A chemotactic agent is added to the lower part whilst cells are introduced into the upper. The chamber is incubated for discrete amounts of time during which the cells are allowed to migrate, the filters are then subsequently washed, stained with histology dyes and cells counted to assess chemotaxis. Transwell inserts however have allowed the study of chemotaxis to move from the Boyden chamber to more common tissue culture plates (6 well to 96 well plate formats are commercially available).

These inserts require an equilibration step, in which the migration buffer is allowed to fill every pore of the filter to avoid the formation of microscopic air bubbles, which during cell migration, can prevent the passage of the cells themselves. After the equilibration step, the TC plates are filled with the chemotactic stimuli, the transwells are added with cells, and the transfer of the inserts each well and subsequent incubation of the plate in the TC incubator at 37°C determines the starting point of the migration.

The evaluation of cell migration is done counting the cells in the lower part of the system.

- The inserts were equilibrated with migration buffer (RPMI + 1% (v/v) FBS) for 30 minutes at 37°C

- The stimuli were diluted in 600 μL of migration buffer
- Jurkat T cells were resuspended 2×10^6 cells/mL in migration buffer
- After the equilibration, migration buffer was removed from the inserts and from the plate
- 600 μL of diluted stimuli were added to the plate, and 100 μL of cells added inside the transwell
- The inserts were put on the corresponding well and the plate was incubated 6 hours at 37°C

2.3.9 Flow cytometry

Flow cytometry is a standard laboratory technique fundamental to the evaluation of hematopoietic cells, including the identification and discrimination of leukocyte populations, and for the thorough characterisation and description of the immunophenotype of cells derived from a specific tissue.

The flow cytometer is the device through which this technique has been developed and improved, and consists of a light source, photodetectors, optical light filters, electronics and a computer. Fluidics is the heart of this technique. Briefly, the characterisation starts with a fluorophore-tagged antibody-labelled sample consisting of cells in suspension in a buffer that ideally prevents them from sticking together. The cell sample is injected into the fluidic systems flowing sheath fluid, which allows the establishment of a hydrodynamically-focused single-file flow of cells that streams to the analysis point maintaining the cells at the centre of the flow. The analysis point is the site of the fluidic system in which the procession of cells is exposed to the light of different lasers with different wavelengths, allowing the simultaneous excitation of all the fluorophores attached to each cell. The illumination of the cells generates both nonfluorescent and fluorescent signals, which are collected by a detection system consisting of optical filters which select each wavelength band of interest and photodetectors which transform the light into electricity according to its intensity. This way the intensity of each fluorophore and the data relative to the scattering of non-fluorescent light is transformed into data fitting scatterplots or histograms, that give information relative to each cell in the sample

about the size (forward scatter), the granularity of the cytoplasm (side scatter) and the expression of each labelled antigen (specific fluorescence).

Fluorescence-activated cell sorting (FACS)

Fluorescence-activated cell sorting is a special flow cytometry technique. This flow cytometer is provided with an ultrasonic nozzle vibrator just upstream the size/fluorescence detection system, while right downstream said system there is an electrical charging ring that gives an electrical charge to the droplet that corresponds to the cell population chosen on the software. The stream of droplets then passes through an electric field, that deviate the trajectory of the charged droplets only.

2.3.10 Chemokine uptake assay

The chemokine uptake assay involves the use of a C-terminally fluorescently-labelled chemokine: for this thesis project, AlexaFluor647-CXCL12 (Almac) was used at pEC_{80} concentration. Cells fluorescence was read in a flow cytometer, internalisation of the chemokine is proportional to the fluorescence in the fluorophore channel (Le Brocq et al., 2014).

Uptake medium was RPMI1640 + 0.5% (w/v) BSA.

The protocol was the following:

- In a round-bottom 96-well plate, 90 μ L of sample cells were seeded, suspended in uptake medium at the concentration of 1.1×10^6 /mL (100,000 cells per well)
- 10 μ L of 10x concentrated fluorescent chemokine resuspended in uptake medium was added to the cells
- Cells were incubated at 37°C for 1 hour
- Cells were washed twice (300g, 5 min, room temperature) with PEB buffer, resuspended in 150-200 μ L of PEB, and at this stage were ready for flow cytometry

2.3.11 Ki67 proliferation assay

Ki-67 is a protein which is located in the nucleus of eukaryotic cells. Antibodies against Ki-67 are regularly employed as prognostic tools in the diagnosis of cancer as its level strongly correlates with the proliferative capability of cells, making it a widely used biomarker (Bullwinkel et al., 2006). The name Ki-67 was derived from the city where the first monoclonal antibody against it was generated, namely Kiel, Germany, and the number 67 refers to the number of the antibody clone that first detected it in a 96 well plate (Gerdes et al., 1983).

The proliferation rate of the cells was detected by flow cytometry; the cells were stained using the following protocol:

- 70% (v/v) ethanol was prepared and chilled overnight at -20°C
- Cells were prepared
- Cells were washed with FACS buffer 300g 5 min 4°C
- Supernatant was discarded and the pellet loosened by vortex mixing
- 1 drop of cold ethanol was added to the pellet, and the pellet vortexed; a micro-centrifuge tube per sample was placed on ice
- 500 μL of ice cold ethanol was added to the pellet and vortexed
- Another 500 μL of ice cold ethanol was added to the pellet and the sample vortexed for 30 seconds
- Cells were incubated for 1 hour at -20°C
- Cells were washed 300g 5 min 4°C and resuspended in FACS buffer
- Anti-Ki67 antibody was added to the samples at 1:220 dilution (15 μL in 3 mL)
- Samples were incubated in the dark at RT for 30 min
- Samples were washed 300g 5 min 4°C and resuspended in FACS buffer before reading in the flow cytometer

2.4 Biochemistry

2.4.1 Membrane preparation

To generate an optimal working amount of membrane proteins 8 dishes per condition were required. The protocol used was the following:

- Harvest the cells with 10 mL cold sterile PBS, and pipette to detach cells from the dish
- Spin the cell solutions at $\geq 5400 g$ 4°C for 5 min
- Wash and resuspend the pellet in 20 ml cold PBS
- Spin again $\geq 5400 g$ 4°C for 5 min
- Remove the supernatant and store the pellet for at least 1h at -80°C
- Thaw pellet on ice for 30 min
- Prepare membrane buffer (TE buffer (Sigma-Aldrich) + protease inhibitor cocktail (Roche)) vortexing 1 tablet in 50 mL. It takes 5-10 minutes to dissolve
- Add 2 mL of membrane buffer to the thawed pellet, then homogenise in a teflon-glass homogeniser (50 strokes)
- Transfer the homogenates into 7 mL bijoux, then use a 2 mL syringe with a 25G needle to further disaggregate the homogenates by passing it through the needle 5 times
- Transfer the homogenates to 15 mL falcon tubes, then spin at $150 g$, 4°C 5 min to remove cellular debris
- Transfer the supernatant to glass ultracentrifuge tubes, then spin at $80,000 g$ 4°C for 45 min to pellet the membrane
- Discard the supernatant, and resuspend each pellet in $700 \mu\text{L}$ of membrane buffer

- Pass the resuspended pellet a further 10 times through a 25G needle using a 2 mL syringe, then use the BCA assay to quantitate the concentration of the total membrane protein
- The protein can be stored for up to 3 months at -80°C . Usually $5\ \mu\text{g}$ of total protein per point is required

2.4.2 BCA assay

The Bicinchonic Acid assay, informally referred to as the Pierce assay, is a biochemical assay that is used to determine the amount of protein in a solution. It consists of two solutions, Solution A is a pH 11.25 solution containing Bicinchonic acid, sodium carbonate, sodium bicarbonate and sodium tartrate, while Solution B is copper sulphate.

The presence of carbo-amidic groups (peptidic bonds) reduces Cu^{2+} ions to Cu^{+} , and this phenomenon is proportional to the amount of total protein. Bicinchonic acid can chelate Cu^{+} ions, and this complex absorbs light at 562 nm.

In practical terms, proteins are quantified in a spectrophotometer calculating the absorbance at 562 nm and comparing this value to a standard curve made with known concentration of BSA.

Operative protocol was the following:

- Dispense in duplicate $10\ \mu\text{L}$ of BSA standards (0 to 2 mg/mL, steps of 0.2 mg/mL) in a 96-well microplate
- Dispense in duplicate $10\ \mu\text{L}$ of sample lysates in different wells of the same microplate
- Add $200\ \mu\text{L}$ of 50:1 Solution A:Solution B mixture to each well and incubate at 37°C for 30 minutes in the dark
- Read the 562 nm absorbance and, after correlating through known concentrations the standards and their absorbance value, calculate the concentration of the sample lysates

2.4.3 Radioligand saturation binding assay

The radioligand saturation binding assay allows the calculation of the affinity of a radiolabeled ligand for a receptor. The operative protocol was the following:

- Disaggregate the previously prepared membranes by passing them through a 25G needle using a 2 mL syringe
- Prepare an high capacity 96 well plate and add 5 μg of membrane protein per point, adding cold PBS to 75 μL final volume
- In Eppendorf tubes, make 75 μL /point of 4x concentrated radioligand ($[^{125}\text{I}]$ -Adrenomedullin, specific activity 17 Ci/mg, stock of 10 μCi) for each point of the dose-curve (values shown in Figure 5.7)
- Add 25 μL of radioligand to the plate
- Incubate plates on a shaker at room temperature at 600 rpm for 2 hours
- During incubation, pre-soak GF/C filters in a solution of 0.5% (w/v) PEI in distilled water
- Harvest the membranes and wash 2 times with 500 μL of cold wash buffer (300 mM HEPES, 6 mM CaCl_2 , 30 mM MgCl_2 , 3 M NaCl, pH 7.4)
- Dry the filters overnight at room temperature
- Put the filters into scintillation vials, label them, then add 3 mL of scintillation fluid
- Count the vials in a gamma counter

2.4.4 Western blotting

The transfer of macromolecules from gels to microporous membranes is referred to as “blotting”. The first blotting technique described was Southern blotting, by which electrophoresis-resolved DNAs were transferred from an agarose gel to a nitrocellulose membrane and hybridised with a radio-labelled RNA probe. The result of which was

then acquirable through the process of autoradiography (Southern, 1975). A similar process has been applied to RNAs, and named Northern Blotting as “geographically” opposed to Southern. Protein blotting evolved later in time and was named Western Blotting to maintain the “geographic” naming tradition.

Proteins are resolved through a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to their molecular weight. SDS is a detergent, and its presence gives all the proteins in the sample a negative charge, allowing them all to migrate towards the anode. The resolved gel is then juxtaposed with a membrane, usually nitrocellulose or polyvinylidene fluoride (PVDF), and put into a “sandwich” made of absorbent paper and sponges; the orientation of the gel and the membrane are such that the membrane is closer to the anode plate, allowing the transfer of the proteins to the membrane and not away from it. Subsequently unused binding sites on the membrane are blocked and the membrane stained with antigen-specific antibodies, whose Fc is later targeted by secondary antibodies that can be radiolabelled, enzyme-labelled or fluorophore-labelled, and the blotting can then be detected by autoradiograph, photosensitive film chemiluminescence or by scanning the membrane in an infrared detector.

Sample preparation for SDS-PAGE:

Membrane protein samples were prepared as follows:

- The protein was diluted to a final concentration of 2 mg/mL in TE membrane buffer
- The protein sample was then diluted in Laemmli buffer (5M urea, 0.17M SDS, 0.4M dithiothreitol, 50mM Tris-HCl, pH 8.0 and 0,01% (w/v) bromophenol blue) to a final concentration of 1 mg/mL
- The samples were boiled for 5-10 minutes
- 10-20 μ g of protein were loaded to each well of a precast SDS-PAGE protein gel (Mini Protean II, BioRad)

Cell lysate samples were prepared as follows:

- Cells from a 10 cm Petri dish were washed in 1x PBS after the appropriate treatment

- Cells were lysed in 500 μ L of ice cold RIPA buffer (50 mM HEPES, 150 mM NaCl, 1% (v/v) TritonX-100, 0.5% (w/v) Sodium Deoxycholate, 0.1% (w/v) SDS, 10 mM NaF, 5 mM EDTA, 10 mM NaH₂PO₄, 5% (v/v) Ethylenglycol, pH 7.3 + protease inhibitor cocktail (Roche))
- Cells were then rotated 20 min in an Eppendorf tube at 4°C
- The samples were centrifuged at 4°C for 10 minutes at 15,000 *g* and diluted 1:4 in Laemmli buffer
- The samples were then boiled for 5-10 minutes and 20 μ L loaded onto the gel

Samples were run at 200V 400 mA until the dye reached the bottom of the gel.

Following SDS-PAGE, proteins were electrophoretically transferred onto a nitrocellulose or PVDF membrane at 100V 400 mA in transfer buffer (0.2 M Glycine, 0.025 M Tris and 20 % (v/v) methanol). The membrane was then blocked for 1 hour in 5% (w/v) fat-free milk in 1x PBS-T (PBS + 0.2% (v/v) Tween-20) or pure Licor blocking solution. After 3 washes with 1x PBS-T, the membrane was incubated overnight at 4°C with an appropriate dilution of primary antibodies (goat anti-GFP (ABCAM, clone ab5450), mouse anti-hACKR3 (R&D Systems, clone 11G8) and rabbit anti-hCXCR4 (ABCAM, clone ab124824) were used according to the manufacturer indications) in 1x PBS-T containing 1% (w/v) fat-free milk or Licor blocking buffer (Licor Biosciences).

On the next day the primary antibodies were removed and the membrane was washed 3 times with 1x PBS-T, then incubated with the secondary antibodies diluted 1:10,000 for 2 hours at room temperature. The membrane was washed 3 times with 1x PBS-T and according to the secondary antibody labelling the blot was acquired on a photosensitive film if labelled with an HRP-labelled secondary, or its immunofluorescence was read if Licor secondary antibodies were used.

Chemiluminescence was detected using Pierce ECL Western Blotting Substrate (ThermoFisher Scientific). The substrate consisted in two solutions (Mix Detection Reagents 1 and 2) to be mixed 1:1 before applying to the membrane. The development of the photographic film (Kodak) by the reaction happening onto the membrane took place in a dark room inside a black box. Exposure times may vary according to the strength of the signal given by the bands.

For fluorescence detection method in Licor, IRDye secondary antibodies (Licor Biosciences) 800CW donkey anti-mouse and anti-goat and 680DR donkey anti-rabbit were used according to the manufacturer's instructions.

2.4.5 GTP- γ -³⁵S

GPCR activation involves the formation of a complex of ligand/receptor/G protein in which GDP bound to G protein is replaced by GTP, allowing the G $_{\alpha}$ subunit to dissociate from the G $_{\beta\gamma}$ heterodimer and activate downstream effectors. GTP bound to G $_{\alpha}$ is then hydrolysed to GDP to allow the G protein to reassemble and hence to be used again for signalling. GTP- γ ³⁵S (specific activity: 539.24 Ci/mol) is a GTP analogue whose third phosphate group is substituted by a dihydroxyphosphothiol, containing sulphur as a radioactive isotope. The advantage of using this GTP analogue is that the bond is resistant to hydrolysis and the lifetime of the nucleotide-bound GTP is longer. A dose response to agonist results in a sigmoidal curve which reveals the potency and efficacy of the agonist in G protein recruitment.

- GTP- γ -³⁵S buffer (HEPES 20 mM, MgCl₂ 5 mM, NaCl 160 mM, 0.05% (w/v) BSA) was prepared, and the water bath set at 30°C
- The ligand dilutions were prepared
- Glass tubes were prepared and labelled
- 60 μ L of GTP- γ -³⁵S buffer were added to each tube
- 20 μ L of ligand dilutions were dispensed into each tube
- Membranes were diluted to 0.5 μ g/ μ L, vortexed 3 times and 20 μ L of diluted membranes were added to each tube
- Assay mix (GTP- γ -³⁵S 0.1 nM (50 nCi), GDP 1 μ M in GTP- γ -³⁵S buffer) was prepared
- 100 μ L of Assay mix was added to each tube, the tubes were vortexed and covered with foil

- The tubes were incubated for 45 minutes in the water bath (30°C)
- During the incubation, ice cold 1x PBS was prepared, Whatmann paper filters were soaked in PBS and the harvester made ready
- The contents of each tube were harvested, together with 3 tube washes, onto the filters
- Filters were labelled and allowed to dry for at least 3 hours
- The dried filter discs were separated and put into scintillation vials
- 3 mL of scintillant fluid was added to each vial, the vials closed and the lids labelled
- The tubes were transferred to the TriCarb racks and put into the TriCarb scintillation counter (PerkinElmer) to count the radioactivity

2.5 In vivo

In vivo research is a fundamental process in the development of science, both basic and applied. Basic *in vivo* experiments allow us to better understand the mechanisms of physiological processes that are too complex to be studied *in vitro*, helping as well to provide proofs of concept about the roles of different macromolecules. Furthermore, drugs tested and validated *in vitro* need to proceed to *in vivo* models to have their pharmacokinetics and toxicity profile characterised before being subject to human safety trials.

Since these models are whole living organisms, *in vivo* experimentation is strictly regulated by the government and ethics committees in order to prevent procedures that might cause suffering without the support of a reasonable rationale behind.

2.5.1 C57BL/6 mice

C57BL/6, often called “C57” or “black 6”, refers to an inbred strain of mice widely used in labs all over the world. Reasons for the success of this model are the fact that they are bred easily, they are robust and a lot of congenic strains are available for crossings.

The strain C57BL/6 was created in 1921 at the Bussey Institute, and its genome was the second whole genome ever sequenced.

Subcutaneous tumour cells injection

Wild type C57BL/6 mice back skin was shaved the day before injections. Male and female animals were used, at least 12 weeks old (adults). Prior to injection, mice were anaesthetised with isoflurane, then 200,000 LLCs per mouse were injected directly beneath their dorsal skin.

Tumors were measured every day from when a visible blister became visible on the mice skin until their largest radius reached the length of 12 mm (in compliance to the project license). At the end of the growth, mice were subjected to Schedule 1 procedures and tumours were weighed.

All mice were housed in a 12-h light dark cycle with access to food (normal chow diet) and water ad libitum. All experimental procedures were performed in accordance with UK Home Office Guidance on the operation of the Animals (Scientific Procedures) Act (1986), the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (eighth edition) and institutional ethical approval (PLL number 70//8377; PIL number ID34B8D13).

2.6 Statistical analysis

All data were presented as mean \pm SEM of three or more independent experiments. All statistical analysis of data was conducted using the software GraphPad Prism 5.0. Assuming the normal distribution of the data, data were analysed either through two-tailed unpaired student’s t-test or one-way analysis of the variance (ANOVA) if the analysed groups were respectively two or more than two. Tukey’s or Dunnet’s multiple comparison test followed respectively t-tests or one-way ANOVAs to determine the level of significance between groups. Statistical significance was considered reached when p-values were less than 0.05.

Chapter 3

Results 1: Characterisation of ACKR3 and CXCR4 BRET sensors

Molecules active against either CXCR4 or ACKR3, especially blockers, can be developed virtually to contribute to the targeting of tumour growth and metastasis when used alongside current chemotherapeutic drugs. In order to develop drugs, a high-throughput screening assay whose main characteristics are that it is statistically robust, relatively cheap, rapid in terms of time and down-scalable are desirable.

For this project, BRET sensors for detecting either receptor-G protein or receptor- β -arrestin2 interactions were generated by molecular cloning, and the experimental conditions optimised for CXCR4 and ACKR3 responses. Furthermore, a BRET-based β -arrestin2 recruitment assay has been used to characterise the antagonism, the potency and the specificity of brand new anti-ACKR3 nanobodies. These nanobodies were synthesised and supplied by Vladimir Bobkov, ArgenX, Belgium.

3.1 Procedures and results

3.1.1 Generation of constructs

Sequence details of vector constructions have been described previously in Materials and methods. pcDEF3-hACKR3 and pcDEF3-3xHA-hCXCR4 were used as templates for PCR and restriction site sequences were introduced to both the extremities of the polynucleotide: in particular, *HindIII* was inserted 5' of the ATG start sequence, and

KpnI 3' after the last amino acid, making sure not to include the stop codon. Backbone vectors were pcDNA5/FRT/TO hGPR35 SPASM sensors or hGPR35-eYFP, from which hGPR35 was cleaved by endonucleases *HindIII* and *KpnI* and substituted with *HindIII*-hCXCR4(no-stop)-*KpnI* and *HindIII*-hACKR3(no-stop)-*KpnI*. Ligation products were then sent for sequencing to confirm the success in the construction of the vectors. All the sensors were produced both N-terminally FLAG-tagged and without a N-terminal tag (Figure 3.1).

The rationale behind the use of this sensor is schematically illustrated in Figure 3.2. Upon ligand treatment and in presence of the luciferase substrate, if the sensor carries the α_5 helix of the G protein elicited by said ligand, through BRET the fluorophore should get lit in a concentration-dependent manner.

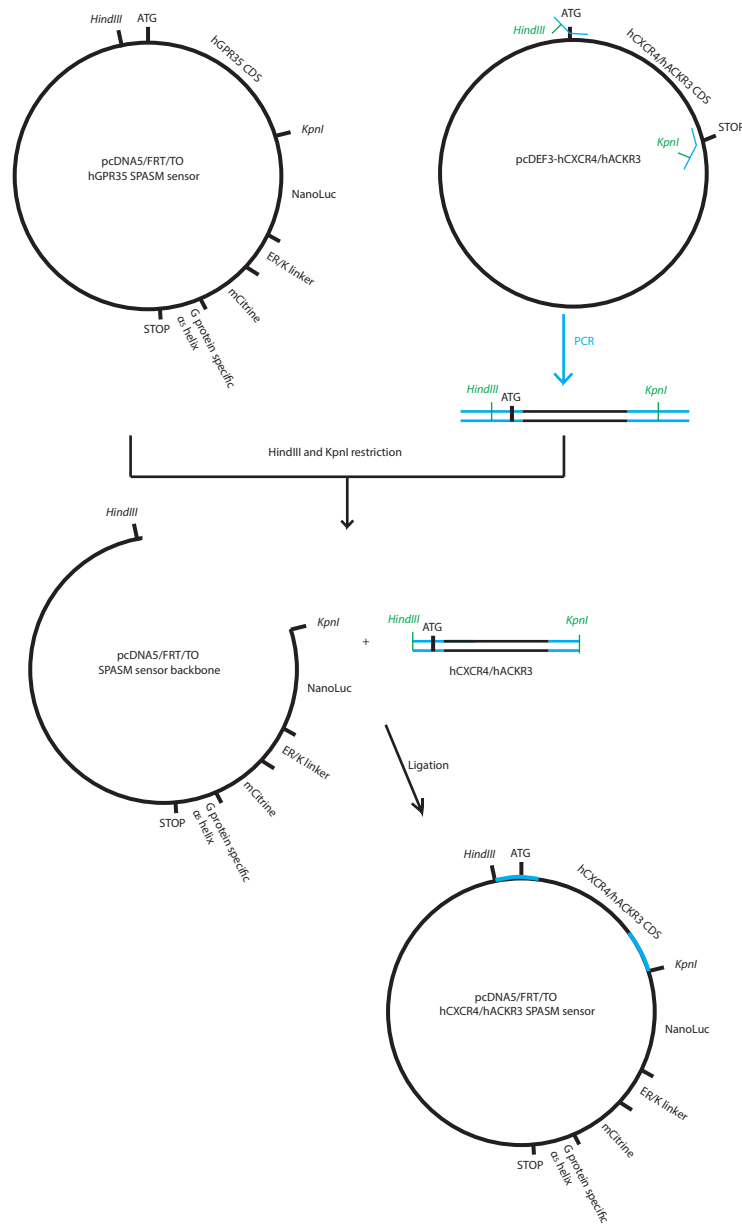


Figure 3.1: **Concept of SPASM vectors generation.** Both SPASM sensors and C-terminally eYFP-labelled receptors were produced from the same template by PCR, adding the restriction sites onto the primer and were then ligated into the SPASM vectors or the eYFP-carrying vectors respectively that had been produced previously for other receptors. The ligation products were validated by sequencing.

3.1.2 G protein-recruitment sensors development

The SPASM sensors were obtained from ready backbone vectors generated previously for different GPCRs by Dr. Brian Hudson. The list is in Appendix 2.

Even though hACKR3 has so far never been shown to elicit G protein-dependent responses upon stimulation, these sensors were created to investigate whether the observation of basal hACKR3-G protein interaction described in the work of Levoye et al (Levoye et al., 2009) could be shown in this system, which could then constitute an interesting target for drugs.

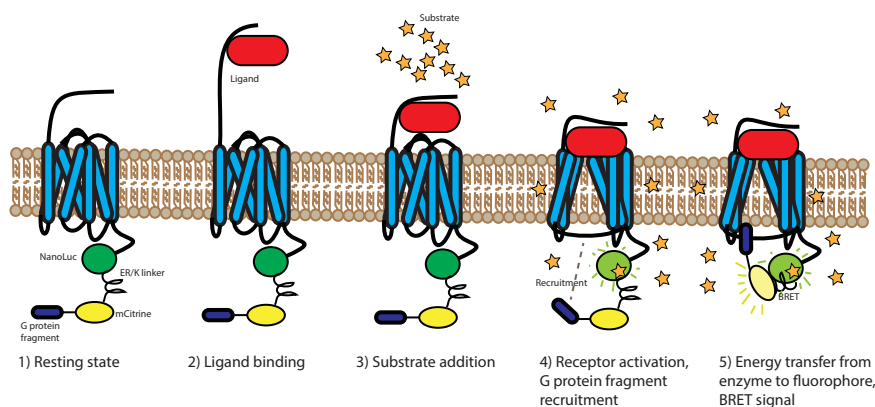


Figure 3.2: **Schematics of SPASM sensor function.** Upon ligand binding and substrate addition, if the G protein of interest gets recruited to the activated receptor mCitrine and NanoLuciferase can generate a BRET signal.

The constructs were used to generate stable cell lines using Flp/In T-REx 293 cells, by a co-transfection 1:1 of pOG44 (which encodes for FLP recombinase) and the pcDNA5/FRT/TO SPASM sensors, followed by selection in blasticidin S HCl and hygromycin B medium. The protocol dictates a selection timeframe of 20-30 days, during which fresh antibiotic-containing medium should be used to replace the old medium every third day. After this month-long selection process, the cells were maintained under selective conditions and were used for the experiments.

Once obtained, the cells were validated for expression and correct membrane localisation of the receptors: the cells were indeed lysed and the whole lysates run on an SDS-PAGE gel to separate the proteins according to their molecular weight. The membranes were then stained with goat anti-GFP primary antibodies, which were targeted with an infrared dye-labelled donkey anti-goat secondary antibody (Figure 3.3).

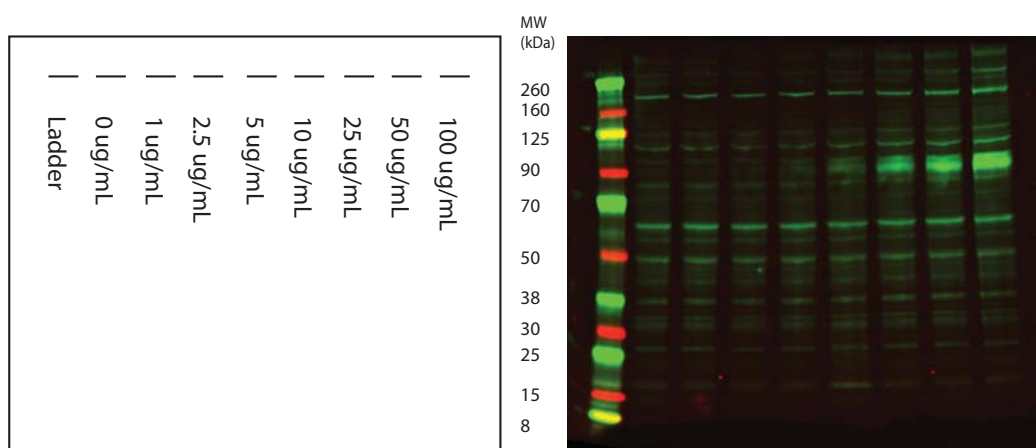


Figure 3.3: **Doxycycline titration curve.** Stable transfectants of hCXCR4-G_{α1/2} SPASM were treated with increasing concentrations of doxycycline. The sensor has approximately a molecular weight slightly higher than the 90 kDa band, which is compliant with the theoretical molecular weight calculation of 101.706 kDa. Panel A shows the loading order of the lysates in the SDS-PAGE, Panel B shows the Western Blot obtained from a single experiment.

