Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Context-dependent signalling of CXC chemokine receptor 4 (CXCR4) and atypical chemokine receptor 3 (ACKR3)

Joyce Heuninck*, Cristina Perpiñá Viciano*, Ali Işbilir*, Birgit Caspar, Davide Capoferri, Stephen J. Briddon°, Thierry Durroux°, Stephen J. Hill°, Martin J. Lohse°, Graeme Milligan°, Jean-Philippe Pin°, Carsten Hoffmann#

* These autors contributed equally to this work.

° These authors have been listed alphabetically.

Corresponding author

Institut de Génomique Fonctionnelle (IGF), CNRS, Montpellier, France (JH, TD, JPP)

Institute of Pharmacology and Toxicology, University of Würzburg, Würzburg, Germany (CPV, CH, AI, MJL)

Institute for Molecular Cell Biology, Centre for Molecular Biomedicine (CMB), University Hospital Jena, Friedrich Schiller University Jena, Jena, Germany (CPV, CH)

Max Delbrück Center for Molecular Medicine, Berlin, Germany (AI, MJL)

Centre for Translational Pharmacology, Institute of Molecular, Cell, and Systems Biology, College of Medical, Veterinary and Life Sciences, University of Glasgow, Glasgow, UK (DC, GM)

Division of Pharmacology, Physiology and Neuroscience, School of Life Sciences, University of Nottingham, Nottingham, U.K. (BC, SJB, SJH)

Centre of Membrane Proteins and Receptors (COMPARE), University of Birmingham and University of Nottingham, The Midlands, U.K. (BC, SJB, SJH)

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Running Title: Context-dependent signalling of CXCR4 and ACKR3

Corresponding author:

Carsten Hoffmann; Institute for Molecular Cell Biology, Centre for Molecular Biomedicine (CMB), University Hospital Jena, Friedrich Schiller University Jena, Hans-Knöll-Str. 2, D-07745, Jena, Germany; Tel.: 0049 03641/9395600; Fax: 0049 03641/9395602; carsten.hoffmann@med.uni-jena.de

Text pages: 22

Tables: 2

Figures: 2

References: 142

Abstract word count: 248

Introduction word count: 744

Discussion word count: 888

Abbreviations:

ACKR3: Atypical Chemokine Receptor 3; AR: Adrenergic Receptor; BRET: Bioluminescence Resonance Energy Transfer; CCR: CC chemokine Receptor; CD: cluster of differentiation; CFP: Cyan Fluorescent Protein; CXCL: CXC chemokine Ligand; CXCR: CXC chemokine Receptor; EGFR: Endothelial Growth Factor Receptor; ERK: Extracellular signal-Regulated Kinase; FRET: Förster Resonance Energy Transfer; GFP: Green Fluorescent Protein; GPCR: G Protein-Coupled Receptor; GRK: G protein Receptor Kinase; HEK293: Human Embryonic Kidney cells 293; HER2: Human Epidermal Growth factor Receptor 2; HIV: Human Immunodeficiency Virus; ICL: Intracellular Loop; JAK2: Janus Kinase 2; MAPK: Mitogen-Activated Protein Kinase; NHERF1: Na^+/H^+ Exchanger Regulatory Factor 1; RLuc: Renilla Luciferase; SAPK: Stress-Activated Protein Kinase; STAT3: Signal Transducer and Activator of Transcription 3; TM: Trans-Membrane; VSMCs: Vascular Smooth Muscle Cells; WHIM: Warts Hypogammaglobulinemia, Infections, Myelokathexis; YFP: Yellow Fluorescent Protein

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Abstract

G protein-coupled receptors (GPCRs) are regulated by complex molecular mechanisms, both in physiological and pathological conditions, and their signalling can be intricate. Many factors influence their signalling behaviour, 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 CXCR4 and ACKR3, both members of the GPCR superfamily, are important and established therapeutic targets in relation to cancer, HIV infection and inflammatory diseases. Therefore, it is crucial to understand how the signalling of these receptors works to be able to specifically target them. In this review, we discuss how the signalling pathways activated by CXCR4 and ACKR3 can vary in different situations. G protein signalling 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 signalling spectrum upon heteromerisation with other receptors, such as CXCR4, endothelial growth factor receptor (EGFR) or the α_1-adrenergic receptor (α_1-AR). Also, CXCR4 forms heteromers with CCR2, CCR5, the Na^+/H^+ exchanger regulatory factor 1 (NHERF1), CXCR3, α_1-AR and the opioid receptors, which results in differential signalling to 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 signalling properties. In this review, we also provide an overview of the currently known critical amino acids involved in CXCR4 and ACKR3 signalling.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Introduction

G protein-coupled receptor (GPCR) signalling involves numerous factors that influence cellular functions. These include (i) the variety of ligands binding to the receptor, (ii) the kinetics of the processes, (iii) the location of the GPCR and (iv) the available interactome or cellular context.

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

(ii) GPCR activation is also influenced by the kinetics of both ligand binding and receptor signalling, which can possibly lead to the observation of bias profiles, such as in the case of the dopamine D_2 receptor (Klein Herenbrink et al., 2016).

(iii) Most GPCRs signal from the plasma membrane, where they gather in separate compartments rich in G proteins (Haung et al., 1997) and interact with other partners (Hur and Kim, 2002). Nevertheless, increasing evidence suggests that GPCRs also signal after internalisation (Calebiro et al., 2010; Vilardaga et al., 2014; Eichel and von Zastrow, 2018) and from subcellular sites, including the endoplasmic reticulum, Golgi apparatus and nucleus (Boivin et al., 2008; Godbole et al., 2017; Rebois et al., 2006). These internalised receptors could activate distinct signalling pathways to those activated by the same receptors at the cell surface.

(iv) Different cellular contexts contain different sets of proteins that may directly or indirectly interact with the GPCR and hence alter its signalling. Therefore, the signalling 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 signalling properties compared to 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.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Is this complexity in signalling 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 signalling 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 physiological conditions, CXCR4 is involved in vascularisation (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).