The stable transfectants were grown on coverslips and induced with 50 $\mu\text{g}/\text{mL}$ of doxycyclin. The fluorescence of mCitrine was detected by fluorescence microscopy using a

Zeiss inverted microscope. (Figure 3.4).

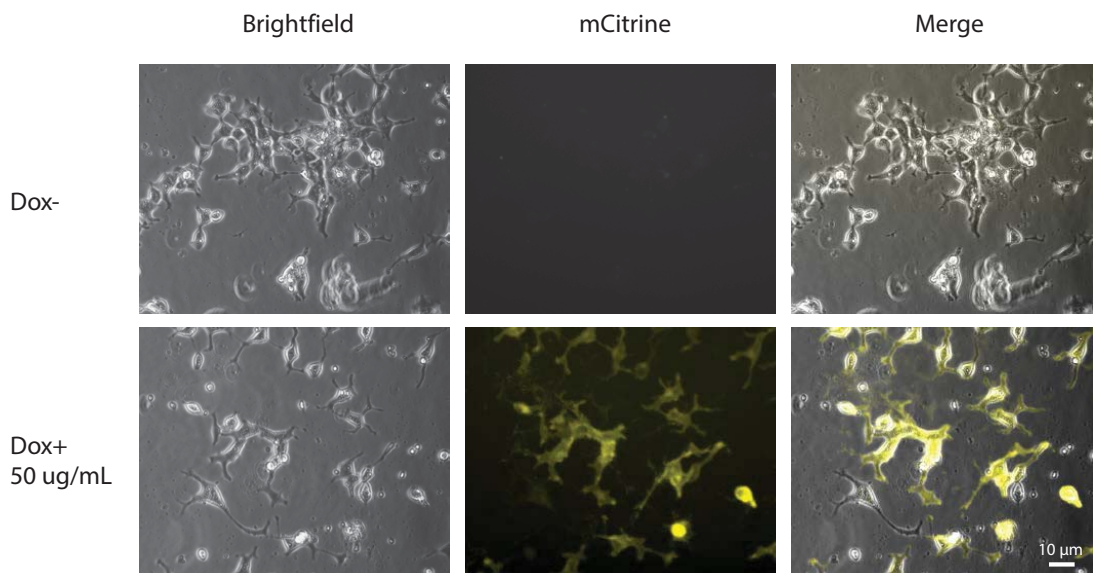


Figure 3.4: **SPASM sensor expression assessment by fluorescence microscopy.** Fluorescence microscopy of stable transfectants upon doxycyclin-induced SPASM sensor expression. mCitrine showed a strong signal only in doxycyclin-treated cells. Representative images of 3 adjacent optic fields from one experiment.

Thus the expression of the transfected proteins was validated, so a first BRET screening using the protocol that worked for GPR35 was performed (Figure 3.5). In order not to waste reagents, the assay was performed on hCXCR4- $G_{\alpha_{i1/2}}$ and hCXCR4-nopeptide SPASM sensors only, as the literature describes clearly hCXCR4 as the only receptor involved in this study able to recruit G proteins, and that most of the signal is transduced by G_{α_i} family proteins.

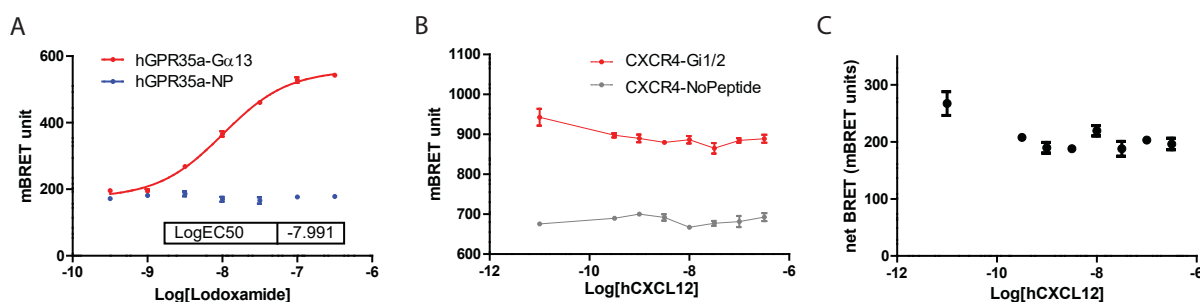


Figure 3.5: Panel A: concentration-response curve of hGPR35a-G $_q$ SPASM to Lodoxamide. The protocol used with this sensor was applied in Panels B and C to hCXCR4 SPASMS (G $_{\alpha i1/2}$ and NoPeptide) upon stimulation with 500 nM hCXCL12 (EC $_{80}$). Far left point in each panel represent the vehicle-treated condition; the plots show the average of three independent replicates (n=3).

Initial screening made it clear that further optimisation was needed.

The kinetics of the sensors were then investigated. A time frame of 30 minutes with 5 minutes intervals was considered, showing that the G $_{\alpha i1/2}$ recruitment seemed to have a peak after 10 minutes upon ligand addition (Figure 3.6).

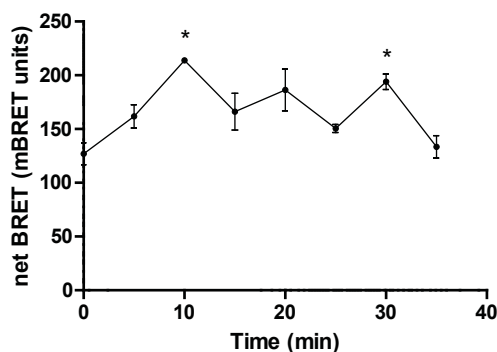


Figure 3.6: **Kinetic G $_{\alpha i1/2}$ protein recruitment to hCXCR4.** Cells transfected with hCXCR4-G $_{\alpha i1/2}$ SPASM sensor were stimulated with 500 nM hCXCL12 for different amounts of time. The plot obtained showed two significant peaks compared to time zero (basal activity), the earlier 10 minutes after the stimulation, the later at 30 minutes. Statistics were calculated using Student's t-test; biological triplicates were run (n=3). *: p<0.05 .

G $_{\alpha i1/2}$ recruitment was therefore investigated after 10 minutes stimulation, but the dif-

ference between the BRET ratios of chemokine-treated and -untreated samples did not differ significantly (Figure 3.7).

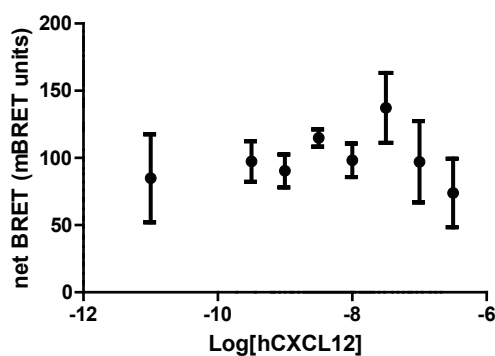


Figure 3.7: Concentration-response curve of $G_{\alpha i1/2}$ protein recruitment to hCXCR4 after 10 minutes stimulation with 500 nM hCXCL12. Three independent experiments were run ($n=3$). A curve could not be fitted by the software as the points do not converge. Far left point represents the vehicle-treated condition.

$G_{\alpha i}$ protein activation is sensitive to pertussis toxin (PTX), a toxin secreted by the gram-negative bacterium *Bordetella pertussis*. When PTX crosses the plasma membrane, its subunit A catalyzes the ADP-ribosylation of $G_{\alpha i}$ locking the G protein in their inactive state (GDP bound) and preventing its activation. Since the sensors lack the guanosine nucleotide binding site, PTX treatment would impair only native G protein recruitment. The rationale for treating with PTX was that, since almost 30% of total G_{α} protein in HEK293 derived cell lines belongs to the $G_{\alpha i}$ family, the BRET ratio would become higher if only the linked peptide was able to interact with the receptor.

PTX treatment was performed but the $G_{\alpha i}$ sensor and the no-peptide control did not show any significant difference in their activation (Figure 3.8).

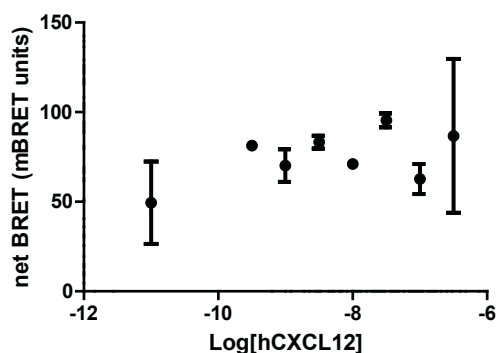


Figure 3.8: Concentration-response curve of $G_{\alpha i1/2}$ protein recruitment to hCXCR4 after 10 minutes stimulation with 500 nM hCXCL12 following pretreatment with PTX. Three independent experiments were run ($n=3$). A curve could not be fitted by the software as the points do not converge. Far left point represents the vehicle-treated condition.

To further enhance the BRET ratio, the receptors were cloned into sensors expressing mNeonGreen (mNG) instead of mCitrine, as mNG has a higher Quantum Yield (QY) (0.8 of mNG versus 0.72 of mCitrine), meaning that the photon transmission efficiency (QY) is higher (0.8 photons are emitted from mNG for every photon that excites it), as well as an higher brightness (92.8 of mNG versus 49.68 of mCitrine). Stimulation was applied to whole cells, PTX-treated whole cells, crude membranes and PTX-treated crude membranes. However, the results were as before with no significant difference from the non-peptide sensor (Figure 3.9).

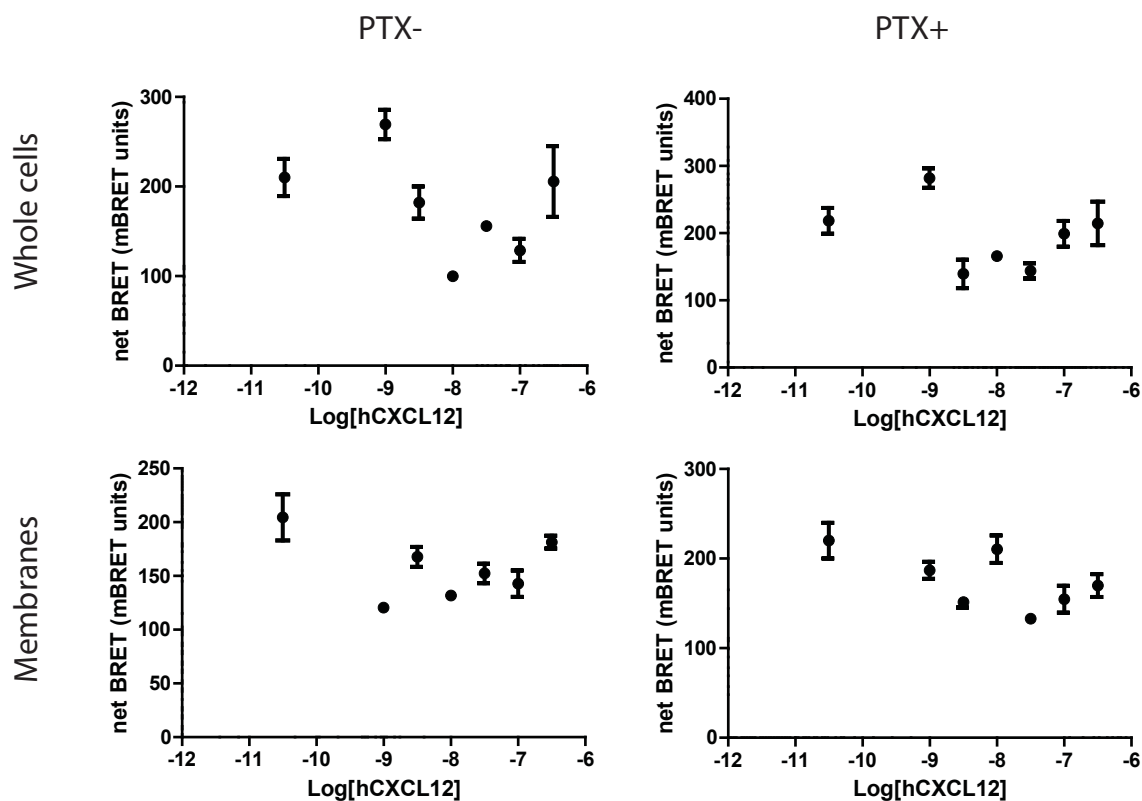


Figure 3.9: Concentration-response curve of $G_{\alpha i1/2}$ protein recruitment to hCXCR4 after 10 minutes stimulation with hCXCL12. The mNeonGreen SPASM sensors were tested both as whole cell transfectants and as crude membranes, in presence of PTX or not. Three independent experiments were run ($n=3$). Curves could not be fitted by the software as the points do not converge. Far left point of each panel represents the vehicle-treated condition.

At this point, the suspicion arose that the sensor was not working because of the receptor functionality itself, even though the sequencing showed that the cloning did not in any way alter its cDNA.

To rule out this possibility, GTP- $\gamma^{35}\text{S}$ G protein recruitment assays were performed as described in Materials and Methods. Upon stimulation, both the $G_{\alpha i1/2}$ and the non-peptide sensor showed activation downstream of hCXCR4-hCXCL12 interaction (Figure 3.10).

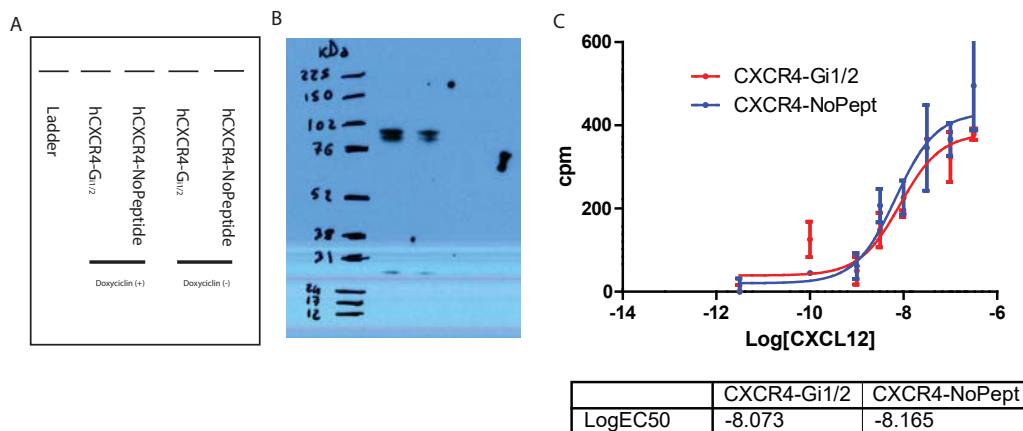


Figure 3.10: The expression and functionality of hCXCR4 in transduced cell lines were assessed by western blot and GTP- $\gamma^{35}\text{S}$ assay respectively. Panel A illustrates the loading order of the samples in the gel: the samples that actually expressed the transfectants, namely the doxycyclin-induced, have been successfully detected by the western blot antibodies anti-GFP (Panel B). Panel C shows the net concentration-response curve for G protein activation upon treatment with hCXCL12. Since both the $G_{\alpha i1/2}$ and the control SPASM sensor encode for hCXCR4 and express it, both the samples behave similarly showing extremely close potency values. Far left point in Panel C represents the vehicle-treated condition; the plot represents the average of three independent replicates ($n=3$).

3.1.3 β -arrestin2 recruitment assay

While hCXCR4 is the only receptor, of those involved in this project, that initiates a G protein response, both hCXCR4 and hACKR3 are described as being able to recruit β -arrestins.

To assess β -arrestin2 recruitment to the receptor upon stimulation, RLuc6- β -arrestin2 was used as BRET donor, and the receptor constructs hCXCR4-eYFP and hACKR3-eYFP were used as luminescence acceptors. Because of Resonance Energy Transfer, if receptor-eYFP construct stimulation results in β -arrestin2 recruitment, the enzyme and the fluorophore come into close proximity, in presence of RLuc6 substrate Coelenterazine-h, the enzyme itself emits light, and the energy of its emitted photons can then excite an adjacent eYFP molecule, generating BRET signals that can be calculated as ratio between eYFP fluorescence and RLuc6 luminescence (Figure 3.11).

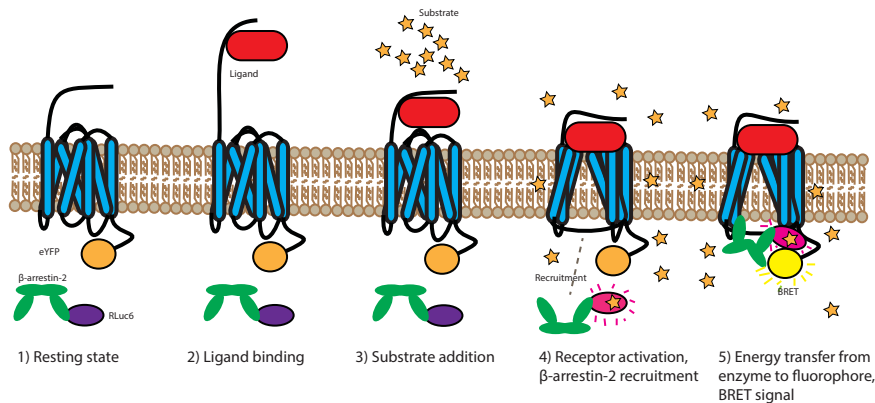


Figure 3.11: **Schematics of β -arrestin2 recruitment principle.** Upon ligand binding and substrate addition, if β -arrestin2 gets recruited to the activated receptor, eYFP and RenillaLuciferase can generate a BRET signal.

hCXCL12 was first interrogated for β -arrestin2 recruitment, in order to develop and optimise the assay if needed. Kinetic curves were first obtained with a concentration of 500 nM of each chemokine ligand (EC_{80}) (Figure 3.12 Panel A). Five minutes after the stimulation with hCXCL12 the BRET signal reached the plateau, meaning that this could be used as a reliable timepoint for the assay. Analogously, the inhibition of hCXCL12 signal by 200 nM (IC_{80}) AMD3100 gave information that the 5 minutes timepoint could abrogate to basal values the β -arrestin2 recruitment extent (Figure 3.12 Panel B).

Similar conclusions could be drawn using the same concentrations of hCXCL12 (Figure 3.13 Panel A) and hCXCL11 (Figure 3.13 Panel B) on hACKR3.

In conclusion, this assay was used to characterise the potency of the following lig-

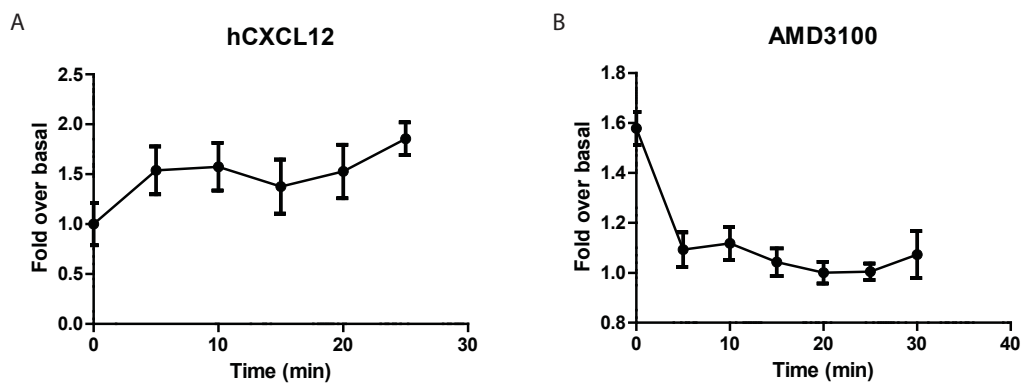


Figure 3.12: β -arrestin2 recruitment kinetics were run for hCXCR4 using hCXCL12 (Panel A) and hCXCL12 inhibited by AMD3100 (Panel B) as ligands. The plots represent the average of three independent replicates (n=3).

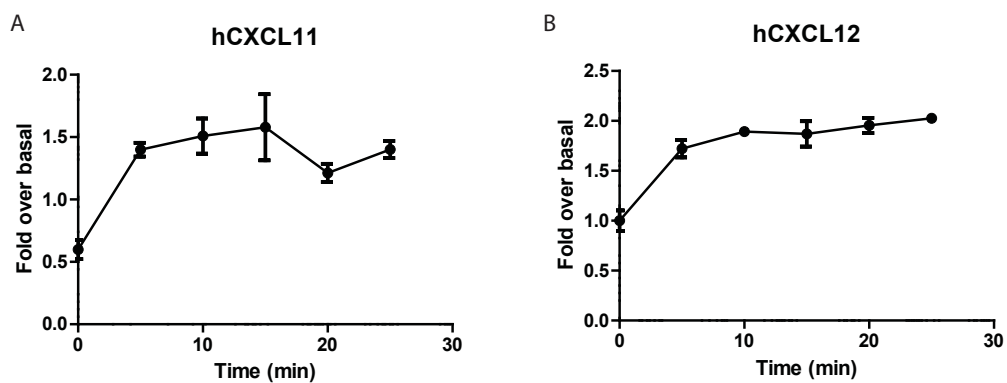


Figure 3.13: β -arrestin2 recruitment kinetics were run for hACKR3 using hCXCL11 and hCXCL12 as ligands. The plots represent the average of three independent replicates (n=3).

ands: hCXCL12 against hCXCR4 and hACKR3, AMD3100 against hCXCR4, hCXCL11 against hACKR3, VUF11207 against hACKR3, VUF11403 against hACKR3 (Figure 3.14).

VUF11207 and VUF11403 were two small molecules gently gifted by Prof. Dr. Martine Smit which bind selectively hACKR3 with an high affinity, used to further assess the robustness of the assay.

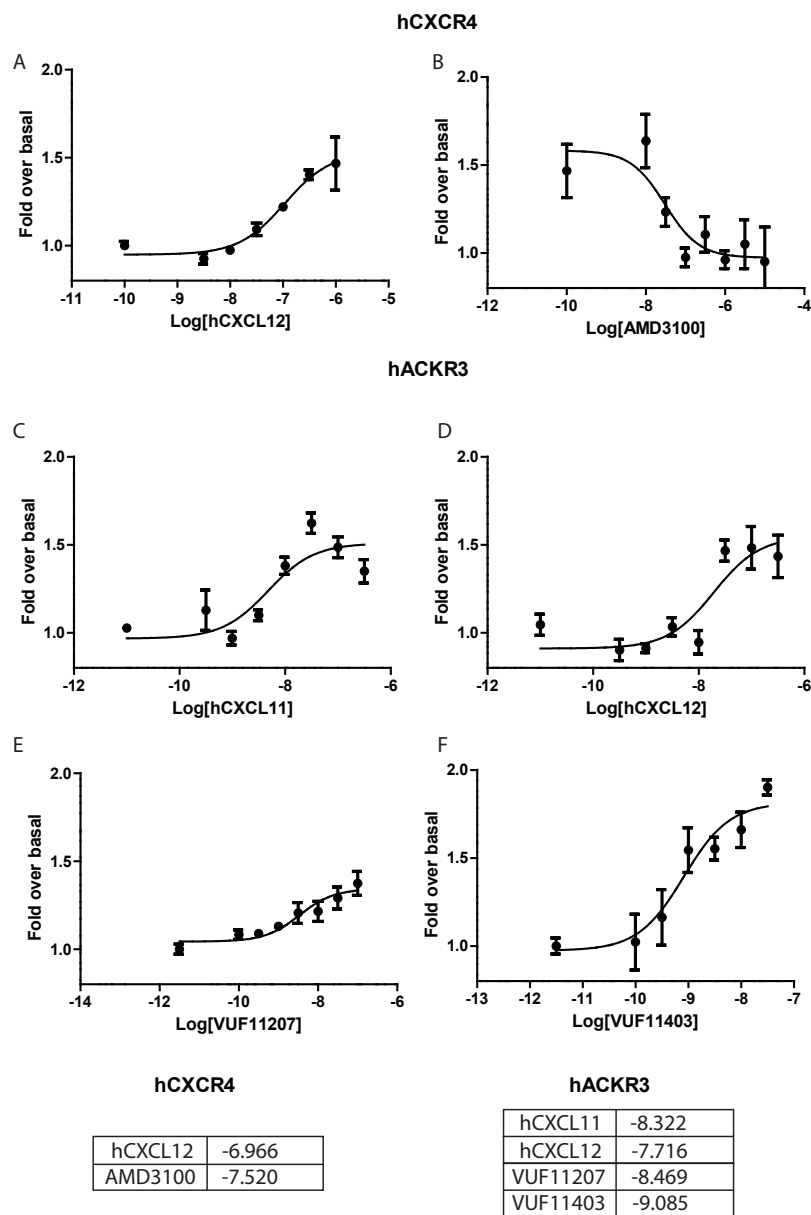


Figure 3.14: β -arrestin2 recruitment assays were performed using both hCXCR4 (A-B) and hACKR3 (C-D-E-F), observing an increase (or decrease for AMD3100) in the BRET signal in a concentration-dependent manner. Far left point in each Panel represents vehicle treated condition. All the plots in the Panels represent the average of three independent replicates (n=3); potencies in the tables are expressed as LogEC_{50} .

3.1.4 ACKR3 nanobody screening

Nanobodies, or single domain antibodies, are 12-15 kDa proteins able to selectively bind antigens. Called also V_HH because of their lack of antibody light chain, they were first characterised in camelids. Due to their small size, they have several basic and translational advantages: they are more simple to manufacture in large amounts, they are chemically more stable than full antibodies and hence can be stored under less strict conditions, for longer, and can be delivered to targets in patients without the need for injection into the bloodstream. In addition they have a longer and more flexible CDR3 region that allows them to reach cryptic epitopes or even enzyme active sites and finally the bare V_HH is safer as the absent Fc is not able to trigger any Fc-Receptor-dependent immune reaction (Jahnichen et al., 2010).

The industrial partner of this project is a company named ArgenX BVA (Zwijnaarde, BE), whose main manufactured products are nanobodies targeting GPCRs, and hACKR3 is a target in their pipeline.

The nanobodies received from the company were both V_HH and bivalent nanobodies (two V_HH linked together by a human Fc) characterised for hACKR3 targeting action, potency and specificity.

All the clones were found to be neutral antagonists (Figure 3.15 Panel D), and their potency against hACKR3 was characterised.

V_HH (Figure 3.15 Panel B) and bivalent nanobodies (Figure 3.15 Panel C) showed good neutral antagonistic activity on hCXCL12-induced β -arrestin2 recruitment to hACKR3, with similar affinity to the receptor of the two forms.

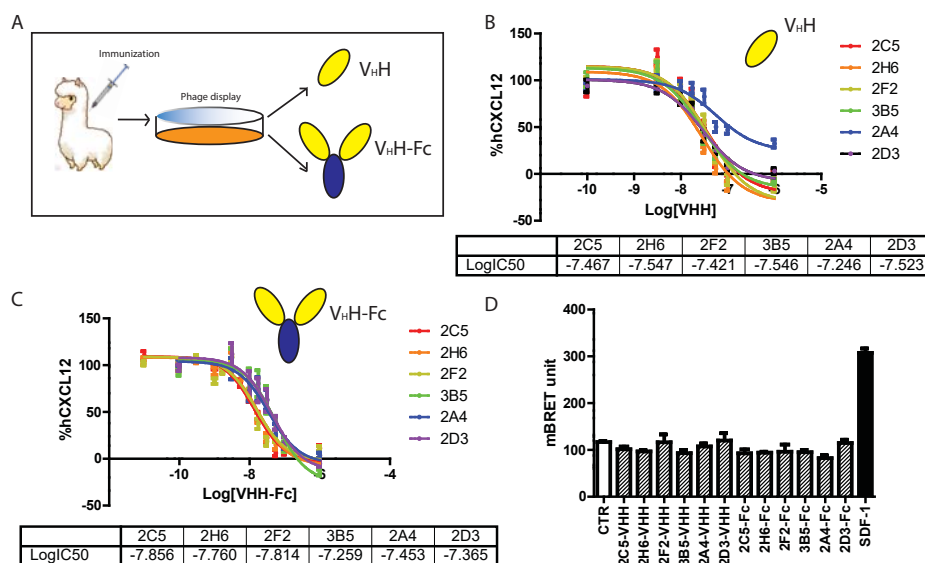


Figure 3.15: Panel A illustrates the nature of the engineered llama immunoglobulins used in this screening. Both V_HH and V_HH-Fc clones come from optimization phage display rounds: the former are purified as nanobodies, the latter as bivalent nanobodies genetically linked to a human crystallizable fragment. The different clones were named with a code, i.e. 2C5, and their bivalent form consist of two copies of the same nanobody expressed on a human Fc scaffold. Panels B and C were obtained challenging with different concentrations of the nanobodies 500 nM (EC₈₀) of hCXCL12. Panel D instead showed that the effect of 1 μM (saturating) concentration of each nanobody construct alone against hCXCR4 doesn't elicit any β-arrestin2 recruitment to hACKR3. Panels B, C and D show the average of five independent replicates (n=5). Far left point of Panel B and C represent the vehicle-inhibited condition.

3.2 Discussion

Although different rounds of optimisation were tried, hCXCR4 SPASM sensors did not show any significant BRET signal upon treatment with hCXCL12. The final goal foreseen for SPASM sensor use was ambitious, since if they managed to give a significant signal they might have been used for further studies. For example the study of biased signalling, namely to understand the priority that a particular downstream pathway has upon receptor stimulation, which has been shown to be ligand-dependent in many GPCRs. Furthermore, by introducing mutations which abolish such interactions on each receptor, heteromerisation could have been studied functionally.

β -arrestin2 recruitment instead showed a high reproducibility of the results, which were generally robust. The optimisation of this assay did not require particular effort. The assay can be easily miniaturised to 384-well plate (not shown), making it an even more highly cost-effective signalling output assay.

All the ligands of hACKR3 and hCXCR4 were responding with potencies against the receptors consistent with literature values (Figure 3.16).

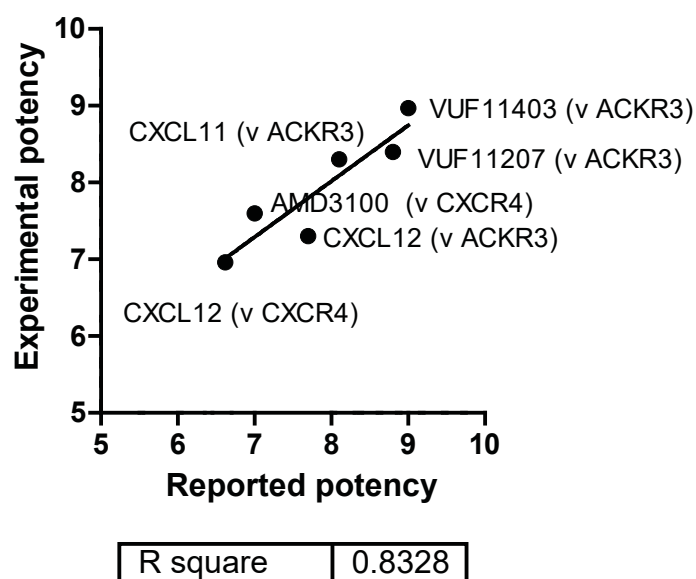


Figure 3.16: Comparison between experimental and reported potencies. The values obtained experimentally correlate positively with the potencies described in the literature for the ligands used in this project, highlighting the suitability of the experimental methodology. References for ACKR3: CXCL11 (Benredjem et al., 2017), CXCL12 (Gravel et al., 2010), VUF11207 (Wijtmans et al., 2012), VUF11403 (Wijtmans et al., 2012). References for CXCR4: CXCL12 (Gravel et al., 2010), AMD3100 (Charo et al., 2019)

During the thesis project, β -arrestin2 recruitment was used to study adrenomedullin interaction with the receptor hACKR3, and all the characterisation relative to this ligand will be described thoroughly in Chapter 5. Further, the kinetics of agonist-induced β -arrestin2 recruitment (5' timepoint) and inhibitor-induced signal blockade (30' timepoint) were employed as well for the investigations described in Chapter 5.

The reliability of β -arrestin2 assay was essential for the characterisation of the nanobodies, that together with radioligand displacement data (not shown) obtained at VU Amsterdam (NL) contributed to the choice of the clone 2C5 as the best candidate. Subsequently its production has been upscaled and it has been re-named VUN700.

Chapter 4

Results 2: Generation of CRISPR ACKR3-KO in Jurkat cells and LLC cells

To gain a better understanding of the implications of the role of ACKR3 in CXCR4 biology, functional studies are crucial. Jurkat T cells were chosen as the model for these experiments, as they are well described for their expression of both hCXCR4 and hACKR3, and for their extensive characterisation in chemotaxis assays.

Indeed, in the first part of this chapter chemotaxis was used as the final readout for the study of the impact of hACKR3 on hCXCR4-dependent migration, in an attempt to define physiologically the pharmacological effects that a specific inhibitor of hACKR3 would have on the motility of tumour cells showing upregulation of both of these receptors.

This aim was pursued thanks to the employ of the genome editing technique CRISPR, that allowed the generation of hACKR3 Knock-Out (KO) clones and their subsequent use in transwell chemotaxis wells.

The second part of this chapter was dedicated to *in vitro* characterization of mACKR3-KO LLC cells, and their subsequent use *in vivo* to study the impact of mACKR3 in tumour growth.

The experiments shown in this chapter were carried on in collaboration with Angeliki Karatza (University of Glasgow), and as such these results are shared.

4.1 Procedures and results

4.1.1 Jurkat T cells and cell migration

Jurkat cells were first characterised for hACKR3 expression by performing western blots and comparing the expression to that seen in THP-1 cells, also described as expressing hACKR3. The latter cell line was used as a positive control, namely to understand whether any eventual negative results were due to antibodies that cannot detect the receptors from cell lysates. Both cell lines express both receptors (Figure 4.1) as described in the literature (Ottoson et al., 2001), (Melo et al., 2014), (Tarnowski et al., 2010). This is also true of the relative abundancy of hACKR3 on Jurkat cells compared to THP-1 (Tarnowski et al., 2010).

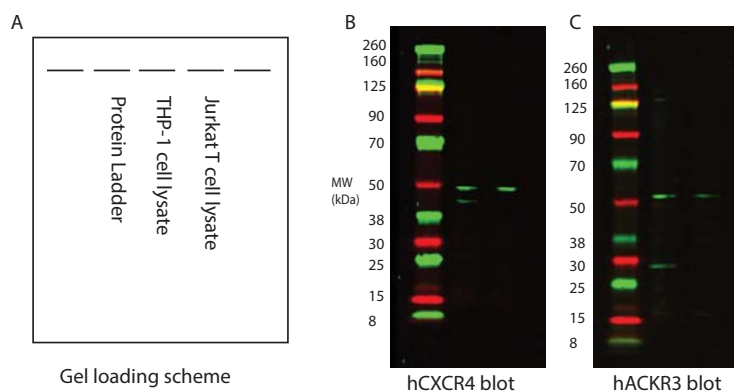


Figure 4.1: Assessment of the expression of hCXCR4 and hACKR3 in Jurkat and THP-1 cells. 5 μ g of THP-1 or Jurkat cell lysates were loaded as shown in Panel A and resolved by SDS-PAGE, and subsequently transferred onto nitrocellulose membranes. Anti-hCXCR4 (Panel B) and Anti-hACKR3 (Panel C) antibodies (see Materials and Methods) were used to detect the expression of these receptors. Representative immunoblot of 3 independent experiments.

Having confirmed that the cell line of choice was compliant to its description in the literature, the next step was to eliminate the expression of hACKR3 from these cells to assess how this would modify the function of hCXCR4.

To obtain this CRISPR technique was chosen. Since all the genome-editing techniques require the genome to be reached by whatever is supposed to edit it, and CRISPR does

not make exception, transfection of the target cell population needed optimisation. Lipofectamine-LTX and electroporation are the most used transfection techniques on Jurkat cells, so they have been compared prior the use of the most suitable on actual samples and subsequent genome editing. Rounds of optimisation using empty px461 D9A Cas9-GFP vector resulted in the choice of electroporation over Lipofectamine-LTX, a low toxicity transfection reagent that killed only half of the transfected cells (versus almost 70% that electroporation killed), but left only 4.5% cells efficiently transfected against the 41% achieved by electroporation. In absolute terms also electroporation was shown to be more efficient than Lipofectamine-LTX, as the latter gave 2% final efficacy, against the 11% of the former (Figure 4.2).

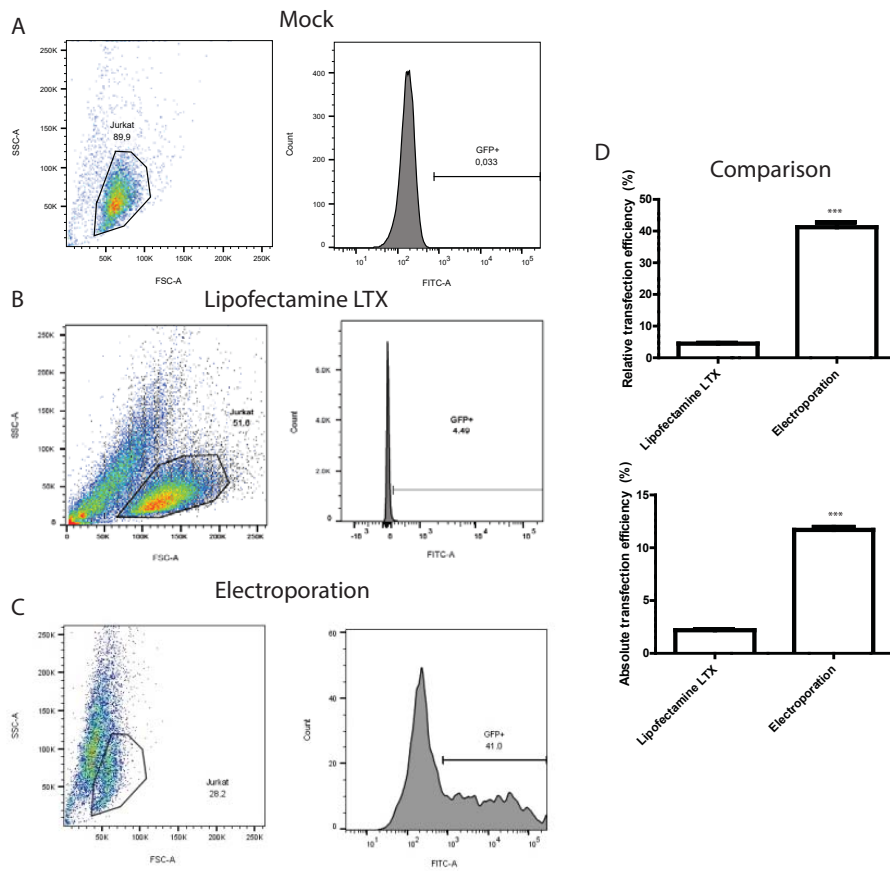


Figure 4.2: Transfection efficiency comparison between electroporation and Lipofectamine LTX on Jurkat cells. Empty plasmid px461 D9A Cas9-GFP was used to compare the two transfection techniques. Mock transfection (Panel A) helps to determine where to set, on the histogram plot, GFP negativity. Forward- (FSC) and side-scattered (SSC) light, which correspond to the physical parameters cell size and cell granularity respectively, help to establish that Lipofectamine-LTX (Panel B) is milder on cell viability than electroporation (Panel C), killing only half of the sample versus the 70% achieved by the latter. Once the population is selected though, the FITC (Fluorescein-Iso-Thio-Cyanide) channel for reflected light, namely the channel in which the intensity of GFP fluorescence is detected, showed that the amount of plasmid that permeated the membrane helped by Lipofectamine-LTX was almost one order of magnitude inferior than what was achieved by electroporation. Electroporation indeed yielded an outstanding efficiency rate both relative only to live cells as well as in absolute terms (Panel D). The experiment was repeated three times with similar results, and a single representative FACS plot is shown for each condition. Student's t-test was used for the statistical analysis; ***: $p < 0.001$

Chapter 4. Results 2: Generation of CRISPR ACKR3-KO in Jurkat cells and LLC cells

Guide RNAs were then designed (Figure 4.3), using the Zhang lab guide design tool (Zhang, n.d.), and cloned into the px461 D9A Cas9-GFP (Nickase) vector.

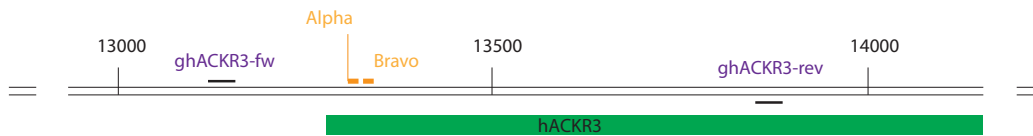


Figure 4.3: Rationale behind the design of the guide RNAs (Alpha and Bravo) and the genomic primers (ghACKR3 for1 and ghACKR3 rev1) on the hACKR3 gene. The software detected a suitable region with two close PAMs straight downstream of the start codon, and the primers were designed in order to be more than 100 bp from the double nick site. Further detail to CRISPR theory and nomenclature is provided in section 1.3 of the Introduction chapter and in subsection 2.3.3 of the Materials and Methods chapter. On the gene, the elements depicted map as described: ghACKR3-fw: 13,142 - 13,161 ; ghACKR3-rev: 13,700 - 13,719 ; Alpha: 13,313 - 13,332 ; Bravo: 13,342 - 13,361

Transfected cells were single-cell sorted by BD FACSAria II for the presence of GFP into 96-well plates, and the surviving clones were screened for genomic editing in proximity of the hACKR3 gene (Figure 4.4).

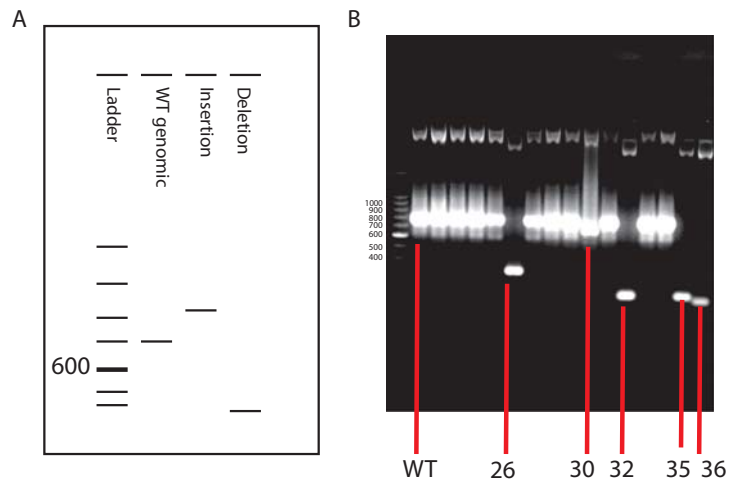


Figure 4.4: **Representative genomic PCR screening.** Since the distance between the two genomic primers is some 600 nucleotides, a double strand break in between would result in non homologous end joining events, which can lead both to random insertions (longer fragments in the gel) or deletions (shorter fragments) (Panel A). Panel B shows the gel from which Clones 26 and 30 were picked (ordered, from the left to the right, from clone 22 to clone 36). Most of the clones were still bearing the wild-type allele, while five clones showed a fragment not aligned with the wild-type control. Clones 32, 35 and 36 died in the selection process.

Three clones were identified as bearing biallelic deletions by fragment sequencing after TA cloning, namely clone 9, clone 26 and clone 30 (Figure 4.5).

Chapter 4. Results 2: Generation of CRISPR ACKR3-KO in Jurkat cells and LLC cells



Figure 4.5: Sequencing results of the genomic amplification fragments. Clones 9, 26 and 30 showed biallelic deletion of the hACKR3 gene in proximity to the region delimited by the guides. The blue box indicates in each allele where the premature stop codon occurs.

The resulting sequences were then translated to assess whether premature stop codons were generated by the deletions (Figure 4.6).

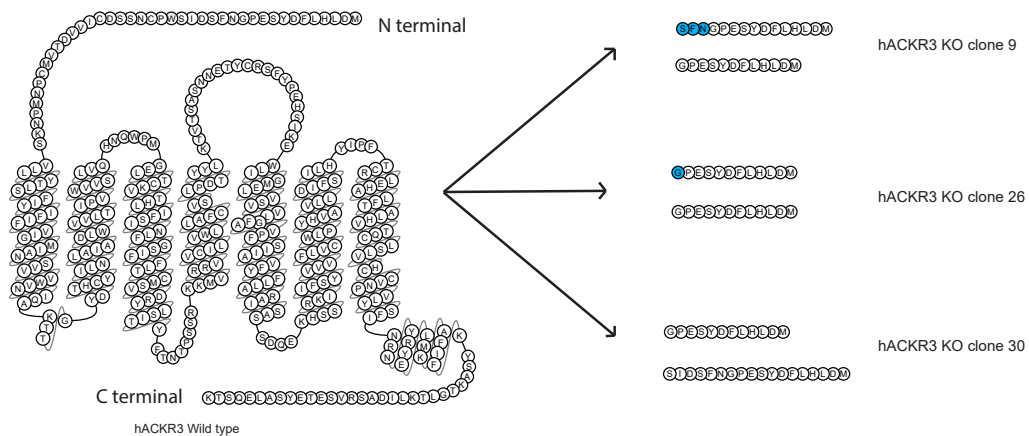


Figure 4.6: Translation of the sequencing results showed that both the alleles in all the three clones resulted in a frameshift that led to the occurrence of a premature stop codon 12-18 amino acids after the methionine start codon. The residues colored in blue highlight the presence of sense mutations.

The clones were then labelled with PE-anti-hACKR3 and APC-anti-hCXCR4, to assess whether any alteration of hCXCR4 expression occurred as a consequence of hACKR3 expression abrogation. Figure 4.7 showed that it did not.

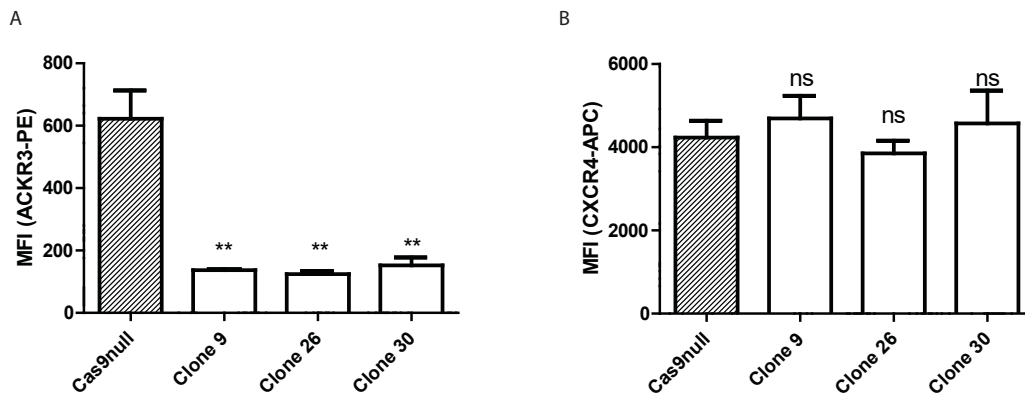


Figure 4.7: Expression of hACKR3 and hCXCR4 in Jurkat cells after CRISPR. Genome editing revealed itself successful also in flow cytometry, as the expression of hACKR3 in the KO clones significantly dropped (Panel A). Nevertheless, hCXCR4 did not show any sign of variation (Panel B), allowing for the statement that any functional difference shown later depends almost exclusively from hACKR3-deficiency. Control was defined Cas9null, which means that underwent the same transfection conditions as KO clones but with an unloaded Cas9. Statistics of three independent replicates (n=3) for each clone were assessed using Student's t-test. **: $p < 0.01$ relative to Cas9null, ns: non significant.

The three clones were then characterised functionally for chemotaxis towards hCXCL12. As a control, Jurkat cells which underwent electroporation with an empty Cas9-GFP plasmid (Cas9 null controls) were employed (Figure 4.8).

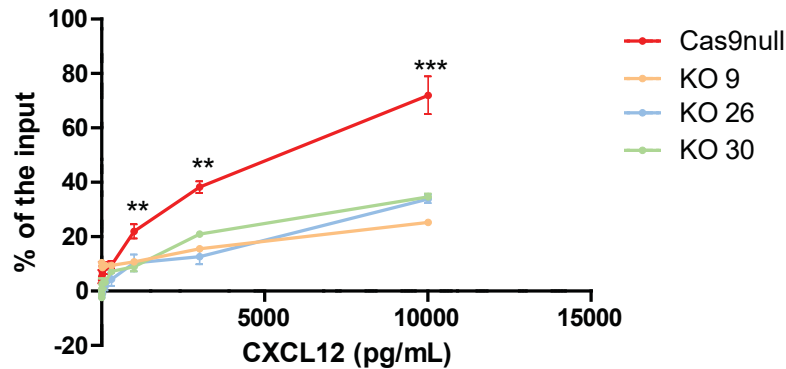


Figure 4.8: Transwell chemotaxis of the three Jurkat hACKR3-KO clones. All the clones showed a reduction in the maximal response to hCXCL12 chemotaxis compared to a Cas9 null control. Migrated cells were plotted as percentage of the input, namely the amount of cells put into the transwell at the beginning of the experiment. Statistics of three independent replicates (n=3) for each clone were assessed using Student's t-test. **: $p < 0.01$, ***: $p < 0.005$. P values are relative to vehicle control (0 pg/mL hCXCL12).

These three clones were then pooled as their migratory behaviour showed consistency, and migration assays in the presence of the hCXCR4 inhibitor AMD3100 (Plerixafor) were performed (Figure 4.9).

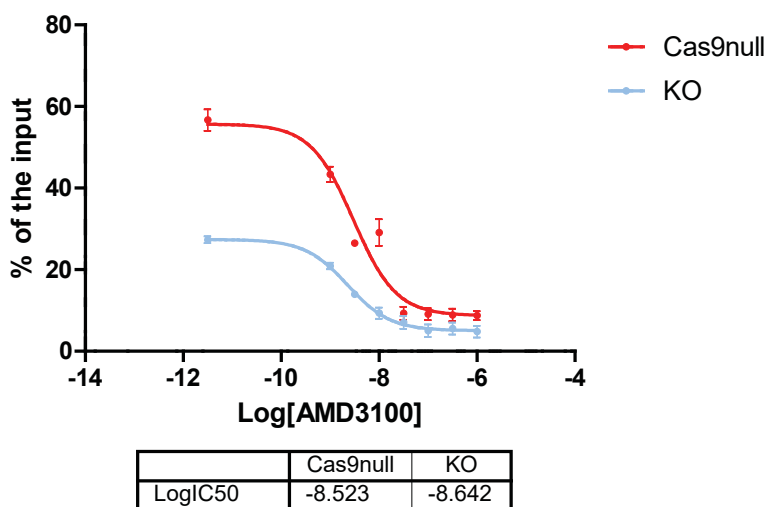


Figure 4.9: The three clones listed in Figure 4.7, namely 9, 26 and 30, were pooled and assayed for AMD3100-inhibited CXCL12-driven chemotaxis. The inhibitor showed equal potency in both hACKR3-KO and Cas9-null cells, indicating that the affinity of AMD3100 for hCXCR4 is not affected by the expression of hACKR3. The curves were obtained using non linear regression assuming a Hill coefficient equal to 1. The average of three (n=3) independent replicates is shown.

4.1.2 LLCs and tumour growth

LLC cells are broadly described to express mACKR3 but not mCXCR4 (Nian et al., 2011), therefore they were chosen as a model to study the role of mACKR3 in tumour growth in a mCXCR4-free system.

The cells showed a good transfection efficiency upon lipofection (not shown). CRISPR guides design and insertion into a px461 D9A Cas9-GFP (Nickase) vector followed the same workflow used for Jurkat cells. Transfected LLCs were single cell-sorted for GFP positivity into 96-well plates, and the surviving clones were screened for genomic editing in proximity of mACKR3 gene (Figure 4.10).

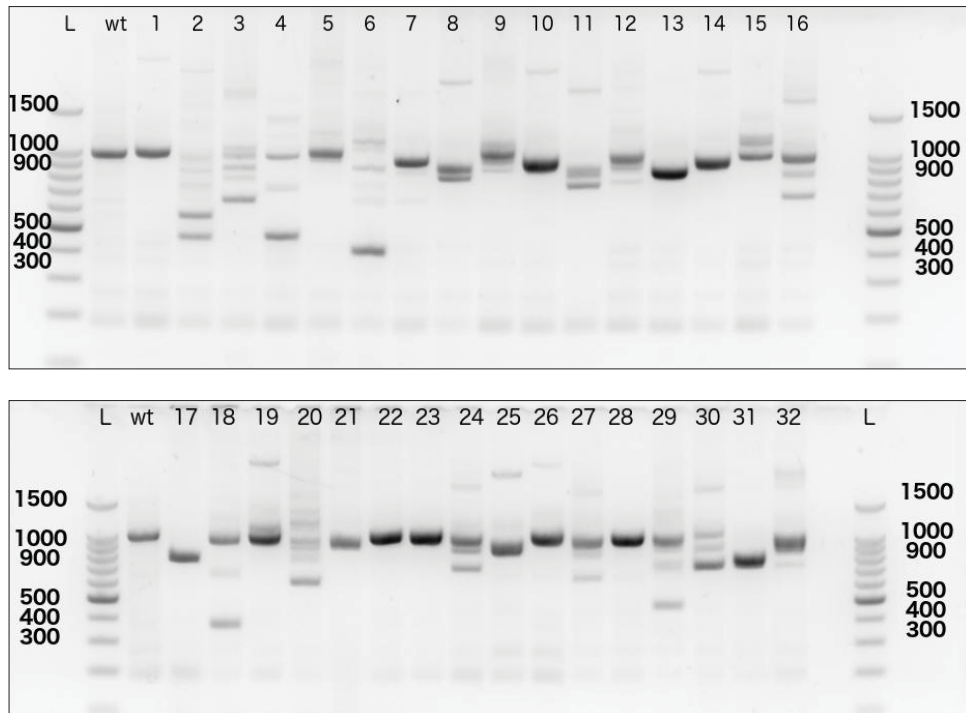


Figure 4.10: **Representative genomic PCR gels.** The same rationale as Figure 4.4 applies here, with the difference that for this screening the wild type fragment is 1000 nucleotides long.

This process led to the selection of a single clone (clone 31) because of a poor survival of the GFP-positive clones. This clone has then been characterised before *in vivo* use. The alleles have then been sequenced and aligned to the mouse receptor, both of them showing the occurrence of a premature stop codon (Figure 4.11).

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ACKR3_WT	MDVHLEFDYAE PGNYS DINWPCNSSDCI VVDTVQCPTMPNK NVLLYTL SFIYIFIFVIGMI	60
C131A111	MDVHLEFDYAE PGNYS DINWPCNSSDCI VVDTVQCPTMPNK NVLLYTL SFIYIFIFVIGMI	60
C131A112	MDVHLEFDYAE PGNYS DINWPCNSSDCI VVDTVQCPTMPNK NVLLYTL SFIYIFIFVIGMI	60

ACKR3_WT	ANSV VV VV VNIQA KTTGYDHCY ILNLA IADLWV VITIPVWV VSLVQH NQWPMGELTCKIT	120
C131A111	ANSV VV A*-----	65
C131A112	ATYVT-----	65
	*. *.	
ACKR3_WT	HLIFS INLFGS IFFLACMS VDRYLSITYFTGTSSYKKKMVRR VVCILVWLLAFFVSLPDT	180
C131A111	-----	66
C131A112	-----GTSSYKKKMVRRVVCILVWLLAFFVSLPDT	95
ACKR3_WT	YYLKT VTSASNNETYCRSFYPEHSI KEWLIGME LVS VILGF AVPFTIIAIF YFL LARAMS	240
C131A111	-----	66
C131A112	YYLSTVTSASCNETYCRSFYPEHSI KEWLIGM*-----	127
ACKR3_WT	ASG DQEKHSSRK IIFS YVVVFLVCWLPYHFVV LLDIFSILHYIPFTCQLENVLF TALHVT	300
C131A111	-----	66
C131A112	-----	127
ACKR3_WT	QCLSLVHCCVNPVLYSFIN RNRYELMKAFIFKYS AKTGLTKLIDASRVSETEYSALEQN	360
C131A111	-----	66
C131A112	-----	127
ACKR3_WT	PK 362	
C131A111	-- 66	
C131A112	-- 127	

Figure 4.11: Allele sequencing translation of mACKR3-KO LLC clone 31. On the wild type sequence, the yellow regions represent the transmembrane domains, in red are highlighted the non-transmembrane regions. Blue boxes represent the site on the reference receptor where the premature stop codon of each allele is located.