As 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; Raschioni et al., 2018; Zheng et al., 2017), and acts as a co-receptor for the HIV to enter host T-cells (Feng, 1996). The discovery of ACKR3 as another CXCL12 receptor added complexity to the understanding of the CXCR4/CXCL12 signalling axis (Balabanian et al., 2005a). ACKR3 is also overexpressed in many cancer types, playing an important role in tumour 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 crosstalk with CXCR4 and they are proposed to heteromerise (Balabanian et al., 2005a; Burns et al., 2006; Decaillot et al., 2011; Levoye et al., 2009). Several other receptors can also alter the function of CXCR4 and ACKR3, either through a functional crosstalk or as a consequence of heteromerisation (Becker et al., 2017; Dinkel et al., 2018; Martínez-Muñoz et al., 2014; Contento et al., 2008).

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 oligomerisation (Meyer et al., 2006). Hence, there is increasing interest in investigating their signalling 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 scope of CXCR4 and ACKR3 signalling in physiological and pathological conditions.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

1. CXCR4 and ACKR3 signalling

A number of signalling 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 signalling.

1.1. G protein-dependent signalling 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 and Förster Resonance Energy Transfer (B/FRET)-based techniques in transfected cells, which provides a very good model to study the possible signalling pathways triggered by a receptor. However, the disadvantage of such studies is the need to transfect cells, which could generate artefacts 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 $G\alpha_{o}$ 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 the $G\alpha_{i3}$ and $G\alpha_{o}$ (Quoyer et al., 2013; Kleemann et al., 2008). No activation of $G\alpha_{z}$, the only member of the $G\alpha_{i}$ family that is resistant to pertussis toxin, has been demonstrated, although the CXCR4/CCR2 hetero-oligomer is capable of stimulating $G\alpha_{z}$-driven Ca^{2+} mobilisation through the CCR2 receptor (Armando et al., 2014).

Besides 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 $G\alpha_{13}$. For example, migration of Jurkat T-cells in response to CXCL12 is not only controlled by $G\alpha_{i}$ through the activation of Rac, but also by $G\alpha_{13}$ 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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 non-cognate 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 polymerisation 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 signalling, 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 signalling properties of this GPCR, although some caution must be taken when comparing the different studies, since the assays employed could differ in their sensitivity and selectivity.

1.2. G protein-independent signalling through CXCR4

Like the majority of GPCRs, CXCR4 can also be regulated by β-arrestins at a number of levels, including CXCR4 internalisation, G protein signalling 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 internalisation of the receptor through clathrin-coated pits. Interestingly, co-expression of CXCR4 with β-arrestin-2 notably increased internalisation 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 internalisation highly depends on the phosphorylation state of CXCR4 (Cheng et al., 2000).

Several studies show that the arrestins attenuate G protein signalling. In human embryonic kidney 293 (HEK293) cells, overexpression of CXCR4 with either β-arrestins reduced inhibition of cAMP

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

production in response to CXCL12, indicating that both β-arrestin-1 and -2 play an important role in signalling regulation (Cheng et al., 2000). In accordance with this, using endogenous levels of CXCR4, lymphocytes isolated from β-arrestin-2 knock-out mice showed a decreased desensitisation and enhanced G protein coupling to CXCR4 (Fong et al., 2002). This attenuating effect on G protein signalling 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 internalisation and 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 ICL-3 of the receptor, as it was also first described in Wu et al. (Wu et al., 1997; Cheng et al., 2000). Overall, β-arrestins appear to regulate CXCR4 signalling 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 are the cause of a rare congenital disease named WHIM syndrome (or Warts, Hypogammaglobulinemia, Immunodeficiency, and Myelokathexis syndrome) (Hernandez et al., 2003; Balabanian et al., 2005b; Luo et al., 2017).

Lastly, 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 kinases (MAPK) pathway (Sun et al., 2002).

1.3. Kinetics of CXCR4 signalling

GPCR activation and downstream signalling kinetics have been extensively studied within the last two decades, with the aid of emerging fluorescence microscopy methods. Unlike for many other receptors (Lohse et al., 2008; Stumpf and Hoffmann, 2016), only few studies have been published on the kinetics of CXCR4 activation and its corresponding downstream signalling 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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 signalling (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 JAK2/3 kinases within a few seconds (Vila-Coro et al., 1999).

1.4. Key residues for signalling in the CXCR4 receptor

The ICL-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 signalling. 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 signalling 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 signalling (Wescott et al., 2016). A schematic summary including important residues relating to the function of CXCR4 is provided in Figure 1.

1.5. G protein-dependent signalling 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 signalling pathways typical of G proteins, in contrast to CXCR4. In fact, ACKR3 lacks the specific DRYLAIV motif in 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 the DRYLSIT is replaced by the corresponding DRYLAIV motif of CXCR4, failed to induce CXCL12-mediated signalling, such as G protein activation, intracellular Ca^{2+} mobilisation, G protein-mediated extracellular signal-regulated kinase (ERK) phosphorylation or chemotaxis (Hoffmann et al., 2012;

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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

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-YFP and $G\alpha_{i1}$-RLuc, which decreased upon treatment with GTP-γS, 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; Banisadr et al., 2016; Ödemis et al., 2012) 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 signalling, there is increasing evidence for a β-arrestin biased receptor in most cell types. Moreover, studies have shown that ACKR3 could modulate other cellular signalling pathways, potentially by forming a heteromeric complex with other receptors, which is discussed in a later section of this review.

1.6. G protein-independent signalling through ACKR3

Many studies show that ACKR3 can act as a "decoy" or "scavenging" receptor, since it can efficiently internalise its chemokine ligands CXCL11 and CXCL12 (Naumann et al., 2010). By internalising CXCL12, ACKR3 finely tunes the CXCL12 gradient necessary for the CXCR4-mediated migration (Boldajipour et al., 2008; Dambly-Chaudière et al., 2007; Donà et al., 2013). Nevertheless, ACKR3 is not only a "decoy" receptor and can also activate downstream pathways via β-arrestins, both in response to CXCL11 and CXCL12, directly promoting Akt and MAPK activity, ERK phosphorylation (Decaillot et al., 2011; Hattermann et al., 2010; Ödemis et al., 2012; Rajagopal et al., 2010; Torossian, 2013), and activation of the JAK2/STAT3 pathway (Hao et al., 2012). CXCL11-dependent ERK

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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

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 internalisation, 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 (Chen et al., 2015; Rajagopal et al., 2010), 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.