Since the plan was to use it in a tumour growth setting, the crucial parameters to evaluate were first of all the success of the mACKR3 knock-out, then the comparison between the proliferation rates of the KO and the Cas9null control. Due to the lack of reliable anti-mACKR3 antibodies in the market, the evaluation of the abrogation of mACKR3 expression was performed through fluorescent CXCL12 uptake: indeed, the KO clone showed a significant decrease of ligand uptake compared to the Cas9null control (Figure 4.12 A). The proliferation rate instead was studied by Ki67 staining: in this case the knock-out clone showed a comparable proliferation rate to its control, which means

that eventual differences in tumour growth would more likely be due to the knock-out itself than to underlying differences between the injected cells (Figure 4.12 B).

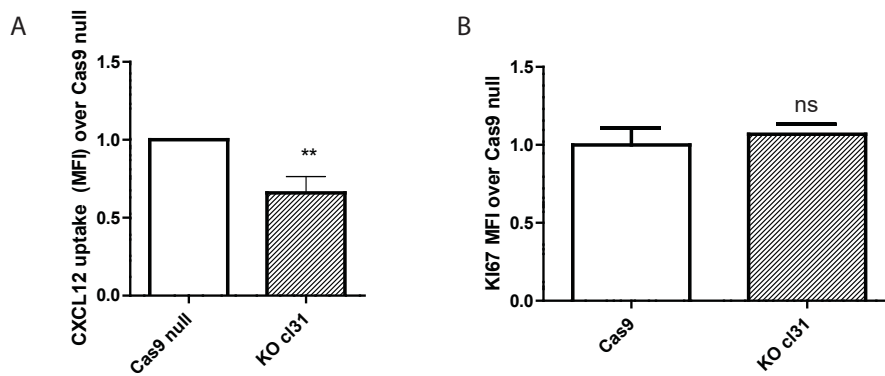


Figure 4.12: Characterisation of LLC cells. The knock-out clone showed a significant decrease of fluorescent CXCL12 uptake (Panel A), indicating the success in the abrogation of mACKR3 expression. The proliferation rate did not show a significant variation in the knock-out compared to its control, showing that the cells proliferate in a similar manner (Panel B). Statistics of three independent replicates (n=3) for each group were assessed using Student's t-test. **: $p < 0.01$, ns: non significant.

Chapter 4. Results 2: Generation of CRISPR ACKR3-KO in Jurkat cells and LLC cells

The Cas9 null control and the mACKR3-KO were then injected subcutaneously into C57BL/6 mice, which do not reject the tumour because of their isogeneity with the cells, and grown until the major radius of the bigger tumour reached 12 mm (according to the project license). Excised tumours were also weighed (Figure 4.13).

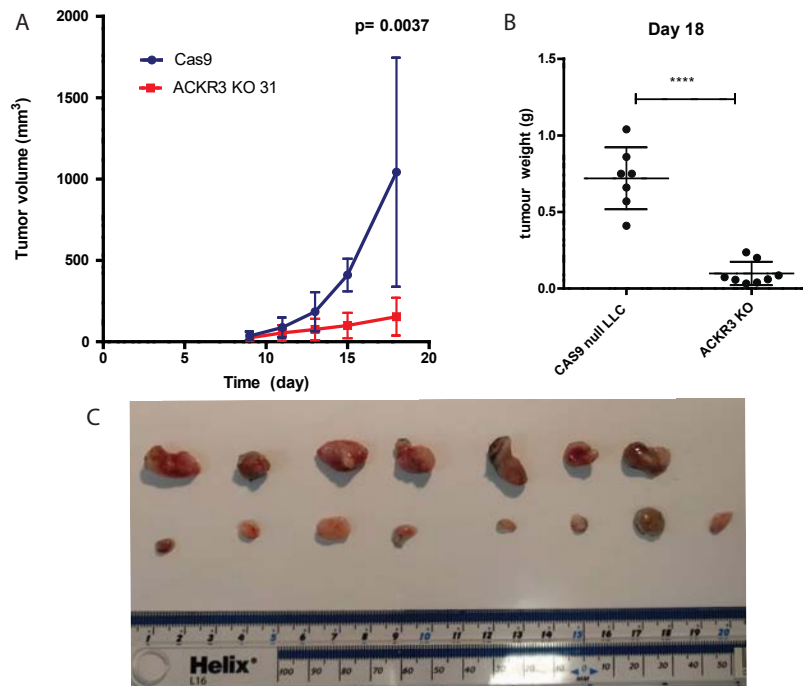


Figure 4.13: Lack of mACKR3 impairs tumour growth in a subcutaneous LLC injection model. mACKR3 KO LLC exhibited significant reduction in tumour growth after 18 days (Panel A), as well as in tumour weight (Panel B). In Panel C, Cas9null derived tumours are displayed in the upper row, while mACKR3 KO derived tumours are in the lower row. Seven and eight mice received a single subcutaneous injection of wild type (n=7) and mACKR3-KO (n=8) LLC cells respectively. One-way analysis of variance (ANOVA) was applied with Tukey's correction for multiple comparisons to assess the significance of the data obtained: **** : $p < 0.0001$; ns: non significant.

4.2 Discussion

The generation of hACKR3 CRISPR knock-outs required significant optimisation but led to robust and significant results. hACKR3 was shown to have a supportive role in hCXCR4-mediated T-cell migration. The fact that the migration is hCXCR4-driven was shown upon inhibition with a hCXCR4-specific small molecule antagonist.

Lack of mACKR3 in a setting in which mCXCR4 is almost absent, such as in the squamous cell carcinoma cell line LLC, showed a significant delay in subcutaneous tumour growth, highlighting the importance of mACKR3 in significant aspects of neoplastic disease development.

Taken together, those two models made clear that ACKR3 has a role in cancer physiology linked to those aspect that make a tumour particularly aggressive: growth and metastatic potential.

Of course more sophisticated experiments must be done in order to address a specific aspect of these phenomena impacted by the overexpression of ACKR3, still these data alone allow for the speculation of encouraging future scenarios.

For example, targeting ACKR3 in order to slow down the growth of a primary tumour can help to improve the efficacy of the surgical asportation and the efficacy of localised radiotherapy. This just because reducing the size of the target site, the easier becomes its excision and the smaller becomes the radiation exposed area. This way, side effects due to these approaches become less impacting on overall patient health.

Targeting ACKR3 in order to reduce metastasis instead might allow to slow down the aggressiveness of a tumour, likely enhancing the effect of current multitherapy anti-neoplastic drugs.

Chapter 5

Results 3: Study of the Adrenomedullin-ACKR3 interaction

Even though a heavily cited paper dates the discovery of the binding of rat adrenomedullin to mouse ACKR3 back to 1995, the article does not actually show this interaction (Kapas and Clark, 1995). The first elegant study of the interaction of mouse adrenomedullin with ACKR3 was reported only in 2014, where Klein et al showed that genetic dosage, obtained both by gene duplication or allele deletion, of these two proteins in transgenic mice led to similar disease and development phenotypes (Klein et al., 2014). Demonstrating the geometry and the specificity of the interaction was outwith the aims of the mentioned paper, but significant *in vivo* evidence resulted in the cue to take this path for the work described in this thesis.

5.1 Procedures and results

The first step for the characterisation of the interaction between human adrenomedullin and human ACKR3 was to study the pharmacology of their interaction. Since the only readout available for hACKR3 was β -arrestin2 recruitment, an adrenomedullin concentration-response curve was compared to those for hCXCL11 and hCXCL12. This demonstrated similar efficacy to the chemokine ligands, but of an order of magnitude weaker in terms of potency (Figure 5.1).

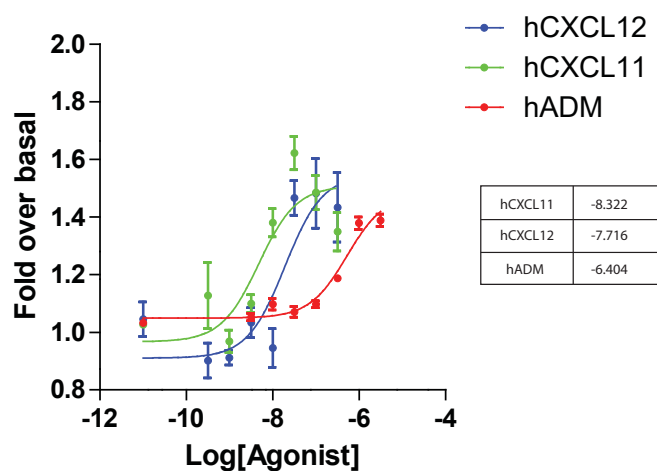


Figure 5.1: β -arrestin2 recruitment concentration-response curves of hACKR3 upon 5 minutes treatment with agonists hCXCL11, hCXCL12 or hAdrenomedullin (hADM). Data were analysed by non linear regression assuming a standard slope with Hill coefficient equal to 1. The model curves were based on four independent experiments ($n=4$). Potencies, expressed as LogEC_{50} were reported in the adjacent table.

To confirm that the interaction was due to a direct activation of hACKR3, a concentration-response curve with a neutralizing immunoglobulin, namely anti-hACKR3 clone 8F11-M16 (Biolegend), was performed, demonstrating that blockade of the receptor did inhibit the recruitment of β -arrestin2 despite the presence of high concentrations (pEC_{80}) of human adrenomedullin (Figure 5.2).

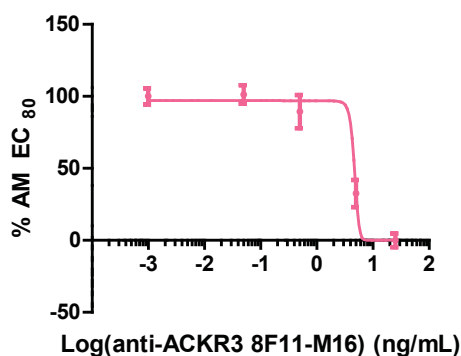


Figure 5.2: β -arrestin2 recruitment concentration-response curve of hACKR3 blockade with anti-hACKR3 8F11-M16 antibody. The effect was calculated using an EC₈₀ concentration of hAdrenomedullin for 5 minutes and an incubation of the neutralising antibody of 30 minutes. Data were analysed by non linear regression of three independent experiments (n=3) assuming a standard slope with Hill coefficient equal to 1.

One of the issues in studying such interactions is that the molecule of interest might give aspecific signalling, thus preventing the first researchers who characterise them to fully understand what is really happening in their system. To partially remove this, the specificity of interaction was investigated by using other chemokine receptors which share the same ligands with hACKR3, namely hCXCR3 and hCXCR4 (Figure 5.3), and targeting hACKR3 with ligands that belong to the same family as adrenomedullin, namely hCGRP (human Calcitonin Gene-Related Protein) and human adrenomedullin2 (Figure 5.4).

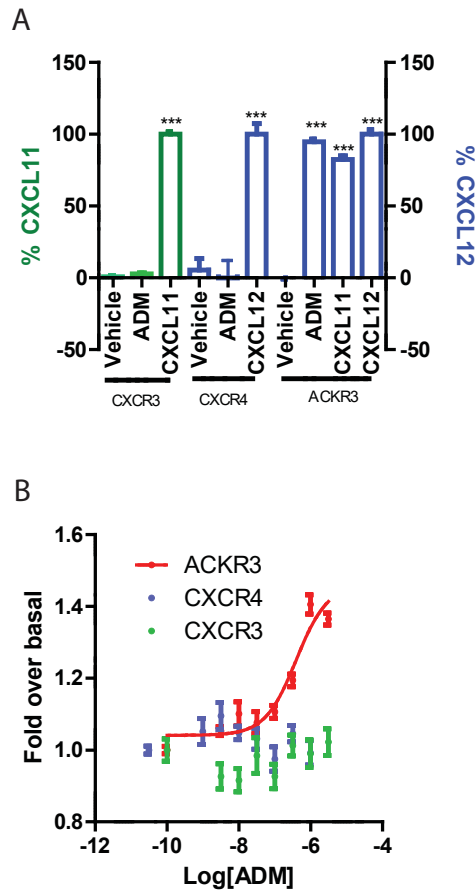


Figure 5.3: EC_{80} concentrations of hCXCL11 and hCXCL12 were used to activate hCXCR3 and hCXCR4 respectively, together with hAdrenomedullin, and all three of the agonists were used to treat for 5 minutes hACKR3, to generate the histogram shown in Panel A. Adrenomedullin failed to promote β -arrestin2 recruitment to hCXCR3 and hCXCR4. The concentration-response curve in Panel B shows that hAdrenomedullin promotes β -arrestin2 recruitment only through hACKR3. Data were analysed by Student's t-test (Panel A) and non linear regression assuming a standard slope with Hill coefficient equal to 1 (Panel B). Statistics were calculated on three independent experiments (n=3); *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.005$.

Neither of the aforementioned typical chemokine receptors gave significant signal over basal upon human adrenomedullin treatment, and equally none of the other ligands belonging to the same family as adrenomedullin gave any signal via hACKR3.

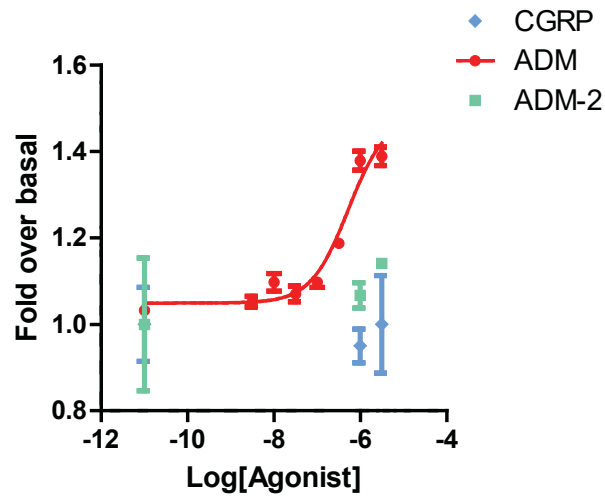


Figure 5.4: The only ligand of the Adrenomedullin family able to stimulate within 5 minutes a response through hACKR3 is hAdrenomedullin. Data were analysed by non linear regression of three independent experiments (n=3) assuming a standard slope with Hill coefficient equal to 1.

There is an extensive literature about hACKR3 downstream signalling subsequent to chemokine ligand treatment that involves MAPK activation (ref). The most accessible assay to detect MAPK activation as part of this work was the ERK phosphorylation kit from Cisbio, which is based upon the use of hTR-FRET. The proximity of the europium-cryptate tag on one of the two antibodies to the fluorophore d2 tag on the second antibody generates a FRET signal in the presence of the appropriate excitation laser. However, in this assay adrenomedullin failed to generate an ERK phosphorylation signal (Figure 5.5).

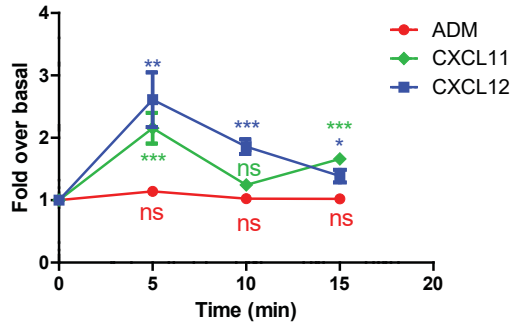


Figure 5.5: hAdrenomedullin treatment fails to produce ERK1/2 phosphorylation in hACKR3-transfected cells at any timepoint up to 15 minutes, whereas hCXCL11 and hCXCL12 both showed phosphorylation of ERK after 5 minutes of treatment; hCXCL12 was able to sustain it until the 10 minute timepoint, while hCXCL11 showed a significant decrease after 10 minutes from the stimulation and a second peak after 15 minutes. Data were analysed by one-way ANOVA. Statistics were calculated on three independent experiments (n=3). ; ns: non significant, **: $p < 0.01$, ***: $p < 0.005$.

In order to strengthen the data, ligand-binding assays were performed, however with disappointing results. Both fluorescently-labelled and radio-labelled ligand approaches were used.

In the case of the fluorescent binding assays, both fluorescent adrenomedullin, specifically N-terminally Fluorescein amidite(FAM)-labelled adrenomedullin sold by Phoenix Pharmaceuticals, and pre-conjugated biotinyl-Adrenomedullin with fluorescently labelled streptavidin were used. The use of these ligands did not give consistent results (Figure 5.6).

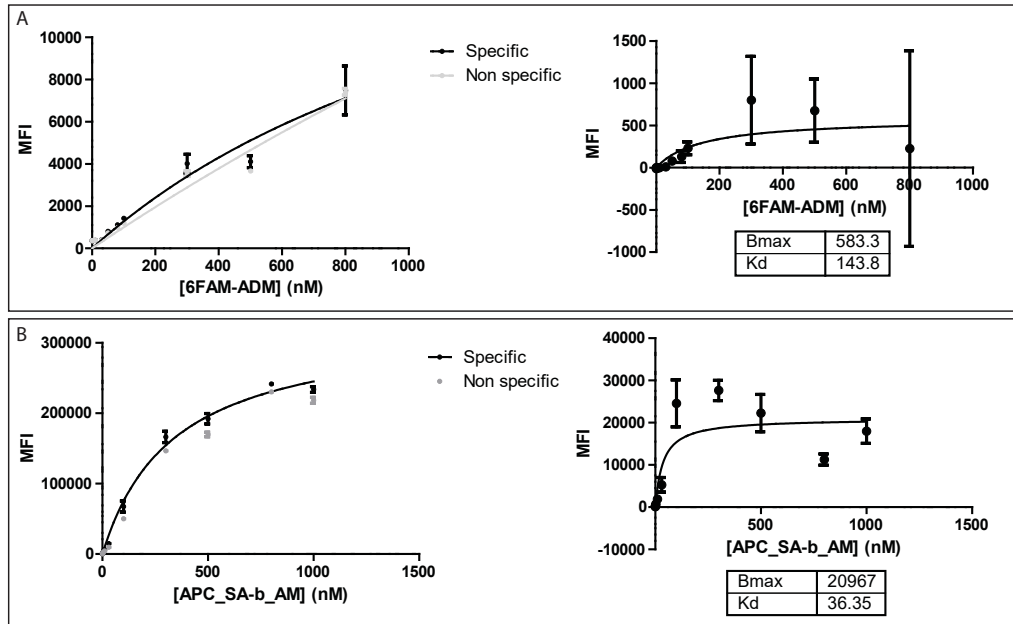


Figure 5.6: Saturation binding of 6-FAM labelled Adrenomedullin (Panel A) and biotinyl-Adrenomedullin preconjugated with APC-Streptavidin (Panel B) to hACKR3-overexpressing HEK293T cells. Results are plotted in function of Mean Fluorescence Intensity (MFI). Representative outcomes are shown but results were inconsistent, as the resulting shape of all of them was far from the expected canonical hyperbola. Three independent experiments were run for all these assays (n=3).

A radioactive approach was then chosen as an alternative readout. [125 I]-Adrenomedullin was purchased and a saturation radioligand binding performed according to the protocol described in Materials and Methods. The curves obtained from hACKR3-overexpressing membranes and mock-transfected membranes were still diverging at the highest concentration used, meaning that the plateau might have been obtainable with concentrations of ligand that are not reachable cost-effectively (Figure 5.7). Indeed, given the modest potency of human Adrenomedullin in functional assays, the saturation curve has shown itself challenging to achieve.

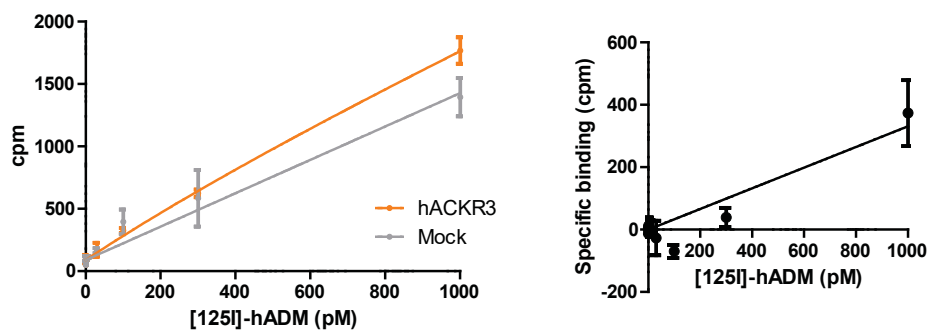


Figure 5.7: Radioactive saturation binding of $[^{125}\text{I}]$ -Adrenomedullin to hACKR3-overexpressing HEK293T lysates. Results are plotted in function of bound radioligand radioactivity, expressed in counts per minute (cpm). Both these plots show that the amount of radioligand used do not allow the curve to reach a plateau. The saturation binding model was applied to three independent experiments ($n=3$).

Despite the failure of the binding assays, the hypothesis that human Adrenomedullin interacts directly with hACKR3 has been demonstrated already by means of functional assays. Hence, to explore exactly where on the receptor does human Adrenomedullin interact, an alanine scanning mutagenesis study was planned.

Site-directed mutagenesis was performed in order to mutate each of the extracellular residues to alanine, with the aim of eliminating specifically any electric charge and/or any steric hindrance. Treating the receptor mutants with high concentrations of human Adrenomedullin and studying their signalling activity, expressed as β -arrestin2 recruitment, would allow the detection of a library of 105 extracellular residue single mutants where each residue was converted to alanine was created. Initially, the mutants were screened in flow cytometry using a PE labelled antibody anti-hACKR3 clone 8F11-M16 to detect their correct expression, and this was cross-checked with their eYFP C-terminal labelling. Then the mutants were screened in 384-well plates for β -arrestin2 recruitment upon pEC₈₀ stimulation with human adrenomedullin. Two mutants, L104A and Q106A, were not correctly expressed, and twelve mutants gave information regarding where the interaction maps (Figures 5.8, 5.9, 5.10, 5.11, 5.12, 5.13).

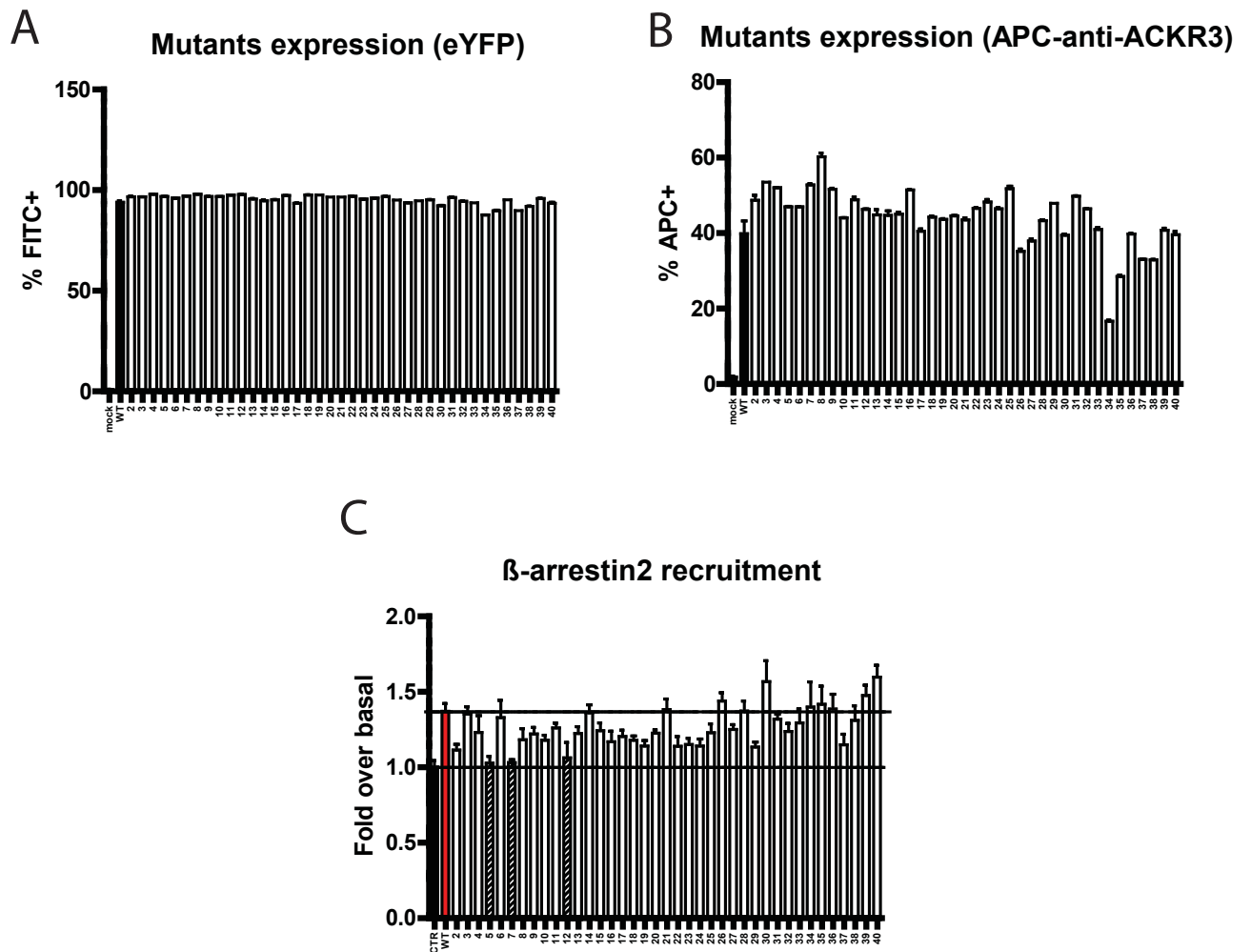


Figure 5.8: Screening of hACKR3 N-terminal region mutants. The expression of each mutant was first screened by FACS for eYFP expression (detected in the FITC channel, Panel A) and hACKR3 labelling by APC-anti-hACKR3 (8F11-M16) antibody (Panel B). β -arrestin2 recruitment after 5 minutes treatment was then investigated for each of the mutants and non-responsive mutants were chosen for further analysis (Panel C). Both expression and functional study statistic were calculated from five independent experiments ($n=5$). The bold black bar represents the basal β -arrestin2 recruitment activity; the bold red bar represents the response upon treatment of wild type hACKR3; the black-striped bars highlight those residues whose activity failed to show any significant difference from the basal activity.

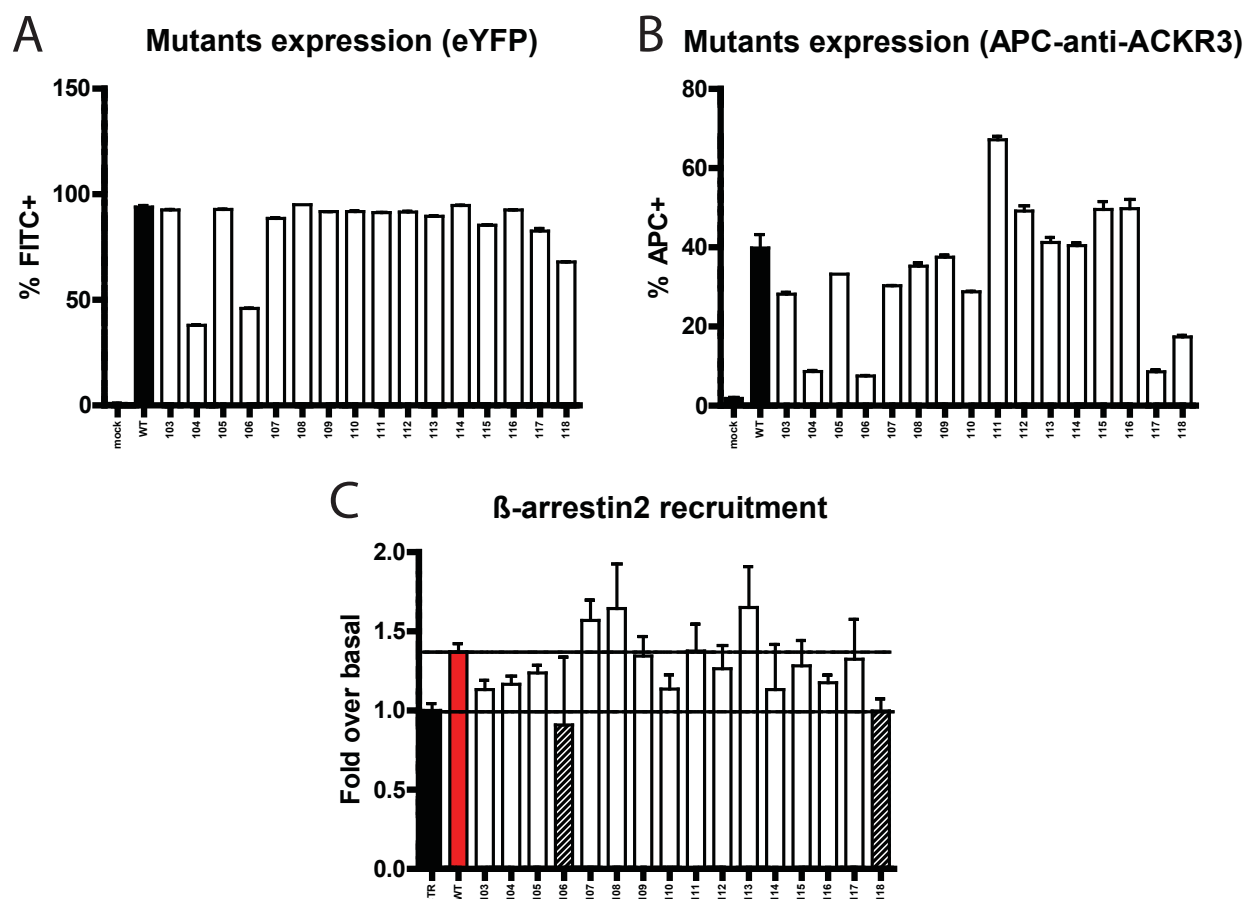


Figure 5.9: Screening of hACKR3 Extracellular Loop 1 (EL1) region mutants. The expression of each mutant was first screened by FACS for eYFP expression (detected in the FITC channel, Panel A) and hACKR3 labelling by APC-anti-hACKR3 (8F11-M16) antibody (Panel B). β -arrestin2 recruitment after 5 minutes treatment was then investigated for each of the mutants and non-responsive mutants were chosen for further analysis (Panel C). Both expression and functional study statistic were calculated from five independent experiments (n=5). The bold black bar represents the basal β -arrestin2 recruitment activity; the bold red bar represents the response upon treatment of wild type hAKR3; the black-striped bars highlight those residues whose activity failed to show any significant difference from the basal activity.

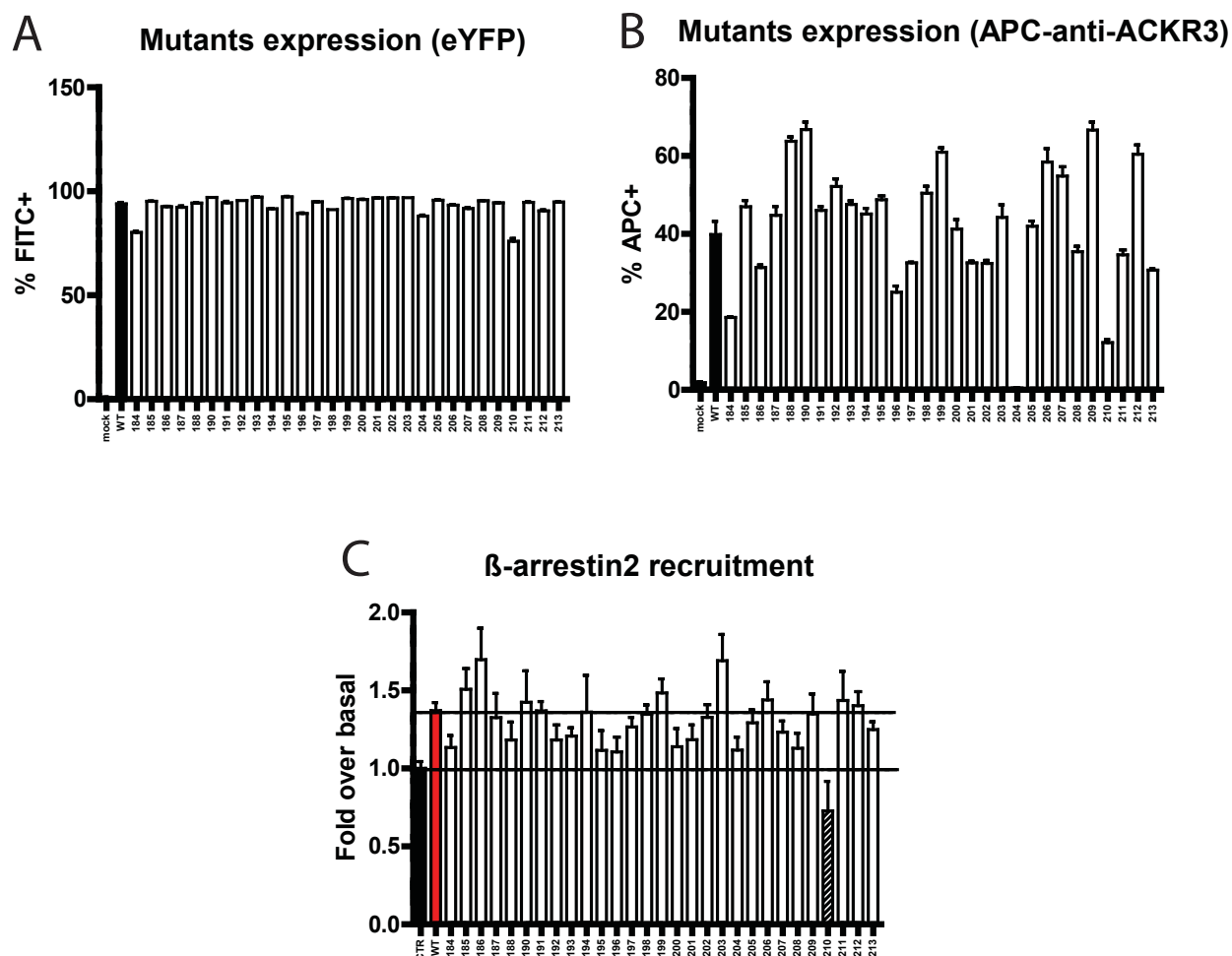


Figure 5.10: Screening of hACKR3 Extracellular Loop 2 (EL2) region mutants. The expression of each mutant was first screened by FACS for eYFP expression (detected in the FITC channel, Panel A) and hACKR3 labelling by APC-anti-hACKR3 (8F11-M16) antibody (Panel B). β -arrestin2 recruitment after 5 minutes treatment was then investigated for each of the mutants and non-responsive mutants were chosen for further analysis (Panel C). Both expression and functional study statistic were calculated from five independent experiments (n=5). The bold black bar represents the basal β -arrestin2 recruitment activity; the bold red bar represents the response upon treatment of wild type hAKR3; the black-striped bars highlight those residues whose activity failed to show any significant difference from the basal activity.

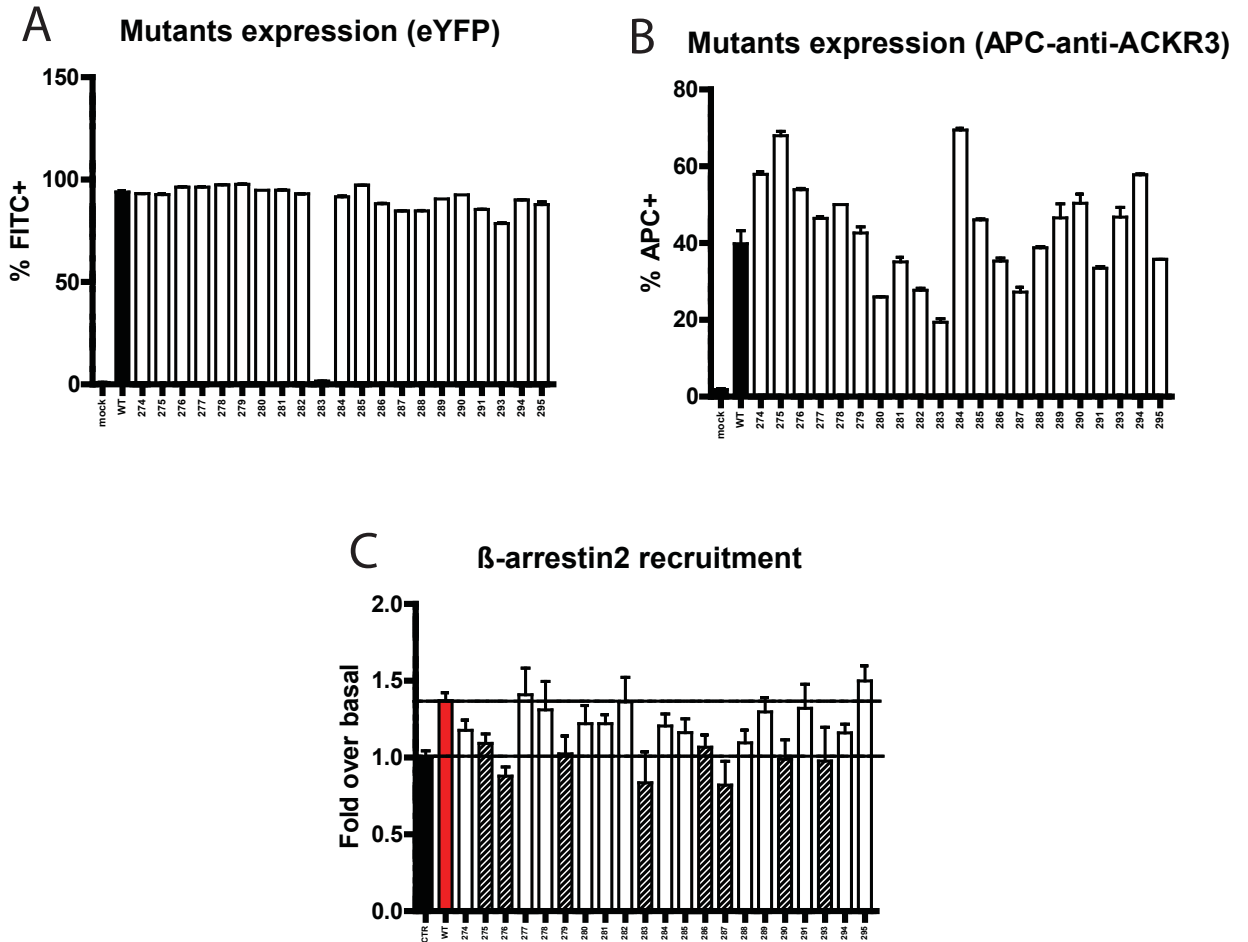


Figure 5.11: Screening of hACKR3 Extracellular Loop 3 (EL3) region mutants. The expression of each mutant was first screened by FACS for eYFP expression (detected in the FITC channel, Panel A) and hACKR3 labelling by APC-anti-hACKR3 (8F11-M16) antibody (Panel B). β -arrestin2 recruitment after 5 minutes treatment was then investigated for each of the mutants and non-responsive mutants were chosen for further analysis (Panel C). Both expression and functional study statistic were calculated from five independent experiments (n=5). The bold black bar represents the basal β -arrestin2 recruitment activity; the bold red bar represents the response upon treatment of wild type hAKR3; the black-striped bars highlight those residues whose activity failed to show any significant difference from the basal activity.

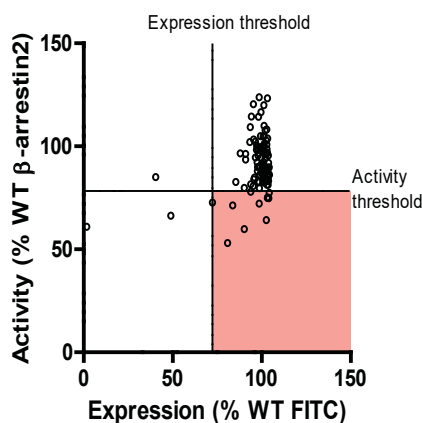


Figure 5.13: Activity versus expression dot plot of the extracellular residues of hACKR3. The red quadrant delineates those residues which exhibited good expression together with low activity. In this region lie the mutants in which the interaction has been impaired by the mutation to alanine. Mean of five independent experiments were plotted.

To obtain confirmation that the lack of signal was not due to incorrect delivery of the mutant to the membrane, these 12 residues (L5, D7, G12, K118, I210, D275, I276, I279, T286, C287, E290 and L293) were screened for β -arrestin2 recruitment upon stimulation with hCXCL11 and hCXCL12 (Figure 5.14). Three mutants (D7, K118 and D275) showed a lack of signal also upon chemokine ligand treatment, and the most likely reason is because of their extremely high basal recruitment activity (Figure 5.15); indeed the basal activity could have been so saturated that the treatment could not increase the β -arrestin2 recruitment level.

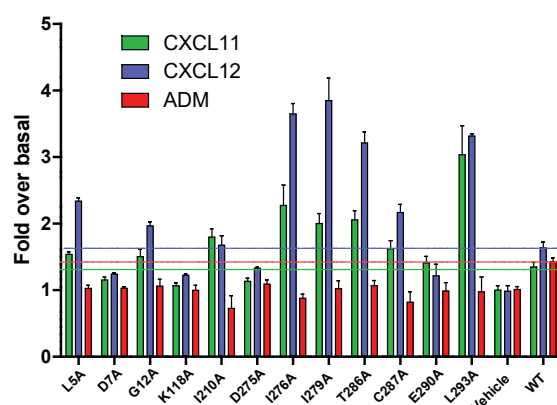


Figure 5.14: hAdrenomedullin-non-responding mutants screening versus pEC₈₀ concentration of chemokine ligands hCXCL11 and hCXCL12 for 5 minutes. Mutants D7A, K118A and D275A, unlike the other mutants, did not show functionality when treated with the chemokine ligands, revealing that the other residues selected are functional for at least one of the chemokine ligands hence their surface expression might be unaffected by the mutation. The plot is the result of three (n=3) independent experiments.

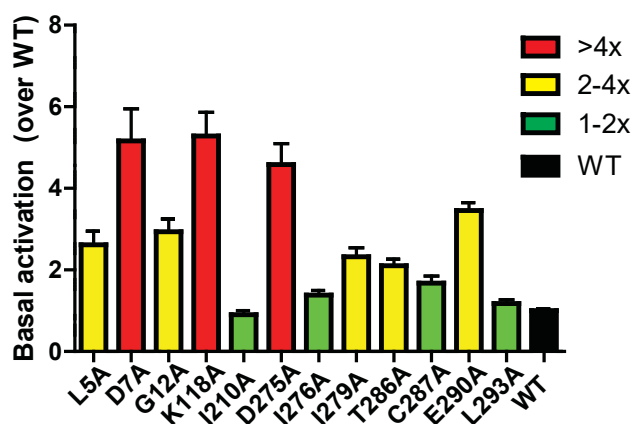


Figure 5.15: Basal β -arrestin2 recruitment of the mutants that do not respond to hAdrenomedullin. Mutants D7A, K118A and D275A did not respond to either chemokine ligands or hAdrenomedullin and it is not possible to distinguish between non interaction with the ligand and an inability of ligands to further increase the high observed basal interactions. The plot has been obtained from three independent experiments (n=3).

From these data, a model of interaction was obtained in collaboration with Dr Martin Gustavsson from the laboratory of Professor Tracy Handel (University of California San

Diego) using molecular docking and protein structure alignment techniques (Figure 5.16, 5.17, 5.18). Based on the known model of CXCL12-ACKR3 interaction, the structural domains of human Adrenomedullin were aligned to the most analogous structures present on CXCL12, and docking was performed according to this analogy, obtaining predictions relative to the most likely interacting residues on the ligand side.

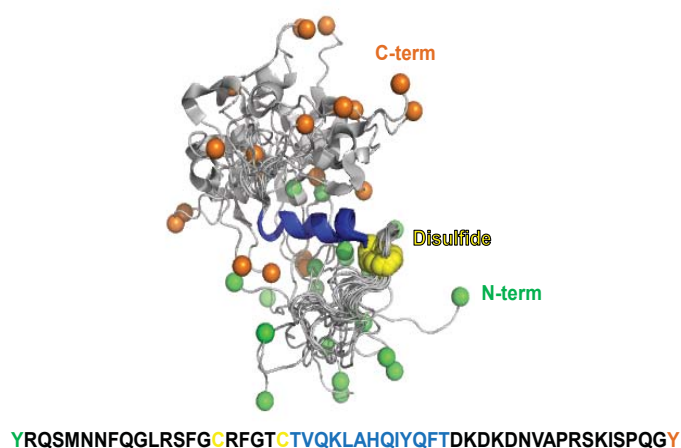


Figure 5.16: The 3-dimensional structure of human Adrenomedullin (PDB: 2L7S) is generally unstructured due to the presence of only two structural domains: a disulphide bridge between the cysteines 16 and 21, and an alpha helix running from threonine 22 to threonine 34. All the sections up- and down-stream of these structural motifs are not spatially constrained, giving different steric configurations. Courtesy of Dr. Martin Gustavsson (UCSD)

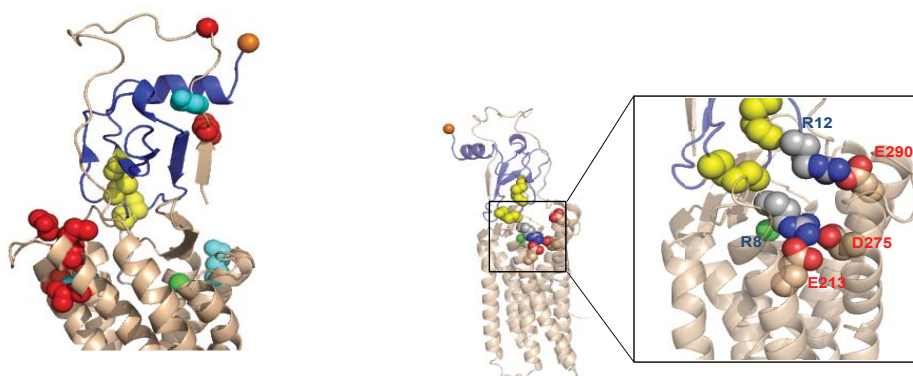


Figure 5.17: Comparison of hAdrenomedullin and hCXCL12 interaction with hACKR3. On the left hand-side, the residues crucial for the interaction of hACKR3 with hAdrenomedullin (red) were mapped onto a model of hACKR3:hCXCL12 interaction. Shown in cyan are the key residues for the interaction with both hAdrenomedullin and chemokine ligands. On the right hand-side three hACKR3 residues are highlighted; of these, D275 and E290 are shared between hCXCL12 and hAdrenomedullin interactions. Courtesy of Dr. Martin Gustavsson (UCSD)

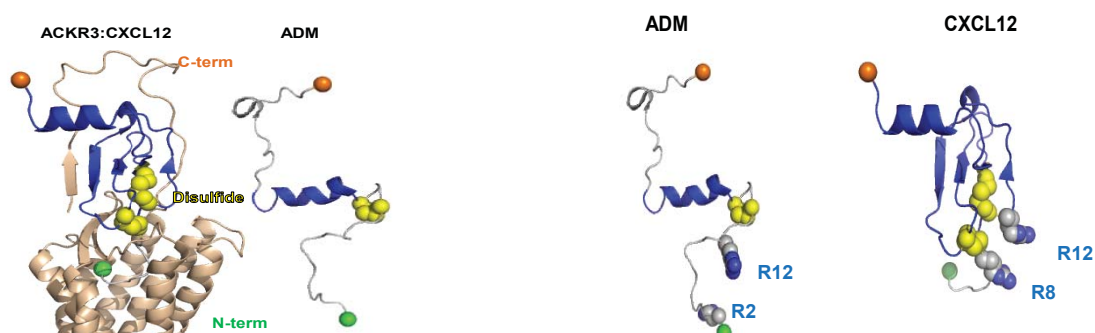


Figure 5.18: Highlighting the likely geometric analogy between the ligands hAdrenomedullin and hCXCL12. The mutagenesis data obtained so far suggest that the N-terminal region of hAdrenomedullin, together with the disulphide bridge, is responsible for the interaction with hACKR3. For the chemokine ligand hCXCL12 the interaction is slightly analogous, as its N-terminal region is also involved in the binding. However the N-terminal region of hCXCL12 is very short and interacts with hACKR3 N-terminal region. Furthermore, hCXCL12 has a more defined structure than hAdrenomedullin, so it is hard to determine from this comparison whether the N-terminal region of hACKR3 is involved in the interaction as well. On the right hand-side, arginines 8 and 12 of hCXCL12 are highlighted, establishing a possible analogy to hAdrenomedullin's arginines 2 and 8 in the interaction with the residues D275 and E290. Courtesy of Dr. Martin Gustavsson (UCSD)

5.2 Discussion

The data presented in this chapter corroborate the initial hypothesis that human Adrenomedullin interacts with hACKR3, and represent the first thorough quantitative characterisation of the interaction between hACKR3 and human Adrenomedullin. hAdrenomedullin has been shown to elicit β -arrestin recruitment to hACKR3 with a potency only one order of magnitude weaker than the receptor's most characterised ligands. This β -arrestin recruitment to hACKR3 due to hAdrenomedullin treatment has been reverted to basal levels when the receptor was occupied by a bulky specific anti-hACKR3 antibody, making it possible to state that this signalling pathway triggering is not dependent on a receptor other than hACKR3.

The unusual nature of this interaction across such different families to which the two interacting partners belong has been established to be unique, at least considering the members of both said families which show between each other the highest degree of homology.

The receptor intracellular interacting partners remain to be discovered, and this might help to have a clearer idea of the biological significance of this interaction. At this point it can only be stated that the activation of hACKR3 upon hAdrenomedullin treatment fails to show ERK phosphorylation within 15 minutes from the administration of the ligand, suggesting that it is likely that other signalling pathways than MAPK are involved in hAdrenomedullin-dependent hACKR3 pathophysiological roles.

Given the modest potency of hAdrenomedullin to the receptor and its relatively high manufacturing costs, the attempts made so far to describe its binding to hACKR3 have revealed themselves unsuccessful and not cost-effective. Competition binding might have been a different way to show binding through labelled ligand displacement, which could have helped to assess whether the highest point reached in both the plots represented in Figure 5.7 was specific or not, though the costs and delivery time of these reagents did not allow these experiments to be performed.

A different kind of study instead has been pursued. Indeed, the interaction between hACKR3 and hAdrenomedullin was investigated through the screening of receptor mutants lacking, each, one of the extracellular aminoacids which likely is crucial for the interaction with the ligand, substituted by an alanine, which lacks of any polarity and gives a reduced steric hindrance.

The mutagenesis study led to the identification of 9 residues which are correctly expressed to the membrane and activate β -arrestin dependent signalling when treated with at least one of the hACKR3 cognate chemokine ligands, but fail to show any activity upon hAdrenomedullin treatment.

These data were used to understand the geometry of the interaction in collaboration with the laboratory of Professor Tracy Handel, based in UCSD and devoted to chemokine receptor structures.

Even though hAdrenomedullin is a protein mostly free of structural constraints (it only has a relatively short α -helical domain and a single disulphide bridge), its only documented conformation involved in an interaction has been compared to the conformation of hCXCL12 bound to hACKR3, showing some analogies.

These data together constitute a robust statement that the interaction does occur, and it can be used as a strong starting point for several projects.

Chapter 6

Final discussion

The project started with the very ambitious goal of finding out exactly how the presence of an atypical receptor such as ACKR3 could impact on CXCR4 signalling. Sharing one of the most important ligands for the development of almost all of the animal species, namely CXCL12, and being often expressed together in those biological processes in which CXCL12 plays a crucial role (i.e. embryo development, haemopoiesis, leukocyte trafficking, immune surveillance, angiogenesis and cell migration, both in a disease-free context as well as during pathological events), it would have been interesting to study ACKR3 contribution.

BRET-based sensors have been developed for this purpose, each one of which was designed to be able to detect a specific G_{α} isoform-dependent signal. Several rounds of troubleshooting and optimisation have been made with the intention of solving the problems that the sensors might have encountered in pursuing the purpose for which they have been generated. Nevertheless, the data obtained from these sensors have been sub-optimal and apparently not amenable to further improvement.

In parallel to the G protein signalling investigation, β -arrestin2 recruitment assays has been optimised for both CXCR4 and ACKR3, and in this case a body of interesting data has been collected regarding the latter. Indeed, the optimisation and the validation of this assay for ACKR3 has allowed the selection of a candidate biological drug, the nanobody VUN700, whose affinity had been previously characterised by another group. Due to their reduced size, compared to a classic antibody, and their comparable antigen-binding affinity, the nanobody-based market is expanding. Their high solubility and

chemical stability makes them desirable for several applications, from diagnostics to the development of drugs with a particularly long shelf life. Scientific research has itself been exploiting nanobodies properties for years; due mainly to their small size, they can easily reach cryptic epitopes and stabilise large protein complexes prior to crystallographic studies, a feature that especially in GPCR structural biology allows also for the study of receptor activation states.

The investigation of the role of ACKR3 in CXCR4-dependent signalling continued with the development of a pair of cancer cell lines defective for ACKR3 expression and the study of their behaviour.

Jurkat cells, or adult T-cell leukemia cells, have been described already in the literature as expressing both of the two chemokine receptors of interest. A western blot confirmed this, and the genome editing through nickase Cas9 CRISPR led to the generation of three different clones of ACKR3-KO Jurkat cells. The sequencing of genomic PCR products confirmed that the two chromosomes underwent mainly deletions, which resulted in all cases in biallelic premature stop codon occurrence a few nucleotides further downstream from the 5'ATG.

Jurkat cells are a suspension cell line, and their migration through transwell membranes has been extensively studied. Hence, a chemotaxis assay was performed to identify behavioural differences between wild type Jurkat cells and their corresponding ACKR3 KO clones, revealing a significantly deficient quantitative response to the chemokine CXCL12 of the latter compared to the wild type cells. Moreover, by blocking CXCR4 with the small molecule AMD3100, the migration was reduced to zero in both the groups, revealing that in both cases the chemotactic response was driven by CXCR4 alone, and the action of ACKR3 only enhances what is driven by CXCR4.

These data agree with what was seen in Burkitt's lymphoma cell line NC-37 by blocking ACKR3 with the proprietary drug CCX771 (Chemocentryx) during transendothelial tumor cell migration (Zabel et al., 2011), and with the behavioural observation that ACKR3-KO VAL cells (B lymphoma model) injected in a mouse model had the tendency not to infiltrate into draining lymph nodes (Puddinu et al., 2017).

LLCs instead are mouse-derived squamous lung cell carcinoma cells, with a really useful peculiarity that served excellently for this study: only a very small portion of these heterogeneous cells express mCXCR4, namely about the 0.18%. This allowed for the study of the action of mACKR3 in a setting in which the main receptor for CXCL12 was

absent.

The validation of the only mACKR3-KO clone required a different method for the quantitation of mACKR3 expression, still it revealed itself a good model for tumour growth and as such gave very important results. The conclusions that can derive from this second chapter were that ACKR3 has an impact on tumour growth and metastasis development, as its vacancy gives strikingly different phenotypes in both the models employed, though the mechanisms that make this happen can only be objects of speculation at this point. Previous analogous works showed that upon RNA silencing of ACKR3 in three different cell lines, namely 4T1 (mouse breast cancer), MDA-MB-435s (human breast cancer) and LLC (mouse lung carcinoma), *in vivo* tumour growth was inhibited compared to the behaviour of the corresponding wild type model (Miao et al., 2007), while the use of ACKR3-KO VAL cells in a model of disseminated xenograft (intravenous injection) in NOD/SCID/common- γ -chain^{ko} mice showed a milder clinical manifestation and a reduced organ invasion compared to wild type VAL cells (Puddinu et al., 2017).