1.7. Key residues for signalling 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 Figure 2.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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 internalisation and recycling (Canals et al., 2012). Recently, the residues protected by CXCL12 were determined by radiolytic footprinting (Gustavsson et al., 2017).

2. Oligomerisation of CXCR4 and ACKR3 influences signalling

2.1. CXCR4 and ACKR3 homomerisation

CXCR4 is known to potentially form dimers and, in accordance, it has been crystallised 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-CFP and CXCR4-YFP could be detected in intact tumour cells and when the energy transfer was decreased, by depletion of cholesterol in lipid rafts or using a transmembrane (TM) 4 peptide analogue, tumour cells significantly lost their capacity to migrate towards 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 signalling (Wang et al., 2006). The observation of ligand-induced conformational changes within the CXCR4 homodimer unit was also reported prior to this work (Percheranier 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 signalling. In the same study, it has been shown that CXCR4 dimers also have more tendency to internalise 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 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

abundantly (Lao et al., 2017), which is consistent with the involvement of dimers in migration (Wang et al., 2006). Recently, also nanoclusters of CXCR4 have been observed in Jurkat T-cells using single molecule tracking and super resolution 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 analogue strongly impaired CXCR4 functioning, suggesting that not only dimers, but also bigger clusters of CXCR4 might be involved in signalling. Co-expression 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 signalling 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 GFP-tagged CXCR4 receptors, indicating that multiple homomerisation interfaces might exist (Hamatake et al., 2009). Since evidence exists that dimerisation has an influence on CXCR4 signalling (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 signalling 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 (Levoye et al., 2009; Kalatskaya et al., 2009). The co-stimulation 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 homomerisation.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

2.2. CXCR4 and ACKR3 heteromerisation

CXCR4 function can be influenced by the interaction with other receptors, as shown by many publications that demonstrate CXCR4 heteromerisation or cross-regulation with/via other chemokine receptors. The occurrence of heterodimers might be feasible, since chemokine receptors are often co-expressed 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 heteromerise with CCR2 and co-activation of both co-expressed 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 again BRET, it has been seen that while the CXCR4 homodimer was able to recruit the $G\alpha_{13}$ 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 be forming 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 in order 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. 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 heteromerisation when targeting CCR5 in HIV infection (Hammad et al., 2010). In the last case, the existence of CXCR3/CXCR4 heteromers has been seen by co-immunoprecipitation, saturation BRET, time-resolved FRET and GPCR-heteromer identification technology. A negative cooperativity for ligand binding was observed as well

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

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-heteromerisation identification technology (Watts et al., 2013).

CXCR4 has also been suggested to heteromerise with other class A GPCRs, such as adrenergic and opioid receptors (Gao et al., 2018; Tripathi et al., 2015; Pello et al., 2008). 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 internalisation of CXCR4, as shown using the PRESTO-Tango assay in HEK293 cells. Both of these effects could not be inhibited by AMD3100 or the 12G5, an antagonist and internalisation-blocking CXCR4 antibody, respectively, but could be abolished by disrupting the heteromer using a peptide analogue 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 analogue 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+} mobilisation 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 on how such crosstalk can affect currently used treatments is the crosstalk between CXCR4 and the opioid receptors. In mice studies, CXCR4 activation by CXCL12 decreased the effect of anti-nociceptive drugs on the μ- and δ-opioid receptors, but activation of these opioid 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 crosstalk between CXCR4 and the other opioid receptors might not

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

necessarily be due to heteromerisation, but rather as a consequence of sharing the same intracellular signalling pathways.

Not only human receptors from the class A GPCRs are able to change the signalling of CXCR4, but also some viruses can take advantage of the alterations in receptor signalling potentially caused by heteromerisation. For example, the Epstein Barr Virus encodes in its genome a viral GPCR named BILF1, which heteromerises with human CXCR4, according to BRET experiments. Co-expression of the constitutively active BILF1 impairs CXCL12 binding to CXCR4 and ultimately the CXCL12-mediated G protein signalling (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 oligomerisation, but can also be due to a crosstalk in signalling pathways. In pathology, the degree of oligomerisation and the type of oligomers could be heavily altered. For example, using BRET, the authors observed that CXCR4-WHIM mutants can oligomerise with the wild-type CXCR4 and possibly retain it at the plasma membrane (Lagane et al., 2008).

Regarding ACKR3 heteromerisation, 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).

2.3. Crosstalk 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 co-expression of CXCR4 and ACKR3 in diverse cell types and the influence of a possible CXCR4:ACKR3 interaction and/or crosstalk on the signalling properties. CXCR4 and ACKR3 are co-expressed 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

(Mazzinghi 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 hypothesised 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 signalling 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. suggested that the modulation of CXCR4 activation via ACKR3 is achieved by the scavenging activity of ACKR3, rather than heterodimerisation, as they did not observe any co-internalisation 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-oligomerisation of CXCR4/ACKR3 in intact HEK293 cells in the absence of CXCL12 was demonstrated using FRET acceptor photobleaching method (Sierro et al., 2007). This study also highlighted that their co-expression potentiated Ca^{2+} flux mediated by CXCR4 activation and delayed ERK phosphorylation.

A follow-up study investigating CXCR4/ACKR3 hetero-oligomerisation confirmed the heteromer formation in HEK293T cells using BRET. However, they showed a negative modulation of the Ca^{2+} flux when both receptors were co-expressed. In accordance with this result, GTP binding potency of $G\alpha_i$ upon CXCR4 activation with CXCL12 decreased in cells co-expressing ACKR3. Moreover, ACKR3 co-expression with CXCR4 in HEK293 cells induced a conformational change between the pre-coupled 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 (Levoye et al., 2009).