All together, these data corroborate the hypothesis by which ACKR3 contributes to tumour malignancy by means of a higher cell motility, a higher capability to enter the bloodstream to give rise to metastases and a greater ability to enhance tumour growth. The final part of the project focused on one of the ligands that, in concert with the presence of ACKR3, might exert a crucial role for tumour vascularisation, which is adrenomedullin.

Adrenomedullin is able to make ACKR3 recruit β -arrestin2, but fails to show MAPK signalling. Several rounds of optimisation of the binding assay using commercial N-terminally labelled adrenomedullin failed to show binding.

A large library of 105 single mutants of the extracellular domains of ACKR3, was interrogated for adrenomedullin interaction, obtaining enough data to simulate a 3-dimensional docking.

So far, the experiments have shown that ACKR3 and adrenomedullin do interact and the characterisation has indicated the crucial residues and regions involved in both the interaction partners.

These data allow speculation about the role of ACKR3 in various ways and each of these areas of speculation can become a supporting hypothesis for future experiments, such as:

- Charge swap experiments. By swapping the crucial aminoacids from hACKR3

to the corresponding interaction site on hAdrenomedullin and viceversa. If no interaction impairment is seen, then this gives indication that the geometry and the charge distribution along the interaction surface is not changed, making it possible to state that this precise interaction occurs.

- Transmembrane residue mutagenesis to alanine. This kind of experiment can give really useful details about the interaction, by establishing to what depth into the receptor the interaction can reach during the docking and if the microswitch domains show analogies with what is already described in the literature for hCXCR4.
- Generation of *in vivo* models able to exclude a single ligand binding impairment. This would require the greatest investment in time, but would show with great detail which ligand exerts the biggest impact on embryo development impairment.
- Generation of ligand selective cell lines by CRISPR knock-in. This would be more oriented to the study of tumour growth, as it would help establish which ligand can determine a more striking phenotype in tumour growth and invasiveness.

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Appendices

Appendix 1 - Mutagenesis primers

Position	Aminoacid	Primer sequence (5' -> 3')
2	D→A	gtcgaagagatgcag <u>agcc</u> atggtggcaagctt aagcttgccaccatg <u>gctct</u> gcatctcttcgac
3	L→A	gagtagtcgaagagatg <u>cg</u> catccatggtggcaagcttaag cttaagcttgccaccatggat <u>gcg</u> catctcttcgactactc
4	H→A	tctgagtagtcgaagag <u>agcc</u> agatccatggtggcaag cttgccaccatggatctg <u>gctct</u> cttcgactactcaga
5	L→A	gctctgagtagtcga <u>agg</u> catgcagatccatggtgg ccaccatggatctgcat <u>gc</u> cttcgactactcagagc
6	F→A	ctggctctgagtagtc <u>ggc</u> gagatgcagatccatgg ccatggatctgcatctc <u>gcc</u> gactactcagagccag
7	D→A	ccctggctctgagta <u>ggc</u> gaagagatgcagatc gatctgcatctcttc <u>gc</u> ctactcagagccaggg
8	Y→A	gatctgcatctcttcgac <u>gc</u> ctcagagccaggaactt aagttcctggctctga <u>ggc</u> gtcgaagagatgcagatc
9	S→A	aagttcctggctct <u>gc</u> gtagtcgaagagatgc gcatctcttcgactac <u>gc</u> agagccaggaactt
10	E→A	cgagaagttcctgg <u>cg</u> ctgagtagtcgaagag ctcttcgactactcag <u>gc</u> ccaggaacttctcg
11	P→A	ccgagaagttccct <u>gc</u> ctctgagtagtcgaa ttcgactactcagag <u>gc</u> aggaacttctcgg

Position	Aminoacid	Primers
12	G→A	ccgagaagtt <u>cgct</u> ggcctctgagtagtcgaa ttcgactactcagagcca <u>gcga</u> acttctcgg
13	N→A	gctgatgtccgaga <u>aggcc</u> cttgctctgagtag ctactcagagccaggg <u>gcct</u> tctcggacatcagc
14	F→A	ccagctgatgtccga <u>ggc</u> gttccttgctctgag ctcagagccaggaac <u>gcct</u> cggacatcagctgg
15	S→A	ccagctgatgtc <u>cg</u> gaagtccctggct agccaggaactc <u>gcg</u> gacatcagctgg
16	D→A	catggccagctgat <u>ggcc</u> gagaagttccctg caggaacttctc <u>g</u> ccatcagctggccatg
17	I→A	gttgcatggccagct <u>ggc</u> gtccgagaagttccct agggaacttctcggac <u>gcc</u> actggccatgcaac
18	S→A	gctggtgcatggcca <u>ggc</u> gatgtccgagaagttc gaacttctcggacatc <u>gcct</u> ggccatgcaacagc
19	W→A	gctgctggtgcatgg <u>cg</u> gctgatgtccgagaag cttctcggacatcagc <u>gc</u> ccatgcaacagcagc
20	P→A	gctgctggtgcat <u>gcc</u> cagctgatgtccg cggacatcagctgg <u>gc</u> atgcaacagcagc
21	C→A	cagtcgctgctggt <u>gg</u> ctggccagctgatgtc gacatcagctggcca <u>gcca</u> acagcagcagctg
22	N→A	gatgcagtcgctgct <u>ggc</u> gcatggccagctgatg catcagctggccatgc <u>gcc</u> agcagcagctgcatc
23	S→A	gctggccatgcaac <u>gcc</u> agcagctgcatcg cgatgcagtcgct <u>ggc</u> ggttgcattggccagc
24	S→A	ccacgatgcagtc <u>ggc</u> gctggttgcattggcc ggccatgcaacagc <u>gcc</u> gactgcatcgtgg
25	D→A	ccaccacgatgca <u>ggc</u> gctgctggttgcatt atgcaacagcagc <u>gcct</u> gcatcgtggtgg
26	C→A	gtgtccaccacgat <u>ggc</u> gtcgtgctggttgcatt tgcaacagcagcagc <u>gcc</u> atcgtggtggacac

Position	Aminoacid	Primers
27	I→A	ccgtgtccaccac <u>ggc</u> gcagtcgctgctgt acagcagcgcactgc <u>gcc</u> gtggtggacacgg
28	V→A	caccgtgtccac <u>cg</u> gatgcagtcgct agcgactgcatc <u>cg</u> ggtggacacgggtg
29	V→A	acatcaccgtgtc <u>gcc</u> acgatgcagtcg cgactgcatcggtg <u>gcg</u> gacacgggtgatgt
30	D→A	gacacatcaccgt <u>ggc</u> caccacgatgcag ctgcatcggtg <u>gcc</u> acgggtgatgtgtc
31	T→A	gggacacatcac <u>cg</u> gtccaccacgatgc gcatcggtggac <u>cg</u> ggtgatgtgtccc
32	V→A	tgttgggacacat <u>cg</u> ccgtgtccaccacg cgtggtggacacg <u>gcg</u> atgtgtccaaca
33	M→A	ggcatgttgggaca <u>cg</u> ccaccgtgtccaccac gtggtggacacgggtg <u>gcg</u> gtgtccaacatgcc
34	C→A	gttgggcatgttggg <u>ag</u> ccatcaccgtgtccacc ggtggacacgggtgatg <u>gct</u> ccaacatgccaac
35	P→A	gggcatgtt <u>gg</u> acacatcaccgtgtcc ggacacgggtgatgtgt <u>gca</u> aacatgccc
36	N→A	cgcttttgttgggcat <u>ggc</u> gggacacatcaccgtgt acacgggtgatgtgtccc <u>gca</u> tgccaacaaaagcg
37	M→A	ggacgcttttgttggg <u>cg</u> ggttgggacacatcaccg cgggtgatgtgtccaac <u>gcg</u> ccaacaaaagcgtcc
38	P→A	aggacgcttttgtt <u>gg</u> ccatgttgggacacatc gatgtgtccaacatg <u>gca</u> aacaaaagcgtcct
39	N→A	gagcaggacgctttt <u>ggc</u> gggcatgttgggacac gtgtccaacatgccc <u>gca</u> aaaagcgtcctgctc
40	K→A	tgtagagcaggacgctt <u>gc</u> gttgggcatgttgggac gtccaacatgccaac <u>gca</u> agcgtcctgctctaca

Position	Aminoacid	Primers
103	S→A	ccagtctgggtggtc <u>gct</u> ctcgtgcagcacia ttgtgctgcacgag <u>agc</u> gaccaccagactgg
104	L→A	gtgctgcac <u>ggc</u> actgaccaccagactgg ccagtctgggtggtcagt <u>gcc</u> gtgcagcac
105	V→A	actggttgctg <u>cg</u> cagactgaccacc ggtggtcagtctc <u>gcg</u> cagcacaaccagt
106	Q→A	ggccactggttg <u>gc</u> cacgagactgaccac gtggtcagtctcgtg <u>gcg</u> cacaaccagtggcc
107	H→A	atgggccaactggtt <u>ggc</u> ctgcacgagactgac gtcagtctcgtgcag <u>gca</u> accagtggcccat
108	N→A	ccatgggccaactg <u>ggc</u> gtgctgcacgagac gtctcgtgcagcac <u>gcc</u> agtggcccatgg
109	Q→A	gcccatagggca <u>cg</u> gttgctgcacg cgtgcagcacaac <u>gcg</u> gtggcccatgggc
110	W→A	ctcgccatggg <u>gc</u> cctggttgctgc gcagcacaaccag <u>gcg</u> ccatgggag
111	P→A	gctcgccat <u>ggc</u> ccaactggttg cacaaccagtgg <u>gcc</u> atgggag
112	M→A	cgtgagctcgcc <u>cg</u> gggccaactggttg caaccagtggccc <u>gcg</u> ggcgagctcacg
113	G→A	cacgtgagctc <u>ggc</u> catgggccaact agtggccatg <u>gcc</u> gagctcacgtg
114	E→A	ttgcacgtgag <u>cg</u> gcccatgggccc ggccatgggc <u>gcg</u> ctcacgtgcaa
115	L→A	gtgactttgcacgt <u>ggc</u> ctcgccatgggccc ggccatgggag <u>gcc</u> acgtgcaaagtcac
116	T→A	tgtgactttgca <u>cg</u> cagctcgccatgg ccatgggagctc <u>gcg</u> tgcaaagtcaca
117	C→A	gaggtgtgacttt <u>ggc</u> cgtagctcgccatg catgggagctcacg <u>gca</u> aaagtcacacacct
118	K→A	gatgaggtgtgact <u>gcg</u> cacgtgagctcgccc gggagctcacgtg <u>gcg</u> agtcacacacctcatc

Position	Aminoacid	Primers
184	K→A	cgcagacgtgacggt <u>cgcc</u> aggtagtaggtgtca tgacacctactacctg <u>gcg</u> accgtcacgtctgcg
185	T→A	cgcagacgtgac <u>ggc</u> cttcaggtagtagg cctactacctgaag <u>gcc</u> gtcacgtctgcg
186	V→A	tggacgcagacgt <u>ggc</u> ggtcttcaggtag ctacctgaagacc <u>gcc</u> acgtctgctcca
187	T→A	gttggacgcaga <u>cg</u> gacggtcttcaggt acctgaagaccgtc <u>gcg</u> tctgctccaac
188	S→A	ctgaagaccgtcacg <u>gct</u> gctccaacaatg cattggtggacgc <u>agcc</u> gtgacggtcttcag
189	A	
190	S→A	ggtctcattggt <u>ggc</u> gcagacgtgacgg ccgtcacgtctgcg <u>gcca</u> acaatgagacc
191	N→A	gcagtaggtctcatt <u>ggc</u> ggacgcagacgtgacg cgtcacgtctgctcc <u>gcca</u> aatgagacctactgc
192	N→A	ccggcagtaggtctc <u>agc</u> gttgacgcagacgtg cacgtctgctccaac <u>gct</u> gagacctactgccgg
193	E→A	gaccggcagtaggt <u>cg</u> cattggtggacgcag ctgctccaacaat <u>gcg</u> acctactgccggtc
194	T→A	ggaccggcagta <u>ggc</u> ctcattggtggacg cgtccaacaatgag <u>gc</u> ctactgccggtcc
195	Y→A	ggtagaaggaccggca <u>ggc</u> ggtctcattggtggacg cgtccaacaatgagacc <u>gcct</u> gccggtccttctacc
196	C→A	ggggtagaaggaccg <u>ggc</u> gtaggtctcattggtg caacaatgagacctac <u>gccc</u> ggtccttctacccc
197	R→A	ctcgggtagaaggac <u>cg</u> cgcagtaggtctcattg caatgagacctactgc <u>gcg</u> tcttctaccccag
198	S→A	tcgggtagaa <u>ggc</u> ccggcagtaggtc gacctactgccgg <u>gcct</u> tctaccccga
199	F→A	tgtgctcgggtaga <u>ggc</u> ggaccggcagtagg cctactgccggtcc <u>gcct</u> accccagacaca

Position	Aminoacid	Primers
200	Y→A	gctgtgctcggg <u>ggc</u> gaaggaccggcag ctgccggtccttc <u>gcccc</u> gagcacage
201	P→A	atgctgtgctc <u>ggc</u> gtagaaggaccgg ccggtccttctac <u>gcc</u> gagcacagcat
202	E→A	ccttgatgctgtg <u>cg</u> gggtagaaggac gtccttctacccc <u>g</u> gcacagcatcaagg
203	H→A	ccactccttgatgct <u>ggc</u> ctcgggtagaaggac gtccttctaccccag <u>gcc</u> agcatcaaggagtgg
204	S→A	cagccactccttgat <u>ggc</u> gtgctcgggtagaag cttctaccccagcac <u>gcc</u> atcaaggagtggctg
205	I→A	gatcagccactcctt <u>ggc</u> gtgctcgggtag ctaccccagcacagc <u>gcca</u> aggagtggctgatc
206	K→A	ccgatcagccactc <u>gcg</u> gatgctgtgctcggg cccagcacagcatc <u>gcg</u> gagtggctgatcgg
207	E→A	tgccgatcagcca <u>cg</u> ccttgatgctgtgc gcacagcatcaagg <u>cg</u> tggctgatcggca
208	W→A	ctccatgccgatcag <u>cg</u> cctccttgatgctgtgc gcacagcatcaaggag <u>gcg</u> ctgatcggcatggag
209	L→A	cagctccatgccgat <u>cg</u> cccactccttgatgctg cagcatcaaggagtgg <u>gcg</u> atcggcatggagctg
210	I→A	gaccagctccatgcc <u>ggc</u> cagccactccttgatg catcaaggagtggctg <u>gcc</u> ggcatggagctggtc
211	G→A	gaccagctccat <u>ggc</u> gatcagccactc gagtggctgatc <u>gcc</u> atggagctggtc
212	M→A	cggagaccagctc <u>cg</u> gccgatcagccact agtggctgatcggc <u>gcg</u> gagctggtctccg
213	E→A	caacggagaccag <u>cg</u> ccatgccgatcagc gctgatcggcatg <u>gcg</u> ctggtctccgttg

Position	Aminoacid	Primers
274	L→A	atggagaagatgtcc <u>gcc</u> cagcaccgccacgtg cacgtggcggtgctg <u>gcg</u> gacatcttctccat
275	D→A	ggatggagaagat <u>ggc</u> cagcagcaccgcc ggcggtgctgctg <u>gcc</u> atcttctccatcc
276	I→A	tgcaggatggagaa <u>ggc</u> gtccagcagcaccgc gcggtgctgctggac <u>gct</u> tctccatcctgca
277	F→A	gtagtgcaggatgga <u>ggc</u> gatgtccagcagcacc ggtgctgctggacatc <u>gct</u> ccatcctgcactac
278	S→A	atgtagtgcaggat <u>ggc</u> gaagatgtccagcagc gctgctggacatctt <u>gcc</u> atcctgcactacat
279	I→A	aaagggatgtagtgcag <u>ggc</u> ggagaagatgtccagcag ctgctggacatcttctcc <u>gcc</u> ctgcactacatcccttt
280	L→A	gtgaaagggatgtagt <u>gcg</u> gatggagaagatgtccag ctggacatcttctccatc <u>gcg</u> cactacatccctttcac
281	H→A	caggtgaaagggatgta <u>ggc</u> caggatggagaagatgtc gacatcttctccatcctg <u>gct</u> acatccctttcacctg
282	Y→A	ggcaggtgaaagggat <u>ggc</u> gtgcaggatggagaaga tcttctccatcctgcac <u>gcc</u> atecctttcacctgcc
283	I→A	ccggcaggtgaaagg <u>ggc</u> gtagtgcaggatggag ctccatcctgcactac <u>gcc</u> cctttcacctgccgg
284	P→A	ccggcaggtgaa <u>agc</u> gatgtagtgcagga tcttgcactacatc <u>gct</u> ttcacctgccgg
285	F→A	ctgcactacatccctgccacct <u>gcc</u> ggctgga tccagccggcaggt <u>ggc</u> agggatgtagtgcag
286	T→A	ctccagccggca <u>ggc</u> gaaagggatgtagt actacatccctttc <u>gct</u> gccggctggag
287	C→A	cgtgctccagcc <u>ggc</u> gggtgaaagggatgt acatccctttcacc <u>gcc</u> ggctggagcagc
288	R→A	ggcgtgctccag <u>gcg</u> caggtgaaagg ccctttcacctgc <u>gcg</u> ctggagcagcgc
289	L→A	gagggcgtgctc <u>gcg</u> ccggcaggtgaaa tttcacctgccgg <u>gcg</u> gagcagcgcctc

Position	Aminoacid	Primers
290	E→A	agagggcgtg <u>cgcc</u> cagccggcag ctgccggctg <u>gcg</u> cacgcctct
291	H→A	cgtgaagagggc <u>ggc</u> ctccagccggcag ctgccggctggag <u>gcc</u> gcctcttcacg
292	A	
293	L→A	cagggccgtgaa <u>ggc</u> ggcgtgctccagc gctggagcacgc <u>gc</u> cttcacggcctg
294	F→A	atgcagggcgtg <u>ggc</u> gagggcgtgctcc ggagcacgcctc <u>gcg</u> acggcctgcat
295	T→A	catgcagggc <u>cg</u> gaagagggcgtg cacgcctcttc <u>gcg</u> gcctgcatg
296	A	

List of SPASM sensors generated for this project

All these vectors had been validated by sequencing (not shown).


- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine-nopeptide
- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i1/2}}$
- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i3}}$
- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine- G_{α_s}
- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine- G_{α_q}
- FLAG-hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{13}}$
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine-nopeptide
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i1/2}}$
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i3}}$
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine- G_{α_s}
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine- G_{α_q}
- FLAG-hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{13}}$
- hCXCR4-NanoLuc-ER/K-mCitrine-nopeptide
- hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i1/2}}$
- hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i3}}$
- hCXCR4-NanoLuc-ER/K-mCitrine- G_{α_s}
- hCXCR4-NanoLuc-ER/K-mCitrine- G_{α_q}
- hCXCR4-NanoLuc-ER/K-mCitrine- $G_{\alpha_{13}}$
- hACKR3-NanoLuc-ER/K-mCitrine-nopeptide
- hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i1/2}}$

- hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{i3}}$
- hACKR3-NanoLuc-ER/K-mCitrine- G_{α_s}
- hACKR3-NanoLuc-ER/K-mCitrine- G_{α_q}
- hACKR3-NanoLuc-ER/K-mCitrine- $G_{\alpha_{13}}$
- hCXCR4-NanoLuc-ER/K-mNeonGreen-nopeptide
- hCXCR4-NanoLuc-ER/K-mNeonGreen- $G_{\alpha_{i1/2}}$
- hACKR3-NanoLuc-ER/K-mNeonGreen-nopeptide
- hACKR3-NanoLuc-ER/K-mNeonGreen- $G_{\alpha_{i1/2}}$

Publications

MINIREVIEW

Context-Dependent Signaling of CXC Chemokine Receptor 4 and Atypical Chemokine Receptor 3

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ABSTRACT

G protein-coupled receptors (GPCRs) are regulated by complex molecular mechanisms, both in physiologic and pathologic conditions, and their signaling can be intricate. Many factors influence their signaling behavior, including the type of ligand that activates the GPCR, the presence of interacting partners, the kinetics involved, or their location. The two CXC-type chemokine receptors, CXC chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3), both members of the GPCR superfamily, are important and established therapeutic targets in relation to cancer, human immunodeficiency virus infection, and inflammatory diseases. Therefore, it is crucial to understand how the signaling of these receptors works to be able to specifically target them. In this review, we discuss how the signaling pathways activated by CXCR4 and ACKR3 can vary in different situations. G protein signaling of CXCR4

depends on the cellular context, and discrepancies exist depending on the cell lines used. ACKR3, as an atypical chemokine receptor, is generally reported to not activate G proteins but can broaden its signaling spectrum upon heteromerization with other receptors, such as CXCR4, endothelial growth factor receptor, or the α_1 -adrenergic receptor (α_1 -AR). Also, CXCR4 forms heteromers with CC chemokine receptor 2 (CCR2), CCR5, the Na^+/H^+ exchanger regulatory factor 1, CXCR3, α_1 -AR, and the opioid receptors, which results in differential signaling from that of the monomeric subunits. In addition, CXCR4 is present on membrane rafts but can go into the nucleus during cancer progression, probably acquiring different signaling properties. In this review, we also provide an overview of the currently known critical amino acids involved in CXCR4 and ACKR3 signaling.

Introduction

G protein-coupled receptor (GPCR) signaling involves numerous factors that influence cellular functions. These

include: 1) the variety of ligands binding to the receptor, 2) the kinetics of the processes, 3) the location of the GPCR, and 4) the available interactome or cellular context:

1. Different ligands can induce a variety of conformational changes in a receptor and, therefore, adopt several conformations (Kim et al., 2013; Manglik et al., 2015; Masureel et al., 2018). These conformations could preferentially activate different pathways, which is

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ABBREVIATIONS: ACKR3, atypical chemokine receptor 3; AR, adrenergic receptor; BRET, bioluminescence resonance energy transfer; CCR, CC chemokine receptor; CD, cluster of differentiation; CXCL, CXC chemokine ligand; CXCR, CXC chemokine receptor; EGFR, endothelial growth factor receptor; ERK, extracellular signal-regulated kinase; FRET, Förster resonance energy transfer; GPCR, G protein-coupled receptor; GRK, G protein receptor kinase; HEK293, human embryonic kidney 293; NHERF1, Na^+/H^+ exchanger regulatory factor 1; RLuc, Renilla luciferase; TM, transmembrane; VSMC, vascular smooth muscle cell; YFP, yellow fluorescent protein.

known as biased agonism (Vaidehi and Kenakin, 2010; Lane et al., 2017).

2. GPCR activation is also influenced by the kinetics of both ligand binding and receptor signaling, which can possibly lead to the observation of bias profiles, such as in the case of the dopamine D₂ receptor (Klein Herebrink et al., 2016).
3. Most GPCRs signal from the plasma membrane, where they gather in separate compartments rich in G proteins (Huang et al., 1997) and interact with other partners (Hur and Kim, 2002). Nevertheless, increasing evidence suggests that GPCRs also signal after internalization (Calebiro et al., 2010; Vilardaga et al., 2014; Eichel and von Zastrow, 2018) and from sub-cellular sites, including the endoplasmic reticulum, Golgi apparatus, and nucleus (Rebois et al., 2006; Boivin et al., 2008; Godbole et al., 2017). These internalized receptors could activate signaling pathways distinct from those activated by the same receptors at the cell surface.
4. Different cellular contexts contain different sets of proteins that may directly or indirectly interact with the GPCR and hence alter its signaling. Therefore, the signaling pattern of one GPCR can strongly vary between cell types. For instance, although class A GPCRs can function as monomers (Whorton et al., 2007), they can also form and function as homo- and hetero-oligomers, which might result in altered signaling properties compared with those of the individual monomers (Jordan and Devi, 1999; Ferré et al., 2014). In this respect, the existence of membrane compartments can facilitate the interaction between different partners and result in a variety of cellular outcomes.

Is this complexity in signaling also applicable to the GPCRs' CXC chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3)? Both receptors bind the same chemokine, CXC motif ligand 12 (CXCL12), but interestingly their signaling outcomes are different (Busillo and Benovic, 2007; Rajagopal et al., 2010). In addition, ACKR3 also binds CXCL11, although with lower affinity (Burns et al., 2006). Under physiologic conditions, CXCR4 is involved in vascularization (Tachibana et al., 1998), neurogenesis (Cui et al., 2013), angiogenesis (Salcedo and Oppenheim, 2003) and homing of immune cells in the bone marrow (Sugiyama et al., 2006), while ACKR3 has a role in the development of the central nervous system (Wang et al., 2011), angiogenesis (Zhang et al., 2017), neurogenesis (Kremer et al., 2016), and cardiogenesis (Ceholski et al., 2017).

Similar to most chemokine receptors, CXCR4 and ACKR3 are important therapeutic targets due to their involvement in immune-related diseases and cancer. The CXCL12/CXCR4 axis is involved in over 23 types of cancer, including breast, lung, colon, and ovary cancer (Guo et al., 2014; Panneerselvam et al., 2015; Zheng et al., 2017; Raschioni et al., 2018), and acts as a coreceptor for the human immunodeficiency virus (HIV) to enter host T cells (Feng et al., 1996). The discovery of ACKR3 as another CXCL12 receptor added complexity to the understanding of the CXCR4/CXCL12 signaling axis (Balabanian et al., 2005a). ACKR3 is also overexpressed in many cancer types, playing an important role in tumor development and metastasis by promoting cell survival and

adhesion (Burns et al., 2006; Miao et al., 2007; Wang et al., 2008). Importantly, ACKR3 has a functional cross-talk with CXCR4, and they are proposed to heteromerize (Balabanian et al., 2005a; Burns et al., 2006; Levoe et al., 2009; Decaillot et al., 2011). Several other receptors can also alter the function of CXCR4 and ACKR3, either through a functional cross-talk or as a consequence of heteromerization (Contento et al., 2008; Martínez-Muñoz et al., 2014; Becker et al., 2017; Dinkel et al., 2018).

Studies regarding CXCR4 and ACKR3 have been performed using a variety of cellular systems in which interacting proteins may not necessarily be identical, and often in transfected conditions, which could lead to the artificial induction of oligomerization (Meyer et al., 2006). Hence, there is increasing interest in investigating their signaling in a native-like context. In this review, we discuss these issues and the importance of location, kinetics, and interactions with other receptors/effectors in the scope of CXCR4 and ACKR3 signaling in physiologic and pathologic conditions.

CXCR4 and ACKR3 Signaling

A number of signaling pathways are known to be activated by CXCR4 and ACKR3, with outcomes differing depending on the cellular context. Generally, CXCR4 is able to signal through multiple G proteins and is also regulated by β -arrestins through different interacting regions. Conversely, ACKR3 signals predominantly via β -arrestins and is generally not able to activate G proteins. Nevertheless, as discussed in the following section, there is still conflicting evidence in relation to the precise details of their signaling.

G Protein-Dependent Signaling through CXCR4. CXCR4 couples predominantly to G proteins of the $G_{\alpha_{i/o}}$ family. Upon activation of the receptor, this family of G proteins generally leads to the inhibition of adenylyl cyclase, and as a consequence, cAMP production and the activity of cAMP-dependent protein kinases are reduced.

Many G protein activation studies are performed using bioluminescence resonance energy transfer (BRET)-based and Förster resonance energy transfer (FRET)-based techniques in transfected cells, which provide a very good model to study the possible signaling pathways triggered by a receptor. However, the disadvantage of such studies is the need to transfect cells, which could generate artifacts as a result of overexpression of the corresponding proteins (Meyer et al., 2006). Studies using these recombinant systems have shown that CXCR4 can engage and activate different $G_{\alpha_{i/o}}$ proteins, including $G_{\alpha_{i1}}$, $G_{\alpha_{i2}}$, $G_{\alpha_{i3}}$, and G_{α_o} , in response to CXCL12 stimulation. In particular, it seems that CXCR4 might couple more efficiently to the $G_{\alpha_{i1}}$ and $G_{\alpha_{i2}}$ subtypes than to $G_{\alpha_{i3}}$ and G_{α_o} (Kleemann et al., 2008; Quoyer et al., 2013). No activation of G_{α_z} , the only member of the G_{α_i} family that is resistant to pertussis toxin, has been demonstrated, although the CXCR4/CC chemokine receptor 2 (CCR2) hetero-oligomer is capable of stimulating G_{α_z} -driven Ca^{2+} mobilization through the CCR2 receptor (Armando et al., 2014).

In addition to its coupling to the $G_{\alpha_{i/o}}$ subfamily, CXCR4 can also signal through other G proteins. Studies using a more endogenous-like setting suggested that CXCR4 mediates some of its functions through $G_{\alpha_{i3}}$. For example, migration of Jurkat T cells in response to CXCL12 is controlled not only by G_{α_i} through the activation of Rac but also by $G_{\alpha_{i3}}$ through

the activation of Rho (Tan et al., 2006). Importantly, it seems that the coordinated activation of these two pathways is also essential for the CXCR4-induced migration of metastatic basal-like breast cancer cells in vitro and in vivo in response to CXCL12 (Yagi et al., 2011). The coupling of CXCR4 to the noncognate G protein $G\alpha_{13}$ might be relevant in specific contexts, such as in metastatic breast cancer cells, where $G\alpha_{13}$ is potentially overexpressed (Yagi et al., 2011; Rasheed et al., 2015). In addition, CXCR4 trafficking into Rab11+ vesicles upon CXCL12-induced endocytosis in T cells is known to be dependent on $G\alpha_{13}$, which, together with Rho, mediates the polymerization of actin necessary for this process. It is thought that in this subcellular compartment, CXCR4 forms heterodimers with the T lymphocyte Ag receptor (Kumar et al., 2011).

CXCL12 stimulation of CXCR4 also led to activation of $G\alpha_q$ (Soede et al., 2001), a strong activator of members of the phospholipase C- β subfamily. However, this was only the case in dendritic cells and granulocytes, but not in T and B cells, where CXCR4 signaling and, ultimately, chemotaxis were shown to be $G\alpha_i$ -dependent (Shi et al., 2007). Altogether, these examples suggest that the cellular context can potentially have an impact on the signaling properties of this GPCR, although some caution must be taken when comparing the different studies, since the assays used could differ in their sensitivity and selectivity.

G Protein-Independent Signaling through CXCR4. Similar to the majority of GPCRs, CXCR4 can also be regulated by β -arrestins at a number of levels, including CXCR4 internalization, G protein signaling, and chemotaxis.

Following activation of a receptor, G protein-coupled receptor kinases (GRKs) phosphorylate the intracellular side of the receptor, resulting in the recruitment of β -arrestins-1/2 and subsequent internalization of the receptor through clathrin-coated pits. Interestingly, coexpression of CXCR4 with β -arrestin-2 notably increased internalization of CXCR4 upon CXCL12 stimulation in contrast to β -arrestin-1. However, this difference disappeared when GRK2 was overexpressed, suggesting that β -arrestin-1-mediated internalization highly depends on the phosphorylation state of CXCR4 (Cheng et al., 2000).

Several studies have shown that the arrestins attenuate G protein signaling. In human embryonic kidney 293 (HEK293) cells, overexpression of CXCR4 with either β -arrestin reduced inhibition of cAMP production in response to CXCL12, indicating that both β -arrestin-1 and -2 play an important role in signaling regulation (Cheng et al., 2000). In accordance with this, using endogenous levels of CXCR4, lymphocytes isolated from β -arrestin-2 knockout mice showed a decreased desensitization and enhanced G protein coupling to CXCR4 (Fong et al., 2002). This attenuating effect on G protein signaling could be abolished by truncating the C terminus of the receptor, revealing a functional interaction of the receptor's C terminus with the arrestin. However, receptor internalization and extracellular signal-regulated kinase (ERK) activation were not affected, suggesting that a different region of CXCR4, in addition to the C terminus, is involved in the binding of these proteins with a different functional role (Cheng et al., 2000). This other region appears to be the intracellular loop 3 of the receptor, as it was also first described by Wu et al. (1997) and Cheng et al. (2000). Overall, β -arrestins appear to regulate CXCR4 signaling through at

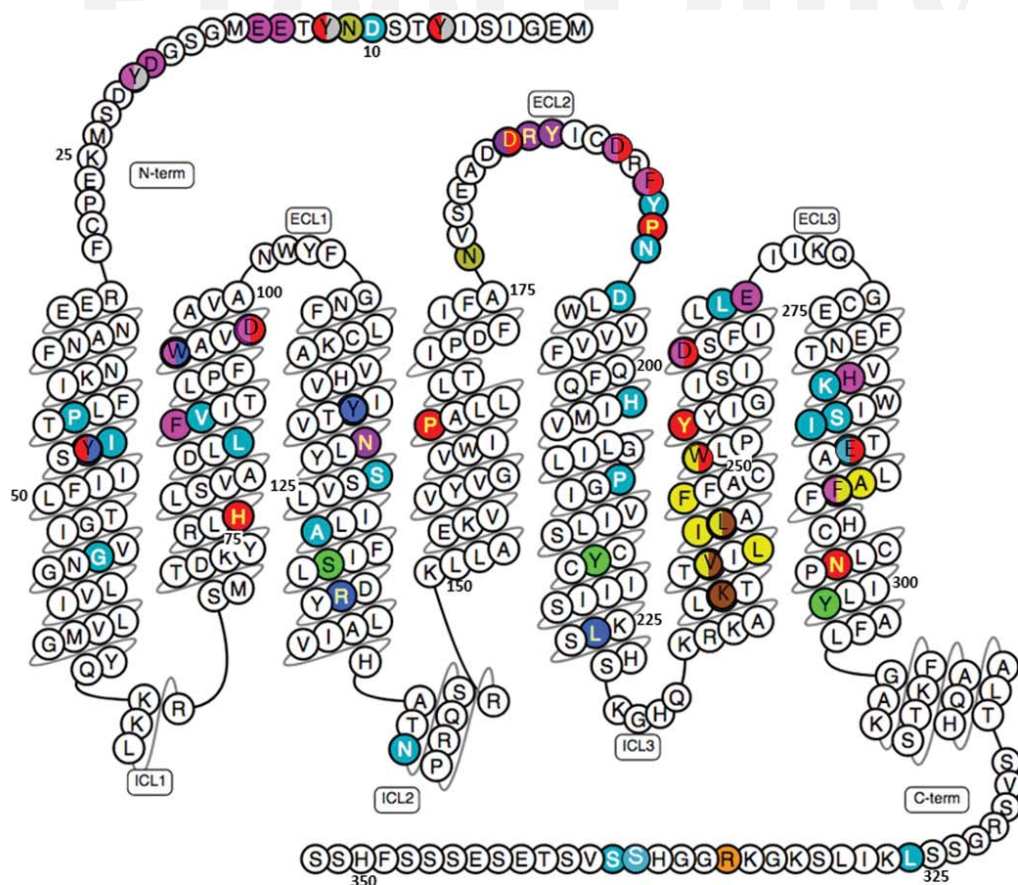
least two different and independent interacting regions on the receptor (Cheng et al., 2000). In accordance, the presence of mutations or truncations in the C terminus of CXCR4 is the cause of a rare congenital disease named warts, hypogammaglobulinemia, immunodeficiency, and myelokathexis syndrome (Hernandez et al., 2003; Balabanian et al., 2005b; Luo et al., 2017).

Last, β -arrestin-2 also plays a key role in CXCR4/CXCL12-mediated chemotaxis of HeLa cells, enhancing the chemotactic efficacy of the ligand mainly through the p38 mitogen-activated protein kinase pathway (Sun et al., 2002).

Kinetics of CXCR4 Signaling. GPCR activation and downstream signaling kinetics have been extensively studied within the last two decades with the aid of emerging fluorescence microscopy methods. Unlike many other receptors (Lohse et al., 2008; Stumpf and Hoffmann, 2016), only a few studies have been published on the kinetics of CXCR4 activation and its corresponding downstream signaling processes. Even so, using BRET studies, activation kinetics by CXCL12 and the pepducin ATI-2341 were compared. CXCL12 has been shown to rapidly induce $G\alpha_i$ protein recruitment to CXCR4 and lead to a full activation with a $t_{1/2}$ value of approximately 32 seconds. The kinetics of $G\alpha_i$ protein recruitment were similar for the pepducin, although activation of $G\alpha_i$ was significantly slower (Quoyer et al., 2013). One study also focused on the phosphorylation kinetics of intracellular sites of CXCR4 in both HEK293 and human astroglial cells and suggested that Ser-324, Ser-325, and Ser-339 were phosphorylated rapidly by GRK6 after CXCL12 exposure, while the kinetics for Ser-330 phosphorylation were significantly slower. Such phosphorylation is directly involved in the association of arrestin to the receptor and hence can finely regulate CXCR4 signaling (Busillo et al., 2010). Another group also demonstrated that $G\alpha_i$ engagement to CXCR4 upon CXCL12 stimulation led to the phosphorylation of Tyr residues in the receptor via the Janus kinase 2/3 kinases within a few seconds (Vila-Coro et al., 1999).

Key Residues for Signaling in the CXCR4 Receptor. The intracellular loop 3 and the C-terminal tail of the receptor seem to be important for β -arrestin recruitment and G protein activation, and accordingly, mutations in these regions have a considerable impact on signaling. Several mutational studies have been performed to unravel how CXCL12 binds to CXCR4, and how the signal is transmitted from the extracellular part of the receptor through the transmembrane regions to the intracellular part, where interactions with protein partners involved in signaling occur. In these regards, previous studies have identified, with nearly atomic resolution, the pathway from the binding of the chemokine to the G protein coupling, and that several mutations in the receptor impair ligand binding and signaling (Wescott et al., 2016). A schematic summary including important residues relating to the function of CXCR4 is provided in Fig. 1.

G Protein-Dependent Signaling through ACKR3. Many studies have shown that ligand binding to ACKR3 does not result in either coupling to or activation of G proteins, or the triggering of signaling pathways typical of G proteins, in contrast to CXCR4. In fact, ACKR3 lacks the specific DRY-LAIV motif on the intracellular side of the receptor that is essential for G protein interaction in other chemokine receptors, and instead presents a DRYLSIT motif (Ulvmar et al., 2011). However, efforts on creating a chimeric ACKR3 where



Function	Residue
Tyrosine sulfation site (CXCR4 dimerisation and increases affinity for CXCL12)	Y7 ⁵ , Y12 ⁵ , Y21 ⁵
Glycosylation site	N11 ² , N176 ²
CXCL12 binding	E14 ⁷ , E15 ⁷ , D20 ³ , Y21 ^{3,7} , F87 ⁸ , W94 ⁴ , D97 ^{1,7} , D187 ^{1,7} , F189 ⁹ , D262 ² , E268 ⁸ , H281 ¹ , F292 ⁸
Initiation residue of the signal transmission by CXCL12 to the intracellular part	Y45 ¹ , W94 ¹ , Y116 ¹
Microswitch residue in CXCL12 signal transmission	S131 ¹ , Y219 ¹ , Y302 ¹
Propagation of the CXCL12 signal transmission	V242 ¹ , L244 ¹ , I245 ¹ , L246 ¹ , F248 ¹ , W252 ¹ , A291 ¹ , F292 ¹
G protein recruitment	R134 ¹ , L226 ¹
G protein signalling	N119 ⁹ , D182 ⁵ , R183 ⁵ , Y184 ⁵
CXCL12-mediated signalling (calcium flux)	D10 ¹ , P42 ¹ , I44 ¹ , G55 ¹ , L86 ¹ , V88 ¹ , S122 ¹ , A128 ¹ , N143 ¹ , Y190 ³ , N192 ¹ , D193 ⁷ , H203 ¹ , P211 ¹ , L267 ¹ , K282 ¹ , S285 ¹ , I286 ¹ , E288 ¹ , L326 ¹ , S338 ¹ , S339 ¹
Implicated in HIV infection	Y7 ⁷ , Y12 ⁷ , Y45 ⁸ , H79 ⁸ , D97 ^{7,8} , P163 ⁸ , D182 ⁸ , D187 ^{7,8} , F189 ⁸ , P191 ⁸ , W252 ⁸ , Y255 ⁸ , D262 ⁸ , E288 ^{7,8} , N298 ⁸
Implicated in WHIM syndrome	R334 ¹⁰
CXCR4 nanoclustering	K239 ¹¹ , V242 ¹¹ , L246 ¹¹

Fig. 1. Snake plot of human CXCR4 with highlighted residues important for receptor function as determined in the following studies: ¹Wescott et al. (2016), ²Berson et al. (1996), ³Zhou et al. (2001), ⁴Cronshaw et al. (2010), ⁵Rapp et al. (2013), ⁶Doranz et al. (1999), ⁷Brelot et al. (2000), ⁸Tian et al. (2005), ⁹Armando et al. (2014), ¹⁰Ballester et al. (2016), and ¹¹Martínez-Muñoz et al. (2018). Snake plot adapted from GPCRdb (Pándy-Szekeres et al., 2018). WHIM, warts, hypogammaglobulinemia, infections, myelokathexis.

the DRYLSIT is replaced by the corresponding DRYLAIV motif of CXCR4 failed to induce CXCL12-mediated signaling, such as G protein activation, intracellular Ca²⁺ mobilization, G protein-mediated ERK phosphorylation, or chemotaxis

(Naumann et al., 2010; Hoffmann et al., 2012). This implies that the missing DRYLAIV motif in ACKR3 is not the only determinant for the lack of G protein-dependent signaling.

Nonetheless, the interaction of ACKR3 with G proteins has been proposed in two studies. In the first case, a specific BRET signal was detected between ACKR3–yellow fluorescent protein (YFP) and $G\alpha_{i1}$ –Renilla luciferase (RLuc), which decreased upon treatment with guanosine 5′-3-O-(thio)triphosphate, suggesting that ACKR3 can interact with G proteins in the absence of an agonist but fails to activate them (Levoye et al., 2009). In the second case, CXCL12 was still able to promote $G_{i/o}$ protein activation in primary astrocytes after CXCR4 depletion but not after ACKR3 depletion. In addition, ACKR3-only-expressing astrocytes also led to ERK and Akt activation in response to both CXCL12 and CXCL11, although only the former appeared to be G protein dependent (Ödemis et al., 2012). Both $G_{i/o}$ and ACKR3 are highly abundant in astrocytes and glioma cells (Schönemeier et al., 2008; Tiveron et al., 2010; Ödemis et al., 2012; Baniadr et al., 2016), and therefore, a hypothesis is that ACKR3 might be able to activate G proteins specifically in these cell types, indicating once again how important the interactome might be for a given GPCR.

Overall, although there is conflicting evidence on the role of ACKR3 in relation to G protein–dependent signaling, there is increasing evidence for a β -arrestin–biased receptor in most cell types. Moreover, studies have shown that ACKR3 could modulate other cellular signaling pathways, potentially by forming a heteromeric complex with other receptors, which is discussed in a later section of this review.

G Protein–Independent Signaling through ACKR3.

Many studies have shown that ACKR3 can act as a “decoy” or “scavenging” receptor, since it can efficiently internalize its chemokine ligands CXCL11 and CXCL12 (Naumann et al., 2010). By internalizing CXCL12, ACKR3 finely tunes the CXCL12 gradient necessary for the CXCR4-mediated migration (Dambly-Chaudière et al., 2007; Boldajipour et al., 2008; Donà et al., 2013). Nevertheless, ACKR3 is not only a “decoy” receptor, it can also activate downstream pathways via β -arrestins, in response to both CXCL11 and CXCL12, directly promoting Akt and mitogen-activated protein kinase activity, ERK phosphorylation (Hattermann et al., 2010; Rajagopal et al., 2010; Decaillot et al., 2011; Ödemis et al., 2012; Torossian et al., 2014), and activation of the Janus kinase 2/signal transducer and activator of transcription 3 pathway (Hao et al., 2012). CXCL11-dependent ERK phosphorylation could be seen in ACKR3-overexpressing HEK293 cells but not in rat vascular smooth muscle cells (VSMCs) that endogenously express ACKR3, again demonstrating the importance of the cellular context (Rajagopal et al., 2010). Interestingly, AMD3100, an antagonistic small-molecule against CXCR4, can have an agonistic effect on ACKR3. In high concentrations, this molecule can induce β -arrestin recruitment to ACKR3 and increase CXCL12 binding to the receptor (Kalatskaya et al., 2009). A similar scenario was observed with the CXCR4 inverse agonist TC14012, which acts as an agonist on ACKR3 (Gravel et al., 2010). Therefore, when considering CXCR4 as a therapeutic target, it should be taken into account that a molecule can have unexpected effects via ACKR3 and vice versa.

Although ACKR3 is constitutively internalized via clathrin-coated pits by β -arrestins (Luker et al., 2010), it has also been described that ACKR3 internalizes in a ligand-dependent manner in response to both CXCL11 and CXCL12, leading to different patterns of receptor internalization (Rajagopal et al., 2010; Canals et al., 2012).

Ubiquitination, a constitutive modification on ACKR3, is the key modification responsible for the correct trafficking of the receptor from and to the plasma membrane (Canals et al., 2012). Also, the phosphorylation of serine and threonine residues at the cytoplasmic C-terminal tail of ACKR3 has been implicated in ACKR3 internalization, chemokine scavenging, and receptor-arrestin interactions (Ray et al., 2012).

There are some controversies regarding the involvement of ACKR3 in chemotaxis. Some reports suggest that ACKR3 induces migration of different cell types via ACKR3 exclusively (Rajagopal et al., 2010; Chen et al., 2015), while others report a role in migration by only modulating the CXCR4 function (Abe et al., 2014). Hence, this role of ACKR3 awaits further clarification.

Key Residues for Signaling in the ACKR3 Receptor.

In two studies, mutational analysis was performed to identify the key residues of ACKR3 in ligand binding (CXCL11 and CXCL12), recruitment of β -arrestins, the scavenging capacity of chemokines (Benredjem et al., 2017), and trafficking of ACKR3 (Canals et al., 2012). These key residues are shown in Fig. 2.

Key residues for CXCL11 and CXCL12 binding were mostly present in the extracellular loops. Surprisingly, no N-terminal residues of the receptor were required for CXCL12 binding in contrast to CXCL11 binding, highlighting the different binding mechanisms of these ligands (Benredjem et al., 2017). Certain C-terminal residues are ubiquitinated and very important for receptor internalization and recycling (Canals et al., 2012). Recently, the residues protected by CXCL12 were determined by radiolytic footprinting (Gustavsson et al., 2017).

Oligomerization of CXCR4 and ACKR3 Influences Signaling

CXCR4 and ACKR3 Homomerization. CXCR4 is known to potentially form dimers, and in accordance, it has been crystallized as a homodimer in the presence of various ligands (Wu et al., 2010; Qin et al., 2015). There is also evidence that CXCR4 might form higher-order oligomers, demonstrated using bimolecular fluorescence complementation (Armando et al., 2014). A FRET signal between CXCR4–cyan fluorescent protein and CXCR4–YFP could be detected in intact tumor cells, and when the energy transfer was decreased, by depletion of cholesterol in lipid rafts or using a transmembrane (TM) 4 peptide analog, tumor cells significantly lost their capacity to migrate toward CXCL12. Although the decrease in FRET signal does not necessarily imply a disruption of the homomer, it does suggest that changing the conformation of a CXCR4 homomer can influence signaling (Wang et al., 2006). The observation of ligand-induced conformational changes within the CXCR4 homodimer unit was also reported prior to this work (Percherancier et al., 2005). In addition, pertussis toxin treatment reduced the amount of CXCR4 oligomers detected by single-molecule microscopy, suggesting that these oligomers play a role in G protein–mediated signaling. In the same study, it was shown that CXCR4 dimers also have more tendency to internalize than monomers (Ge et al., 2017). However, as stated previously in the *Introduction*, increasing CXCR4 expression levels could also increase the amount of homomers present, which should

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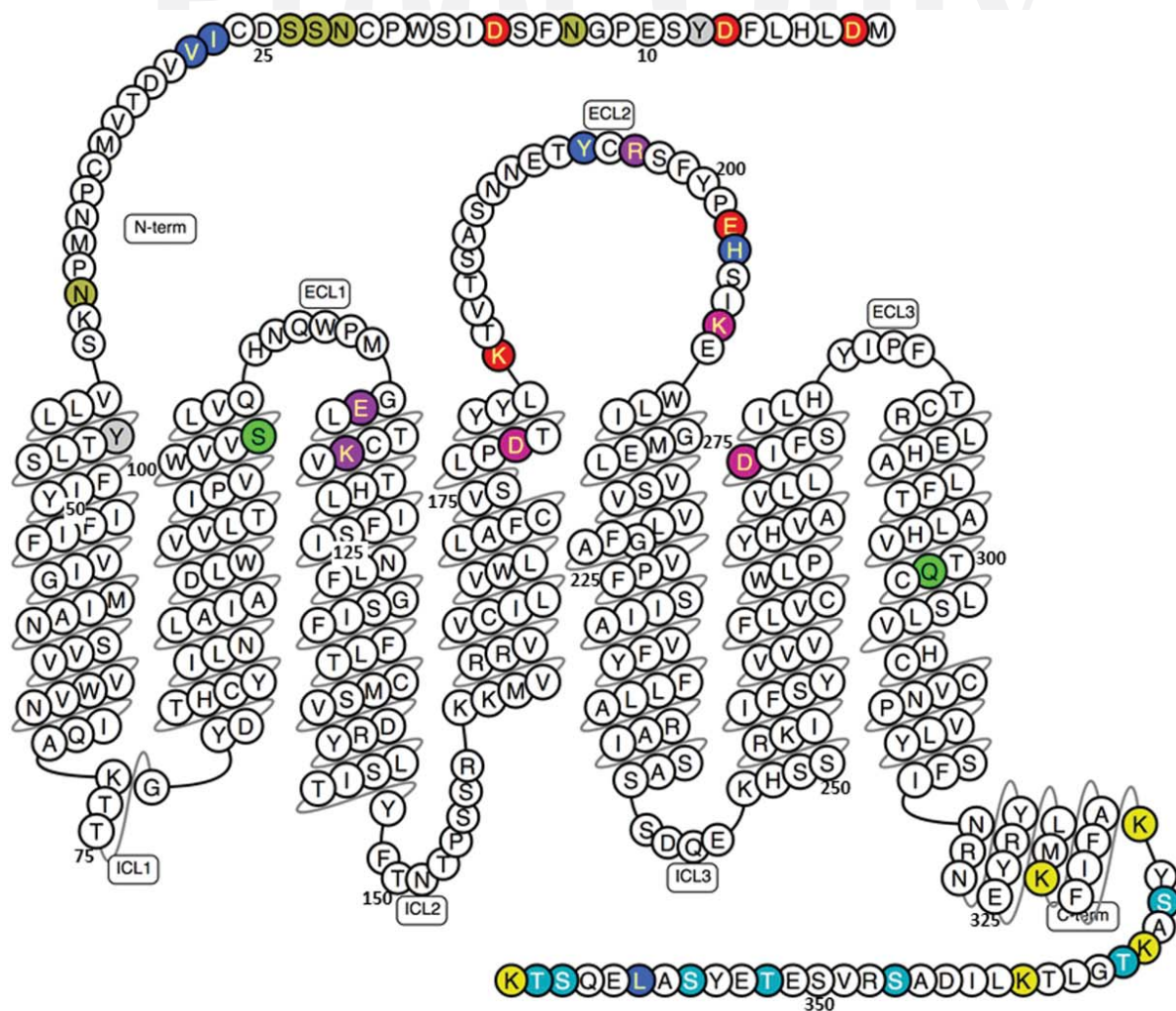
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Function	Residue
Potential sulfation	Y8 ¹ , Y45 ¹
Potential glycosylation	N13 ¹ , N22 ¹ , S23 ¹ , S24 ¹ , N39 ¹
CXCL12 binding	D179 ¹ , K206 ¹ , D275 ¹
Chemokine scavenging	S103 ¹ , Q301 ¹
Ubiquitination sites	K328 ² , K333 ² , K337 ² , K342 ² , K362 ²
ACKR3 activation	E114 ¹ , K118 ¹ , R197 ¹
Phosphorylation sites	S335 ² , T338 ² , S347 ² , T352 ² , S355 ² , S360 ² , T361 ²
CXCL11 binding	D2 ¹ , D7 ¹ , D16 ¹ , K184 ¹ , E202 ¹
Residues protected by CXCL12 (radiolytic footprinting)	I27 ³ , V28 ³ , Y195 ³ , H203 ³ , L357 ³

Fig. 2. Snake plot of human ACKR3 with highlighted residues important for receptor function as determined in the following studies: ¹Benredjem et al. (2017), ²Canals et al. (2012), and ³Gustavsson et al. (2017). Snake plot adapted from GPCRdb (Pándy-Szekeres et al., 2018).

be accounted for when using transfected cell lines. Meanwhile, using single-molecule microscopy, at very low expression levels, CXCR4 was predominantly present in a monomeric state, and increasing its expression levels led to a higher degree of oligomers. This could suggest that higher-order oligomers

might be present in cancer cells, where CXCR4 is expressed abundantly (Lao et al., 2017), which is consistent with the involvement of dimers in migration (Wang et al., 2006). Recently, nanoclusters of CXCR4 were also observed in Jurkat T cells using single-molecule tracking and super-resolution

Q:21 microscopy (Martínez-Muñoz et al., 2018). CXCL12 promoted the formation of these nanoclusters by decreasing the amount of monomers and dimers. The disruption of these nanoclusters using a TM6 analog strongly impaired CXCR4 functioning, suggesting that not only dimers but also bigger clusters of CXCR4 might be involved in signaling. Coexpression of cluster of differentiation 4 (CD4) or inhibition of the actin cytoskeleton reduced the size of CXCR4 nanoclusters and hence reduced the Ca^{2+} flux (Martínez-Muñoz et al., 2018). So, the presence of CD4 in the cellular system seems to be important when interpreting the signaling outcome mediated via CXCR4.

The dimeric interface in the crystal structure of CXCR4 consists of the fifth and sixth transmembrane domains when the receptor is in complex with IT1t (a specific small-molecule antagonist), and of the third and fourth helix when it is in complex with CVX15 (a small cyclic peptide) (Wu et al., 2010). However, mutations in those regions did not significantly decrease the specific BRET signal detected between luciferase- and green fluorescent protein-tagged CXCR4 receptors, indicating that multiple homomerization interfaces might exist (Hamatake et al., 2009). Since evidence exists that dimerization has an influence on CXCR4 signaling (Ge et al., 2017), the dimer conformation might also have important consequences in downstream activation. Since different ligands can induce different conformational changes, it can be speculated that these ligands can also lead to different homodimer interfaces, as could be seen for the crystal structures of CXCR4 (Wu et al., 2010). Hypothetically, these complexes could have different signaling properties (Percherancier et al., 2005).

To our knowledge, two publications suggest the existence of constitutive ACKR3 homomers in transfected HEK293T cells. In both papers, a specific BRET signal was observed between ACKR3-RLuc and ACKR3-YFP (Kalatskaya et al., 2009; Levoye et al., 2009). The costimulation with CXCL12 and AMD3100 caused an increase in the BRET signal between the tagged ACKR3 receptors that was significantly higher than when using CXCL12 alone, which is in accordance with the idea that AMD3100 might be an allosteric agonist for ACKR3 (Kalatskaya et al., 2009). Yet, no other publications focused on ACKR3 homomerization.

CXCR4 and ACKR3 Heteromerization. CXCR4 function can be influenced by the interaction with other receptors, as shown by many publications that demonstrated CXCR4 heteromerization or cross-regulation with/via other chemokine receptors. The occurrence of heterodimers might be feasible, since chemokine receptors are often coexpressed in the same cell types and, in some cases, even bind the same chemokines. For example, several studies using transfected cells showed that CXCR4 is able to form heteromers with CCR2, CCR7, CCR5, and CXCR3, among others.