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Another study also linked direct interactions of CXCR4/ACKR3 with oligomerisation-specific functional outcomes (Decaillot et al., 2011). In this case, the evidence of CXCR4/ACKR3 hetero-oligomerisation comes from the co-immunoprecipitation of overexpressed CXCR4-C9 and ACKR3-FLAG in HEK293 cells. In the same study, co-expression 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 (Decaillot et al., 2011).

Some caution must be taken when studying CXCR4/ACKR3 signalling, 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 heteromerisation or crosstalk with other receptors can strongly impact its signalling.

3. Location of CXCR4 and ACKR3 can influence receptor signalling

3.1. Signalling of CXCR4 in microdomains

As CXCR4 is expressed in diverse tissues, different microenvironments within different cell types play an important role in the manner of CXCR4 signalling. CXCR4 localises 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 internalised and can either be recycled back to the membrane or be 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

nucleus after CXCL12 binding and this nuclear location led to an increased Matrigel matrix invasion. In addition, histological 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 signalling 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 pathological condition, such as cancer.

3.2. Signalling 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-GFP increased membrane localisation by up to 100% when the whole domain was missing. While 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 signalling when localised exclusively to the plasma membrane without the chance to be internalised (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.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Furthermore, through radioligand internalisation, it was demonstrated that ACKR3 brings its chemokine ligands to degradation, confirming its role as scavenger receptor (Naumann et al., 2010). In platelets, where CXCR4 and ACKR3 are both present, CXCL12 induced the internalisation of CXCR4, but at the same time the externalisation of ACKR3. This latter process was CXCR4-mediated, since blocking CXCR4 abolished ACKR3 externalisation (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., 2014; Kumar et al., 2012, Hattermann 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 internalised individually or in proximity. A crosstalk between both receptors was also seen, since CXCL11 could induce the internalisation of CXCR4 (Hattermann et al., 2014).

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Discussion

In this review, we summarised how CXCR4 and ACKR3 signalling can be influenced by their expression levels, localisation and also interacting proteins (crosstalk and oligomerisation) (a summary can be found in Table 1 and Table 2). All 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 signalling in immortalised cell lines, using an expression of reporter/recombinant proteins, often much higher than endogenous expression levels. It is evident that these studies explain several crucial biological 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 signalling behaviour. Chabre et al. 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 signalling 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 biological outcomes of a specific receptor or receptor oligomer activation. Signalling pathways associated to 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 oligomerisation/signalling 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 signalling 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 signalling pathways. In targeting such receptors, for example in cancer, knowing the cellular location

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

of the receptor is relevant - for instance, CXCR4 can localise 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 controverse. This is mostly due to the type of assays that are used, and even the way 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 RET 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 spatio-temporal precision (Sungkaworn et al., 2017). Such methods, combined with fluorescent labeling of endogenous receptors with minimal tags (Coin et al., 2011), 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 recognising the importance of knock-out studies, as these can demonstrate the role of receptors and/or effectors in certain cellular signalling pathways and their consequent biological importance both in health and disease conditions. Advancing CRISPR technologies have recently been used to study signalling bias and cross-activation of signalling pathways (Grundmann et al., 2018). Such studies can also be extended towards GPCR oligomerisation, i.e. knocking-out one of the heteromerising partners, or knocking-out a downstream effector that is believed to be activated only in case of a heteromer activation, and studying the effects of it on downstream signalling.

A heteromer can have completely different signalling properties in comparison to the monomers (Milligan, 2009; Urizar et al., 2011). Thus, therapeutically targeting one particular GPCR might be too simplistic. As evidence on the biological significance of class A GPCR heteromerisation 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

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

option for future investigation into whether it has therapeutic potential. However, determining to what extent oligomerisation is relevant *in vivo* yet remains as a crucial question to be answered.

Overall, in this review, we focused on the advances in the signalling properties of CXCR4 and ACKR3 in health and disease context. Previous studies shed light on distinct outcomes of complex cell-type dependent signalling, receptor-receptor interactions and receptor crosstalk. However, our knowledge for an accurate picture of CXCR4/ACKR3-mediated signalling is still not complete. Since model cells and overexpressing systems might bias receptor location, receptor-receptor interaction and signalling 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 in the near future allow us to study CXCR4 and ACKR3 signalling in a more endogenous and disease-related context.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Acknowledgements

We thank all other colleagues from the ONCORNET consortium for their helpful scientific discussions throughout all the meetings.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Heuninck, Perpiñá Viciano and Işbilir were responsible for the introduction, section 1, section 2 and the discussion. Caspar was responsible for section 3.1. Capoferri was responsible for section 3.2. Briddon, Durroux, Hill, Lohse, Milligan, Pin and Hoffmann provided critical feedback and contributed to the final version of the manuscript.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

References

Abe P, Mueller W, Schütz D, MacKay F, Thelen M, Zhang P, and Stumm R (2014) CXCR7 prevents excessive CXCL12-mediated downregulation of CXCR4 in migrating cortical interneurons. *Development* 141: 1857-1863.

Albee LJ, Eby JM, Tripathi A, LaPorte HM, Gao X, Volkman BF, Gaponenko V, and Majetschak M (2017) Alpha1-Adrenergic Receptors Function Within Hetero-Oligomeric Complexes With Atypical Chemokine Receptor 3 and Chemokine (C-X-C motif) Receptor 4 in Vascular Smooth Muscle Cells. *J Am Heart Assoc* 6(8): 1-17.

Armando S, Quoyer J, Lukashova V, Maiga A, Percherancier Y, Heveker N, Pin JP, Prézeau L, and Bouvier M (2014) The chemokine CXC4 and CC2 receptors form homo- and hetero-oligomers that can engage their signaling G-protein effectors and beta-arrestin. *FASEB J* 28(10): 4509-4523.

Balabanian K, Lagane B, Infantino S, Chow KYC, Harriague J, Moepps B, Arenzana-Seisdedos F, Thelen M, and Bachelerie F (2005a) The chemokine SDF-1/CXCL12 binds to and signals through the orphan receptor RDC1 in T lymphocytes. *J Biol Chem* 280(42): 35760-35766.

Balabanian K, Lagane B, Pablos JL, Laurent L, Planchenault T, Verola O, Lebbe C, Kerob D, Dupuy A, Hermine O, Nicolas JF, Latger-Cannard V, Bensoussan D, Bordigoni P, Baleux F, Le Deist F, Virelizier JL, Arenzana-Seisdedos F, and Bachelerie F (2005b) WHIM syndromes with different genetic anomalies are accounted for by impaired CXCR4 desensitization to CXCL12. *Blood* 105(6): 2249-2257.

Ballester LY, Loghavi S, Kanagal-Shamanna R, Barkoh BA, Lin P, Medeiros LJ, Luthra R, Patel KP (2016) Clinical Validation of a *CXCR4* Mutation Screening Assay for Waldenstrom Macroglobulinemia. *Clin Lymphoma Myeloma Leuk* 16(7): 395-403.

Banisadr G, Podojil JR, Miller SD, and Miller RJ (2016) Pattern of CXCR7 Gene Expression in Mouse Brain Under Normal and Inflammatory Conditions. *J Neuroimmune Pharmacol* 11(1): 26-35.

Becker M, Hobeika E, Jumaa H, Reth M, and Maity PC (2017) CXCR4 signaling and function require the expression of the IgD-class B-cell antigen receptor. *Proc Natl Acad Sci USA* 114(20): 5231-5236.

Benredjem B, Girard M, Rhainds D, St.-Onge G, and Heveker N (2017) Mutational Analysis of Atypical Chemokine Receptor 3 (ACKR3/CXCR7) Interaction with Its Chemokine Ligands CXCL11 and CXCL12. *J Biol Chem* 292(1): 31-42.

Berson JF, Long D, Doranz BJ, Rucker J, Jirik FR, and Doms RW (1996) A Seven-Transmembrane Domain Receptor Involved in Fusion and Entry of T-Cell-Tropic Human Immunodeficiency Virus Type 1 Strains. *J Virol* 70(9): 8.

Boivin B, Vaniotis G, Allen BG, and Hébert TE (2008) G protein-coupled receptors in and on the cell nucleus: a new signaling paradigm? *J Recept Signal Transduct Res* 28(1-2): 15-28.

Boldajipour B, Mahabaleshwar H, Kardash E, Reichman-Fried M, Blaser H, Minina S, Wilson D, Xu Q, and Raz E (2008) Control of Chemokine-Guided Cell Migration by Ligand Sequestration. *Cell* 132(3): 463-473.

Brelot A, Heveker N, Montes M, and Alizon M (2000) Identification of Residues of CXCR4 Critical for Human Immunodeficiency Virus Coreceptor and Chemokine Receptor Activities. *J Biol Chem* 275(31): 23736-23744.

Brown DA and London E (1998) Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* 14: 26.

Burns JM, Summers BC, Wang Y, Melikian A, Berahovich R, Miao Z, Penfold ME, Sunshine MJ, Littman DR, Kuo CJ, Wei K, McMaster BE, Wright K, Howard MC, and Schall TJ (2006) A novel chemokine receptor for SDF-1 and I-TAC involved in cell survival, cell adhesion, and tumor development. *J Exp Med* 203(9): 2201-2213.

Busillo JM and Benovic JL (2007) Regulation of CXCR4 signaling. *Biochim Biophys Acta* 1768(4): 952-963.

Busillo JM, Armando S, Sengupta R, Meucci O, Bouvier M, and Benovic JL (2010) Site-specific Phosphorylation of CXCR4 is Dynamically Regulated by Multiple Kinases and Results in Differential Modulation of CXCR4 Signaling. *J Biol Chem* 285(10): 7805-7817.

Calebiro D, Nikolaev VO, Persani L, and Lohse MJ (2010) Signaling by internalized G-protein-coupled receptors. *Trends Pharmacol Sci* 31(5): 221-228.

Canals M, Scholten DJ, de Munnik S, Han MK, Smit MJ, and Leurs R (2012) Ubiquitination of CXCR7 Controls Receptor Trafficking. *PLoS One* 7(3): e34192.

Ceholski DK, Turnbull IC, Pothula V, Lecce L, Jarrah AA, Kho C, Lee A, Hadri L, Costa KD, Hajjar RJ, and Tarzami ST (2017) CXCR4 and CXCR7 play distinct roles in cardiac lineage specification and pharmacologic beta-adrenergic response. *Stem Cell Res* 23: 77-86.

Chabre M, Deterre P, and Antonny B (2009) The apparent cooperativity of some GPCRs does not necessarily imply dimerization. *Trends Pharmacol Sci* 30(4): 182-187.

Chatterjee M, Seizer P, Borst O, Schönberger T, Mack A, Geisler T, Langer HF, May AE, Vogel S, Lang F, and Gawaz M (2014) SDF-1α induces differential trafficking of CXCR4-CXCR7 involving cyclophilin A, CXCR7 ubiquitination and promotes platelet survival. *FASEB J* 28(7): 2864-2878.

Chen Q, Zhang M, Li Y, Xu D, Wang Y, Song A, Zhu B, Huang Y, and Zheng JC (2015) CXCR7 Mediates Neural Progenitor Cells Migration to CXCL12 Independent of CXCR4. *Stem Cells* 33(8): 2574-2585.

Chen X, Geller EB, Rogers TJ and Adler MW (2007) Rapid heterologous desensitization of antinociceptive activity between mu or delta opioid receptors and chemokine receptors in rats. *Drug Alcohol Depend* 88(1): 6.

Cheng ZJ, Zhao J, Sun Y, Hu W, Wu YL, Cen B, Wu GX, and Pei G (2000) Beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem* 275(4): 7.

Coin I, Katritch V, Sun T, Xiang Z, Siu FY, Beyermann M, Stevens RC, and Wang L (2013) Genetically encoded chemical probes in cells reveal the binding path of urocortin-I to CRF class B GPCR. *Cell* 155(6): 1258-1269.