In the first example, using BRET assays, CXCR4 was shown to heteromerize with CCR2, and coactivation of both coexpressed receptors led to a potentiation in Ca^{2+} release. In addition, this heteromer has been shown to recruit β -arrestin-2 using bimolecular fluorescence complementation. However, using BRET again, it has been seen that while the CXCR4 homodimer was able to recruit the $\text{G}\alpha_{13}$ protein, the CCR2/CXCR4 heteromer completely lost this ability (Armando et al., 2014). Moreover, in radioligand binding assays, binding of the respective chemokines to either CCR2 or CXCR4 impaired chemokine binding to the other receptor,

suggesting a negative cooperativity within the heteromer. This has been shown in recombinant cells as well as in primary leukocytes, where CCR2 and CXCR4 are endogenously present, suggesting that these two receptors might form heteromers even in a native context (Sohy et al., 2007). In the second example, CXCR4 not only formed heteromers with CCR7, as shown by proximity ligation assay, but also required the presence of CXCR4 to be properly expressed on the CD4+ T-cell membrane. When activated by the HIV glycoprotein gp120, CXCR4 enhanced CCR7-mediated migration of CD4+ T cells to the lymph nodes, significantly facilitating HIV infection (Hayasaka et al., 2015). In another study, using bimolecular fluorescence complementation, Hammad et al. (2010) showed that CCR5 homomers could interact with an important GPCR regulatory protein named Na^+/H^+ exchanger regulatory factor 1 (NHERF1). However, upon formation of CCR5/CXCR4 heterodimers, this receptor could no longer interact with NHERF1. Therefore, one should account for heteromerization when targeting CCR5 in HIV infection (Hammad et al., 2010). In the last case, the existence of CXCR3/CXCR4 heteromers has been seen by coimmunoprecipitation, saturation BRET, time-resolved FRET, and GPCR-heteromer identification technology. A negative cooperativity for ligand binding was observed as well for CXCR3/CXCR4 heteromers. Addition of a CXCR3 antagonist impaired CXCL12 binding to CXCR4, but not the other way around. This heteromer could specifically recruit β -arrestin-2 according to an analysis that used GPCR-heteromerization identification technology (Watts et al., 2013).

CXCR4 has also been suggested to heteromerize with other class A GPCRs, such as adrenergic and opioid receptors (Pello et al., 2008; Tripathi et al., 2015; Gao et al., 2018). For example, activation of the α_1 -adrenergic receptor (AR) led to the recruitment of β -arrestin-2 to CXCR4, and a specific agonist of α_1 -AR induced the internalization of CXCR4, as shown using the PRESTO-Tango assay in HEK293 cells. Neither of these effects could be inhibited by AMD3100 or the 12G5, an antagonist and internalization-blocking CXCR4 antibody, respectively, but both could be abolished by disrupting the heteromer using a peptide analog of TM2 of CXCR4, suggesting a tight cross-regulation within the α_1 -AR/CXCR4 complex (Gao et al., 2018). In addition, CXCR4 also influences the adrenergic function (Tripathi et al., 2015). α_1 -AR/CXCR4 heteromers were detected in a completely endogenous context, on the cell surface of rat and human VSMCs, via a proximity ligation assay. Disrupting the α_1 -AR/CXCR4 heteromer with a TM2 analog of CXCR4 or CXCR4 silencing impaired the association of these two receptors, as well as inhibited adrenergic-mediated responses in response to an agonist, such as Ca^{2+} mobilization or myosin light chain 2 phosphorylation. As a result, the authors proposed that targeting the α_1 -AR/CXCR4 heteromer might be an alternative for the current medications against α_1 -AR to modulate blood pressure (Tripathi et al., 2015). The significance of this work comes from it being an exceptional example of detecting oligomers at endogenous expression levels *in vivo*, rather than detection of overexpressed receptor probes with epitope tags.

Another example of how such cross-talk can affect currently used treatments is the cross-talk between CXCR4 and the opioid receptors. In mice studies, CXCR4 activation by CXCL12 decreased the effect of antinociceptive drugs on the μ - and δ -opioid receptors, but activation of these opioid

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receptors did not desensitize CXCR4 (Chen et al., 2007). A cross-desensitization in both directions could be detected only between CXCR4 and the κ -opioid receptor in several cell lines and in vivo (Finley et al., 2008). Such evidence suggests that the effect of painkillers is decreased when CXCR4 is present. Nonetheless, only CXCR4/ δ -opioid receptor heteromers have been observed using FRET experiments (Pello et al., 2008); thus, the cross-talk between CXCR4 and the other opioid receptors might not necessarily be due to heteromerization, but rather as a consequence of sharing the same intracellular signaling pathways.

Not only human receptors from the class A GPCRs are able to change the signaling of CXCR4, but also some viruses can take advantage of the alterations in receptor signaling potentially caused by heteromerization. For example, the Epstein-Barr virus encodes in its genome a viral GPCR named BILF1, which heteromerizes with human CXCR4 according to BRET experiments. Coexpression of the constitutively active BILF1 impairs CXCL12 binding to CXCR4 and, ultimately, the CXCL12-mediated G protein signaling (Nijmeijer et al., 2010).

Altogether, the function of CXCR4 seems to be strongly dependent on the interacting partners found in the cells, and consequently, it significantly varies between cell types. It is important to keep in mind that the change in the CXCR4 function due to the presence of certain proteins is not always due to oligomerization, but can also be due to a cross-talk in signaling pathways. In pathology, the degree of oligomerization and the type of oligomers could be heavily altered. For example, using BRET, the authors observed that CXCR4-warts hypogammaglobulinemia, infections, myelokathexis mutants can oligomerize with the wild-type CXCR4 and possibly retain it at the plasma membrane (Lagane et al., 2008).

Regarding ACKR3 heteromerization, there is evidence of the presence of α_1 -AR:ACKR3:CXCR4 hetero-oligomers in VSMCs, and the activation of ACKR3 can lead to the inhibition of the α_1 -AR activity (Albee et al., 2017). ACKR3 is also known to interact with the epithelial growth factor receptor (EGFR) in a β -arrestin-2-dependent manner and is implicated in the phosphorylation of the EGFR. Together, they are involved in mitosis of breast cancer cells (Salazar et al., 2014).

Cross-Talk between CXCR4 and ACKR3. Upon the discovery of ACKR3 as a receptor that can also bind CXCL12, which was previously known as a CXCR4-exclusive chemokine (Balabanian et al., 2005a), several studies focused on coexpression of CXCR4 and ACKR3 in diverse cell types and the influence of a possible CXCR4:ACKR3 interaction and/or cross-talk on the signaling properties. CXCR4 and ACKR3 are coexpressed in diverse cell types. These include human T and B lymphocytes (Balabanian et al., 2005a), dendritic cells (Infantino et al., 2006), monocytes (Sánchez-Martín et al., 2011), renal progenitor cells (Mazzeinghi et al., 2008), VSMCs (Evans et al., 2016), vascular endothelial cells (Schutyser et al., 2007), and zebrafish primordial germ cells (Boldajipour et al., 2008).

A number of studies hypothesized that ACKR3 might regulate CXCR4 activity by scavenging or segregating CXCL12. ACKR3 generates a gradient of available ligand for CXCR4, thus finely tuning CXCR4-mediated cellular signaling and hence controlling, for example, primordial germ cell

migration in zebrafish (Boldajipour et al., 2008; Naumann et al., 2010). The work of Naumann et al. (2010) suggested that the modulation of CXCR4 activation via ACKR3 is achieved by the scavenging activity of ACKR3, rather than heterodimerization, as they did not observe any cointernalization of these receptors.

Other studies shifted the focus more onto the mechanisms that may be involved, including the physical interaction of both receptors and subsequent modulation of their functions. For example, ACKR3 inhibition can act as a negative modulator of CXCR4-mediated lymphocyte integrin adhesiveness in human T lymphocytes and CD34+ cells (Hartmann et al., 2008). In this case, ACKR3-mediated modulation of CXCR4 activation was suggested to be due to a physical interaction between the two receptors. Indeed, the hetero-oligomerization of CXCR4/ACKR3 in intact HEK293 cells in the absence of CXCL12 was demonstrated using the FRET acceptor photobleaching method (Sierro et al., 2007). This study also highlighted that their coexpression potentiated Ca^{2+} flux mediated by CXCR4 activation and delayed ERK phosphorylation.

A follow-up study investigating CXCR4/ACKR3 hetero-oligomerization confirmed the heteromer formation in HEK293T cells using BRET. However, they showed a negative modulation of the Ca^{2+} flux when both receptors were coexpressed. In accordance with this result, GTP binding potency of $G\alpha_i$ upon CXCR4 activation with CXCL12 decreased in cells coexpressing ACKR3. Moreover, ACKR3 coexpression with CXCR4 in HEK293 cells induced a conformational change between the precoupled CXCR4-YFP and $G\alpha_i$ -RLuc. The same study also demonstrated that knockdown of ACKR3 expression in T lymphocytes resulted in more potent migration at lower CXCL12 concentrations, addressing the scavenging function of ACKR3 (Levoje et al., 2009).

Another study also linked direct interactions of CXCR4/ACKR3 with oligomerization-specific functional outcomes (Decaillet et al., 2011). In this case, the evidence of CXCR4/ACKR3 hetero-oligomerization comes from the coimmunoprecipitation of overexpressed CXCR4-C9 and ACKR3-FLAG in HEK293 cells. In the same study, coexpression of ACKR3 with CXCR4 inhibited CXCR4/ $G\alpha_i$ -mediated inhibition of cAMP production. In addition, activation of ACKR3 with CXCL11 restored CXCR4-dependent inhibition of cAMP production. Moreover, expression of CXCR4 increased the constitutive and ligand-induced recruitment of β -arrestin to ACKR3 heteromers, enhanced β -arrestin-mediated ERK phosphorylation, and increased migration of rat VSMCs (Decaillet et al., 2011).

Some caution must be taken when studying CXCR4/ACKR3 signaling, since their endogenous expression patterns can differ in different cell types and might influence the outcome of the experiments. Regarding drug development, one must acknowledge the complexity of targeting CXCR4 in different diseases and tissues, since heteromerization or cross-talk with other receptors can strongly impact its signaling.

Location of CXCR4 and ACKR3 Can Influence Receptor Signaling

Signaling of CXCR4 in Microdomains. As CXCR4 is expressed in diverse tissues, different microenvironments

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within different cell types play an important role in the manner of CXCR4 signaling. CXCR4 localizes to membrane rafts (Mañes et al., 2000), which are microdomains enriched in cholesterol, sphingolipids, and proteins (Brown and London, 1998). The presence of cholesterol in these rafts seems to play an important role in CXCL12 binding (Nguyen and Taub, 2002), and the activation of CXCR4 can lead to cross-activation of other membrane proteins, such as human epidermal growth factor receptor 2 (HER2) and EGFR in the raft (Conley-LaComb et al., 2016).

Upon activation of CXCR4, the receptor is rapidly internalized and can be either recycled back to the membrane or degraded at the lysosome (Marchese et al., 2003). Evidence suggests that phosphorylation of specific residues is involved in the determination between recycling and degradation (Marchese and Benovic, 2001). In renal cell carcinoma cells, CXCR4 moved to the cell nucleus after CXCL12 binding, and this nuclear location led to an increased Matrigel matrix invasion. In addition, histologic sections showed that CXCR4 was present in the nucleus only in metastatic renal cell carcinoma lesions (Wang et al., 2009). This might have important consequences for targeting CXCR4, since drugs would need to penetrate into the nucleus to attack metastatic cells. While the location of CXCR4 within a cell seems to be important, the location of these CXCR4-expressing cells within an organism might also influence outcomes. During the development of the lateral-line primordium of zebrafish, CXCR4 was present at the front cells while ACKR3 was at the back. This differential spacing might contribute to the establishment of a CXCL12 gradient that is important for the correct development of this species (Valentin et al., 2007; Donà et al., 2013).

Depending on its location, CXCR4 can activate different signaling pathways and can hence trigger different cellular responses. This might explain how CXCR4 has so many different roles in many organs and cell types and how its role might change in a pathologic condition, such as cancer.

Signaling of ACKR3 in Microdomains. In contrast to CXCR4, which is mostly expressed on the plasma membrane and the early and recycling endosomes, ACKR3 is mainly expressed on the membrane of endocytic vesicles in the resting state (Zhu et al., 2012). Shortening the receptor's C-terminal tail in ACKR3–green fluorescent protein increased membrane localization by up to 100% when the whole domain was missing. Although truncating the C terminus did not alter CXCL12 binding to the receptor, it significantly reduced the scavenging of the ligand as well as β -arrestin recruitment and activation of ERK1/2. In the presence of the dominant negative mutant K44A dynamin, all ACKR3 was located on the cell surface (Ray et al., 2012). This did not alter constitutive β -arrestin recruitment, but upon CXCL12 treatment, β -arrestin recruitment significantly increased and ERK phosphorylation lasted significantly longer. Thus, ACKR3 can show thorough signaling when localized exclusively to the plasma membrane without the chance to be internalized (Ray et al., 2012).

Meanwhile, upon chemokine ligand treatment, ACKR3 membrane expression over time did not decrease, as is the case for CXCR4, but after a small decrease, its presence on the membrane was slightly restored and resisted the depletion from the plasma membrane for a prolonged time. Furthermore, through radioligand internalization, it was demonstrated that

ACKR3 brings its chemokine ligands to degradation, confirming its role as a scavenger receptor (Naumann et al., 2010). In platelets, where CXCR4 and ACKR3 are both present, CXCL12 induced the internalization of CXCR4 but, at the same time, the externalization of ACKR3. This latter process was CXCR4-mediated, since blocking CXCR4 abolished ACKR3 externalization (Chatterjee et al., 2014). The same study showed that ERK1/2 phosphorylation was important for the cyclophilin A-mediated ubiquitination of ACKR3, an essential modification for the membrane location of ACKR3.

Some studies have observed ACKR3 predominantly on the membrane (Hattermann et al., 2012, 2014; Kumar et al., 2012). For example, in MCF-7 breast cancer cells, CXCR4 and ACKR3 were mostly observed on the membrane, using immunofluorescence light microscopy and electron microscopy. After CXCL11 or CXCL12 treatment, receptors were internalized individually or in proximity. A cross-talk between both receptors was also seen, since CXCL11 could induce the internalization of CXCR4 (Hattermann et al., 2014).

Discussion

In this review, we summarized how CXCR4 and ACKR3 signaling can be influenced by their expression levels, localization, and interacting proteins (cross-talk and oligomerization) (a summary can be found in Tables 1 and 2). All of these aspects have important consequences, especially when a GPCR is being targeted for drug development.

Many of the examples discussed in this review investigated CXCR4 and ACKR3 location and signaling in immortalized cell lines using an expression of reporter/recombinant proteins that was often much higher than endogenous expression levels. It is evident that these studies explain several crucial biologic outcomes that are governed by CXCR4 and ACKR3. However, it is worth noting that overexpression of receptors and/or downstream effectors might bias the receptor and downstream signaling behavior. Chabre et al. (2009) proposed, for example, a hypothesis for the apparent negative cooperativity between two receptors in ligand binding experiments: overexpression of receptors might lead to an insufficient amount of G proteins available for the receptor, causing receptor heterogeneity; some receptors would be coupled to a G protein, while others would not. These two states might present different affinities for the ligand and hence create an artificial negative cooperativity (Chabre et al., 2009). In addition to this, interaction partners can modulate the signaling properties of a receptor. Moreover, the cellular content (i.e., types and amounts of effector proteins that a receptor can activate) can also greatly influence the biologic outcomes of a specific receptor or receptor oligomer activation. Signaling pathways associated with the activation of CXCR4 and ACKR3 are vast. However, balance and dynamics of these pathways can be different in each tissue type. Thus, choice of a cell type while studying CXCR4 and ACKR3 oligomerization/signaling is crucial, and there is a need for studies in a more endogenous or disease-related context.

In various cell types, receptors can be found in different cellular compartments. Spatial and temporal aspects of chemokine receptor signaling may vary, depending on the receptor location in different cell and tissue types. The location of CXCR4 and ACKR3 can result in the activation of different signaling pathways. In targeting such receptors, such as in

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TABLE 1
Summary of CXCR4 signaling

	Secondary	Function	Effect	Secondary Effect	Studied System	Reference
G protein-dependent signaling Primary $G\alpha_{11}$, $G\alpha_{12}$, $G\alpha_{13}$	PI3K/Akt, inhibition of cAMP production	Cell survival and migration			HEK293 cells	Quoyer et al., 2013
	CXCR4 couples more effectively to $G\alpha_{11}$ and $G\alpha_{12}$ than to $G\alpha_{13}$ and $G\alpha_o$				Metastatic basal-like breast cancer cells T and B cells SF9 cells	Yagi et al., 2011 Shi et al., 2007 Kleemann et al., 2008
	Activation of Rho	Migration			Jurkat T cells Metastatic basal-like breast cancer cells Human PBMC T cells	Tan et al., 2006 Yagi et al., 2011 Kumar et al., 2011
$G\alpha_q$	Activation of PLC- β subfamily	Trafficking of CXCR4 into endosomes			Activation in dendritic cells and granulocytes No activation in T and B cells	Soede et al., 2001 Shi et al., 2007
G protein-independent signaling Primary β -arrestin-1	Attenuation of G protein signaling Increased internalization of CXCR4 (only with GRK2)	Reduced inhibition of cAMP production			HEK293 cells HEK293 cells	Cheng et al., 2000 Cheng et al., 2000
	Attenuation of G protein signaling Increased internalization of CXCR4	Reduced inhibition of cAMP production			HEK293 cells HEK293 cells	Cheng et al., 2000 Cheng et al., 2000
β -arrestin-2	Desensitization p38-MAPK	Decreased G protein signaling Chemotaxis			Lymphocytes HeLa cells	Fong et al., 2002 Sun et al., 2002
Influence on signaling due to coexpression/oligomerization Influence on/oligomer with... CXCR4 (homomer)			Involved in CXCL12-mediated migration		HEK293 cells, HeLa cells, human nonsmall lung carcinoma cell line (NCI-H2126) T-Rex 293 cells	Wang et al., 2006 Ge et al., 2017
			CXCL12-mediated G protein signaling Dimers internalize more than monomers		T-Rex 293 cells	Ge et al., 2017

(continued)

TABLE 1—Continued

	Secondary	Function	Effect	Secondary Effect	Studied System	Reference
CCR2			Nanoclusters implicated in signaling G α_z activation \rightarrow Ca $^{2+}$ mobilization Negative binding cooperativity between CXCR4 and CCR2 Facilitates HIV infection		Human T-cells HEK293 cells	Martinez-Munoz et al., 2018 Armando et al., 2014
CCR7			Heteromer loses interaction with NHERF1 Heteromer recruits β -arrestin-2 Negative binding cooperativity of CXCR3 onto CXCR4 β -arrestin-2 recruitment to CXCR4 Internalization of CXCR4		CHO-K1 cells, primary leukocytes CD4 T-cells HEK293 cells HEK293 cells HEK293 cells HEK293 cells	Sohy et al., 2007 Hayasaka et al., 2015 Hamad et al., 2010 Watts et al., 2013 Watts et al., 2013 Gao et al., 2018
CCR5			Reduced migration of CXCR4 toward CXCL12 Activating CXCR4 potentiates effect of α_1 -AR agonists		Human vascular smooth muscle cells Human vascular smooth muscle cells Rat and human vascular smooth muscle cells In vivo (mice)	Gao et al., 2018 Gao et al., 2018 Gao et al., 2018 Tripathi et al., 2015
α_1 -AR			CXCL12 decreases effect of antinociceptive drugs CXCL12 decreases effect of antinociceptive drugs		In vivo (mice) In vivo (mice)	Chen et al., 2007 Chen et al., 2007
μ -OR					MM-1, IM-9, HEK293, Jurkat, T-cell leukemia cell lines, Human primary monocytes	Pello et al., 2008
δ -OR					Jurkat T cells, primary human neutrophils, murine B cells, mice HEK293 cells	Finley et al., 2008 Nijmeijer et al., 2010
κ -OR			Cross-desensitization between CXCR4 and κ -OR		Zebratfish	Boldajipour et al., 2008
BILF1 (viral)			Impairs G protein signaling by CXCR4 ACKR3 scavenges CXCL12 from CXCR4	Fine tuning of primordial germ cell migration		
ACKR3					Daudi cells, MDCK, HeLa cells, Raji B cells, HUVECs, zebrafish, mouse hearts, human umbilical cords Zebratfish lateral line primordium	Naumann et al., 2010 Donà et al., 2013
		Creating chemokine gradient for migration	ACKR3 defines directionality of migration ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness	Creating chemokine gradient for migration	Zebratfish lateral line primordium T lymphocytes, CD34+ cells	Dambly-Chaudière et al., 2009 Hartmann et al., 2008

(continued)

TABLE 1—Continued

Secondary	Function	Effect	Secondary Effect	Studied System	Reference
		Potentialiation of Ca ²⁺ flux ^a		HEK293 cells	Sierra et al., 2007
		Negative modulation of Ca ²⁺ flux ^a		HEK293 cells	Levoye et al., 2009
		Delay of ERK phosphorylation		HEK293 cells	Sierra et al., 2007
		Enhancement of p38 MAPK and SAPK pathways		HEK293 cells	Decallot et al., 2011
	Enhanced cell migration ^a	Heteromer constitutively recruits β-arrestin-2 ^a	Enhanced cell migration ^a	HEK293 cells, MDA-MB-231, U87 cells	Decallot et al., 2011
		Reduced inhibition of cAMP production		HEK293 cells	Levoye et al., 2009
		Decreased potency for ³⁵ GTP-γS binding after CXCR4 activation		HEK293 cells	Levoye et al., 2009
		ACKR3 inhibits migration by CXCR4 for low CXCL12 dose ^a		T lymphocytes	Levoye et al., 2009

Q:38,39 ³⁵GTP-γS, guanosine 5'-O-(3-³⁵S)thiotriphosphate; MAPK, mitogen-activated protein kinase; OR, XXX; PBMC, XXX; PI3K, XXX; PLC-β, XXX; SAPK, stress-activated protein kinase.

^aControversies between studies.

cancer, knowing the cellular location of the receptor is relevant—for instance, CXCR4 can localize and signal at the nuclear membrane of metastatic cells (Wang et al., 2009).

Despite several reports using diverse types of assays, GPCR oligomerization is still highly disputed. While certain reports demonstrate oligomerization of a certain receptor, others may dispute. This is mostly due to the type of assays that are used and even the manner of setting up the experimental conditions and analysis methods for a certain assay. Despite giving valuable information on receptor-receptor interactions, energy transfer-based methods BRET and FRET lack the ability to elucidate the kinetics of individual events. Since the observed resonance energy transfer signal comes from all of the receptors within a cell or a pool of cells, it is not possible to resolve whether the observed signal is due to a stable or transient interaction. With the help of advanced imaging methods, it is now possible to track the movements and interactions of single receptors with other receptors and interacting partners with high spatiotemporal precision (Sungkaworn et al., 2017). Such methods, combined with fluorescent labeling of endogenous receptors with minimal tags (Coin et al., 2013), can open a new avenue to study receptor-receptor and receptor-effector interactions with superior spatial and temporal resolution at endogenous expression levels in biologically relevant cell types. It is also worth recognizing the importance of knockout studies, as these can demonstrate the role of receptors and/or effectors in certain cellular signaling pathways and their consequent biologic importance in both health and disease conditions. Advancing CRISPR technologies have recently been used to study signaling bias and cross-activation of signaling pathways (Grundmann et al., 2018). Such studies can also be extended toward GPCR oligomerization, i.e., knocking out one of the heteromerizing partners, or knocking out a downstream effector that is believed to be activated only in the case of a heteromer activation, and studying its effects on downstream signaling.

A heteromer can have completely different signaling properties in comparison with the monomers (Milligan, 2009; Urizar et al., 2011). Thus, therapeutically targeting one particular GPCR might be too simplistic. As evidence on the biologic significance of class A GPCR heteromerization is increasing, targeting the pathologically relevant heteromers can be a novel approach to therapy. As allosteric modulators of GPCR dimers, bivalent ligands that could specifically target a heteromer might be an option for future investigation into whether it has therapeutic potential. However, determining to what extent oligomerization is relevant in vivo yet remains as a crucial question to be answered.

Overall, in this review, we focused on the advances in the signaling properties of CXCR4 and ACKR3 in a health and disease context. Previous studies shed light on distinct outcomes of complex cell-type-dependent signaling, receptor-receptor interactions, and receptor cross-talk. However, our knowledge for an accurate picture of CXCR4/ACKR3-mediated signaling is still not complete. Since model cells and overexpressing systems might bias receptor location, receptor-receptor interaction, and signaling outcome, choice of experimental methods and cell types must be well considered. Yet, novel fluorescent labeling, advanced imaging, and genetic engineering in model organisms and primary cells, as well as computational and structural methods, will allow us to

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TABLE 2
Summary of ACKR3 signaling

Primary	Secondary	Function	Effect	Secondary Effect	Studied System	Reference
G protein-dependent signaling Primary $G\alpha_{i1}$	No activation, but a constitutive recruitment No activation ERK/Akt activation				HEK293 cells HEK293 cells Primary rodent astrocytes	Levoye et al., 2009 Hoffmann et al., 2012 Ódemis et al., 2012
$G\alpha_{i6}$ G protein-independent signaling Primary β -arrestin-1/2	Akt and MAPK activation JAK2/STAT3 pathway ERK1/2 phosphorylation No ERK1/2 phosphorylation Internalization Akt phosphorylation				Primary rodent astrocytes Human bladder cancer cells Glioma cells HEK293 cells Rat VSMCs HEK293 cells HEK293 cells Breast cancer cells PB CD34+ cells	Ódemis et al., 2012 Hao et al., 2012 Hattermann et al., 2010 Rajagopal et al., 2010 Rajagopal et al., 2010 Canals et al., 2012 Ray et al., 2012 Luker et al., 2010 Torossian et al., 2014
Influence on signaling due to coexpression/oligomerization Influence on/oligomer with... ACKR3 (homomer)						
α_1 -AR EGFR CXCR4			Unknown Negative regulation of α_1 -AR CXCR7 involved in phosphorylation of EGFR Mitosis of breast cancer cells ACKR3 scavenges CXCL12 from CXCR4	Fine tuning of primordial germ cell migration	HEK293 cells HEK293T cells Human VSMCs Breast cancer cells Breast cancer cells Zebrafish	Levoye et al., 2009 Kalatskaya et al., 2009 Albee et al., 2017 Salazar et al., 2014 Salazar et al., 2014 Boldajipour et al., 2008
				Creating chemokine gradient for migration	Daudi cells, MDCK, HeLa cells, Raji B-cells, HUVECs, zebrafish, mouse hearts, human umbilical cords Zebrafish lateral line primordium	Naumann et al., 2010 Donà et al., 2013
			ACKR3 defines directionality of migration ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness Potentiation of Ca^{2+} flux ^c Negative modulation of Ca^{2+} flux ^c Delay of ERK phosphorylation Enhancement of p38 MAPK and SAPK pathways Heteromer constitutively recruits β -arrestin-2 ^a Reduced inhibition of cAMP production		Zebrafish lateral line primordium T lymphocytes, CD34+ cells HEK293 cells HEK293 cells HEK293 cells HEK293 cells HEK293 cells, MDA-MB-231, U87 cells HEK293 cells	Dambly-Chaudière et al., 2009 Hartmann et al., 2008 Sierra et al., 2007 Levoye et al., 2009 Sierra et al., 2007 Decallot et al., 2011 Decallot et al., 2011 Decallot et al., 2011

(continued)

TABLE 2—Continued

Primary	Secondary	Function	Effect	Secondary Effect	Studied System	Reference
			Decreased potency for $^{35}\text{GTP-}\gamma\text{S}$ binding after CXCR4 activation		HEK293 cells	Levoye et al., 2009
		ACKR3 inhibits migration by CXCR4 for low CXCL12 dose ^a			T lymphocytes	Levoye et al., 2009

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³⁵GTP- γ S, guanosine 5'-O-(3-thio)triphosphate; JAK2, Janus kinase 2; MAPK, mitogen-activated protein kinase; PB, XXX; SAPK, stress-activated protein kinase; STAT3, signal transducer and activator of transcription 3. ^aControversies between studies.

study CXCR4 and ACKR3 signaling in a more endogenous and disease-related context in the near future.

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Wrote or contributed to the writing of the manuscript: Heuninck, Perpiñá Viciano, İşbilir, Caspar, Capoferri, Briddon, Durroux, Hill, Lohse, Milligan, Pin, Hoffmann.

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