Conley-LaComb MK, Semaan L, Singareddy R, Li Y, Heath EI, Kim S, Cher ML, and Chinni SR (2016) Pharmacological targeting of CXCL12/CXCR4 signaling in prostate cancer bone metastasis. *Mol Cancer* 15(1): 68.

Contento RL, Molon B, Boularan C, Pozzan T, Manes S, Marullo S, and Viola A (2008) CXCR4-CCR5: a couple modulating T cell functions. *Proc Natl Acad Sci USA* 105(29): 10101-10106.

Cronshaw DG, Nie Y, Waite J, and Zou YR (2010) An Essential Role of the Cytoplasmic Tail of CXCR4 in G-Protein Signaling and Organogenesis. *PLoS One* 5(11): e15397.

Cui L, Qu H, Xiao T, Zhao M, Jolkkonen J, and Zhao C (2013) Stromal cell-derived factor-1 and its receptor CXCR4 in adult neurogenesis after cerebral ischemia. *Restor Neurol Neurosci* 31(3): 239-251.

Dambly-Chaudière C, Cubedo N, and Ghysen A (2007) Control of cell migration in the development of the posterior lateral line: antagonistic interactions between the chemokine receptors CXCR4 and CXCR7/RDC1. *BMC Dev Biol* 7: 23.

Decaillot FM, Kazmi MA, Lin Y, Ray-Saha S, Sakmar TP, and Sachdev P (2011) CXCR7/CXCR4 Heterodimer Constitutively Recruits β-Arrestin to Enhance Cell Migration. *J Biol Chem* 286(37): 32188-32197.

Dinkel BA, Kremer KN, Rollins MR, Medlyn MJ, and Hedin KE (2018) GRK2 mediates TCR-induced transactivation of CXCR4 and TCR-CXCR4 complex formation which drives PI3Kgamma/PREX1 signaling and T cell cytokine secretion. *J Biol Chem* 239(36): 14022-14039.

Donà E, Barry JD, Valentin G, Quirin C, Khmelinskii A, Kunze A, Durdu S, Newton LR, Fernandez-Minan A, Huber W, Knop M, and Gilmour D (2013) Directional tissue migration through a self-generated chemokine gradient. *Nature* 503: 285-289.

Doranz BJ, Orsini MJ, Turner JD, Hoffman TL, Berson JF, Hoxie JA, Peiper SC, Brass LF, and Doms RW (1999) Identification of CXCR4 Domains That Support Coreceptor and Chemokine Receptor Functions. *J Virol* 73(4): 10.

Eichel K and von Zastrow M (2018) Subcellular Organization of GPCR Signaling. *Trans Pharmacol Sci* 39(2): 200-208.

Evans AE, Tripathi A, LaPorte HM, Brueggemann LI, Singh AK, Albee LJ, Byron KL, Tarasova NI, Volkman BF, Cho TY, Gaponenko V, and Majetschak M (2016) New Insights into Mechanisms and Functions of Chemokine (C-X-C Motif) Receptor 4 Heteromerization in Vascular Smooth Muscle. *Int J Mol Sci* 17(5): 971-991.

Feng Y, Broder CC, Kennedy PE, and Berger EA (1996) HIV-1 Entry Cofactor: Functional cDNA Cloning of a Seven-Transmembrane, G Protein-Coupled Receptor. *Science* 272(5263): 872-877.

Ferré S, Casadó V, Devi LA, Filizola M, Jockers R, Lohse MJ, Milligan G, Pin JP, and Guitart X (2014) G Protein-Coupled Receptor Oligomerization Revisited: Functional and Pharmacological Perspectives. *Pharmacol Rev* 66: 413-434.

Finley MJ, Chen X, Bardi G, Davey P, Geller EB, Zhang L, Adler MW, and Rogers TJ (2008) Bi-Directional Heterologous Desensitization Between the Major HIV-1 Co-Receptor CXCR4 and the kappa-Opioid Receptor. *J Neuroimmunol* 197(2): 114-123.

Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, and Patel DD (2002) Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. *Proc Natl Acad Sci USA* 99(11): 7478-7483.

Gao X, Albee LJ, Volkman BF, Gaponenko V, and Majetschak M (2018) Asymmetrical ligand-induced cross-regulation of chemokine (C-X-C motif) receptor 4 by alpha1-adrenergic receptors at the heteromeric receptor complex. *Sci Rep* 8(1): 2730.

Ge B, Lao J, Li J, Chen Y, Song Y, and Huang F (2017) Single-molecule imaging reveals dimerization/oligomerization of CXCR4 on plasma membrane closely related to its function. *Sci Rep* 7(1): 16873.

Godbole A, Lyga S, Lohse MJ, and Calebiro D (2017) Internalized TSH receptors en route to the TGN induce local G_s-protein signaling and gene transcription. *Nat Commun* 8(1): 443.

Gravel S, Malouf C, Boulais PE, Berchiche YA, Oishi S, Fujii N, Leduc R, Sinnett D, and Heveker N (2010) The peptidomimetic CXCR4 antagonist TC14012 recruits beta-arrestin to CXCR7: roles of receptor domains. *J Biol Chem* 285(49): 37939-37943.

Grundmann M, Merten N, Malfacini D, Inoue A, Preis P, Simon K, Rüttiger N, Ziegler T, Benkel T, Schmitt NK, Ishida S, Müller I, Reher R, Kawakami K, Inoue A, Rick U, Kühl T, Imhof D, Aoki J, König GM, Hoffmann C, Gomeza J, Wess J, and Kostenis E (2018) Lack of beta-arrestin signaling in the absence of active G-proteins. *Nat Commun* 9: 341-356.

Guo Q, Gao BL, Zhang XJ, Liu GC, Xu F, Fan QY, Zhang SJ, Yang B, and Wu XH (2014) CXCL12-CXCR4 Axis Promotes Proliferation, Migration, Invasion, and Metastasis of Ovarian Cancer. *Oncol Res* 22(5-6): 247-258.

Gustavsson M, Wang L, van Gils N, Stephens BS, Zhang P, Schall TJ, Yang S, Abagyan R, Chance MR, Kufareva I, Handel TM (2017) Structural basis of ligand interaction with atypical chemokine receptor 3. *Nat Commun* 8:14135-14148.

Hamatake M, Aoki T, Futahashi Y, Urano E, Yamamoto N, and Komano J (2009) Ligand-independent higher-order multimerization of CXCR4, a G-protein-coupled chemokine receptor involved in targeted metastasis. *Cancer Sci* 100(1): 95-102.

Hammad MM, Kuang YQ, Yan R, Allen H, and Dupre DJ (2010) Na+/H+ exchanger regulatory factor-1 is involved in chemokine receptor homodimer CCR5 internalization and signal transduction but does not affect CXCR4 homodimer or CXCR4-CCR5 heterodimer. *J Biol Chem* 285(45): 34653-34664.

Hao M, Zheng J, Hou K, Wang J, Chen X, Lu X, Bo J, Xu C, Shen K, and Wang J (2012). Role of chemokine receptor CXCR7 in bladder cancer progression. *Biochem Pharmacol* 84(2): 204-214.

Hartmann TN, Grabovsky V, Pasvolsky R, Shulman Z, Buss EC, Spiegel A, Nagler A, Lapidot T, Thelen M, and Alon R (2008) A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J Leukoc Biol* 84(4): 1130-1140.

Hattermann K, Held-Feindt J, Lucius R, Muerkoster SS, Penfold ME, Schall TJ, and Mentlein R (2010) The Chemokine Receptor CXCR7 Is Highly Expressed in Human Glioma Cells and Mediates Antiapoptotic Effects. *Cancer Res* 70(8): 3299-3308.

Hattermann K, Mentlein R, and Held-Feindt J (2012) CXCL12 mediates apoptosis resistance in rat C6 glioma cells. *Oncol Rep* 27(5): 1348-1352.

Hattermann K, Holzenburg E, Hans F, Lucius R, Held-Feindt J, and Mentlein R (2014) Effects of the chemokine CXCL12 and combined internalization of its receptors CXCR4 and CXCR7 in human MCF-7 breast cancer cells. *Cell Tissue Res* 357(1): 253-266.

Haung CH, Hepler JR, Chen LT, Gilman AG, Anderson RGW, and Mumby SM (1997) Organization of G Proteins and Adenylyl Cyclase at the Plasma Membrane. *Mol Biol Cell* 8: 2365-2378.

Hayasaka H, Kobayashi D, Yoshimura H, Nakayama EE, Shioda T, and Miyasaka M (2015) The HIV-1 Gp120/CXCR4 axis promotes CCR7 ligand-dependent CD4 T cell migration: CCR7 homo- and CCR7/CXCR4 hetero-oligomer formation as a possible mechanism for up-regulation of functional CCR7. *PLoS One* 10(2): 1-19.

Hernandez PA, Gorlin RJ, Lukens JN, Taniuchi S, Bohinjec J, Francois F, Klotman ME, and Diaz GA (2003) Mutations in the chemokine receptor gene *CXCR4* are associated with WHIM syndrome, a combined immunodeficiency disease. *Nat Genet* 34(1): 70-74.

Hoffmann F, Müller W, Schütz D, Penfold ME, Wong YH, Schulz S, and Stumm R (2012) Rapid uptake and degradation of CXCL12 depend on CXCR7 carboxyl-terminal serine/threonine residues. *J Biol Chem* 287(34): 28362-28377.

Hur EM and Kim KT (2002) G-protein coupled receptor signalling and cross-talk: Achieving rapidity and specificity. *Cell Signal* 14(5): 9.

Infantino S, Moepps B, and Thelen M (2006) Expression and Regulation of the Orphan Receptor RDC1 and Its Putative Ligand in Human Dendritic and B Cells. *J Immunol* 176(4): 2197-2207.

Jordan BA and Devi LA (1999) G-protein-coupled receptor heterodimerization modulates receptor function. *Nature* 399(6737): 697-700.

Kalatskaya I, Berchiche YA, Gravel S, Limberg BJ, Rosenbaum JS, and Heveker N (2009) AMD3100 is a CXCR7 ligand with allosteric agonist properties. *Mol Pharmacol* 75(5): 1240-1247.

Kim TH, Chung KY, Manglik A, Hansen AL, Dror RO, Mildorf TJ, Shaw DE, Kobilka BK, and Prosser RS (2013) The Role of Ligands on the Equilibria Between Functional States of a G Protein-Coupled Receptor. *J Am Chem Soc* 135(25): 9465-9474.

Kleemann P, Papa D, Vigil-Cruz S, and Seifert R (2008) Functional Reconstitution of the Human Chemokine Receptor CXCR4 with G(i)/G(o)-Proteins in Sf9 Insect Cells. *Naunyn Schmiedebergs Arch Pharmacol* 378(3): 261-274.

Klein Herenbrink C, Sykes DA, Donthamsetti P, Canals M, Coudrat T, Shonberg J, Scammells PJ, Capuano B, Sexton PM, Charlton SJ, Javitch JA, Christopoulos A, and Lane JR (2016) The role of kinetic context in apparent biased agonism at GPCRs. *Nat Commun* 7: 10842.

Kremer D, Cui QL, Göttle P, Kuhlmann T, Hartung HP, Antel J, and Küry P (2016) CXCR7 Is Involved in Human Oligodendroglial Precursor Cell Maturation. *PLoS One* 11(1): 1-12.

Kumar A, Kremer KN, Dominguez D, Tadi M, and Hedin KE (2011) Galpha13 and Rho mediate endosomal trafficking of CXCR4 into Rab11+ vesicles upon stromal cell-derived factor-1 stimulation. *J Immunol* 186(2): 951-958.

Kumar R, Tripathi V, Ahmad M, Nath N, Mir RA, Chauhan SS, and Luthra K (2012) CXCR7 mediates Giα independent activation of ERK and Akt promotes cell survival and chemotaxis in T cells. *Cell Immunol* 272(2): 230-241.

Lagane B, Chow KY, Balabanian K, Levoye A, Harriague J, Planchenault T, Baleux F, Gunera-Saad N, Arenzana-Seisdedos F, and Bachelerie F (2008) CXCR4 dimerization and beta-arrestin-mediated signaling account for the enhanced chemotaxis to CXCL12 in WHIM syndrome. *Blood* 112(1): 34-44.

Lane JR, May TL, Parton RG, Sexton PM, and Christopoulos A (2017) A kinetic view of GPCR allostery and biased agonism. *Nat Chem Biol* 13: 929-937.

Lao J, He H, Wang X, Wang Z, Song Y, Yang B, Ullah Khan N, Ge B, and Huang F (2017) Single-Molecule Imaging Demonstrates Ligand Regulation of the Oligomeric Status of CXCR4 in Living Cells. *J Phys Chem B* 121(7): 1466-1474.

Levoye A, Balabanian K, Baleux F, Bachelerie F, and Lagane B (2009) CXCR7 heterodimerizes with CXCR4 and regulates CXCL12-mediated G protein signaling. *Blood* 113(24): 6085-6093.

Lohse MJ, Nikolaev VO, Hein P, Hoffmann C; Vilardaga JP, and Bünemann M (2008) Optical techniques to analyze real-time activation and signaling of G-protein-coupled receptors. *Trends Pharmacol Sci* 29(3): 159-165.

Luker KE, Steele JM, Mihalko LA, Ray P, and Luker GD (2010) Constitutive and Chemokine-dependent Internalization and Recycling of CXCR7 in Breast Cancer Cells to Degrade Chemokine Ligands. *Oncogene* 29(32): 4599-4610.

Luo J, Busillo JM, Stumm R, and Benovic JL (2017) G Protein-Coupled Receptor Kinase 3 and Protein Kinase C Phosphorylate the Distal C-Terminal Tail of the Chemokine Receptor CXCR4 and Mediate Recruitment of beta-Arrestin. *Mol Pharmacol* 91(6): 554-566.

Mañes S, del Real G, Lacalle RA, Lucas P, Gómez-Moutón C, Sánchez-Palomino S, Delgado R, Alcamí J, Mira E, and Martínez-A C (2000) Membrane raft microdomains mediate lateral assemblies required for HIV-1 infection. *EMBO rep* 1(2): 190-196.

Manglik A, Kim TH, Masureel M, Altenbach C, Yang Z, Hilger D, Lerch MT, Kobilka TS, Thian FS, Hubbell WL, Prosser RS, and Kobilka BK (2015) Structural Insights into the Dynamic Process of β2-adrenergic Receptor Signalling. *Cell* 161(5): 1101-1111.

Marchese A and Benovic JL (2001) Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem* 276(49): 45509-45512.

Marchese A, Chen C, Kim YM, and Benovic JL (2003) The ins and outs of G protein-coupled receptor trafficking. *Trends Biochem Sci* 28(7): 369-376.

Martínez-Muñoz L, Barroso R, Dyrhaug SY, Navarro G, Lucas P, Soriano SF, Vega B, Costas C, Muñoz-Fernández MÁ, Santiago C, Rodríguez Frade JM, Franco R, and Mellado M (2014) CCR5/CD4/CXCR4 oligomerization prevents HIV-1 gp120IIIB binding to the cell surface. *Proc Natl Acad Sci USA* 111(19): E1960-1969.

Martínez-Muñoz L, Rodríguez-Frade JM, Barroso R, Sorzano CÓS, Torreño-Pina JA, Santiago CA, Manzo C, Lucas P, García-Cuesta EM, Gutierrez E, Barrio L, Vargas J, Cascio G, Carrasco YR, Sánchez-Madrid F, García-Parajo MF, and Mellado M (2018) Separating Actin-Dependent Chemokine Receptor Nanoclustering from Dimerization Indicates a Role for Clustering in CXCR4 Signaling and Function. *Mol Cell* 70: 106-119.

Masureel M, Zou Y, Picard LP, van der Westhuizen E, Mahoney JP, Rodrigues JPGLM, Mildorf TJ, Dror RO, Shaw DE, Bouvier M, Pardon E, Steyaert J, Sunahara RK, Weis WI, Zhang C, and Kobilka BK (2018) Structural insights into binding specificity, efficacy and bias of a β_2AR partial agonist. *Nat Chem Biol* 14(11): 1059-1066.

Mazzinghi B, Ronconi E, Lazzeri E, Sagrinati C, Ballerini L, Angelotti ML, Parente E, Mancina R, Netti GS, Becherucci F, Gacci M, Carini M, Gesualdo L, Rotondi M, Maggi E, Lasagni L, Serio M, Romagnani S, and Romagnani P (2008) Essential but differential role for CXCR4 and CXCR7 in the therapeutic homing of human renal progenitor cells. *J Exp Med* 205(2): 479-490.

Meyer BH, Segura JM, Martinez KL, Hovius R, George N, Johnsson K, and Vogel H (2006) FRET imaging reveals that functional neurokinin-1 receptors are monomeric and reside in membrane microdomains of live cells. *Proc Natl Acad Sci USA* 103(7): 2138-2143.

Miao Z, Luker KE, Summers BC, Berahovich R, Bhojani MS, Rehemtulla A, Kleer CG, Essner JJ, Nasevicius A, Luker GD, Howard MC, and Schall TJ (2007) CXCR7 (RDC1) promotes breast and lung tumor growth in vivo and is expressed on tumor-associated vasculature. *Proc Natl Acad Sci USA* 104(40): 15735-15740.

Milligan G (2009) G protein-coupled receptor hetero-dimerization: contribution to pharmacology and cuntion. *Br J Pharmacol* 158: 5-14.

Naumann U, Cameroni E, Pruenster M, Mahabaleshwar H, Raz E, Zerwes HG, Rot A, and Thelen M (2010) CXCR7 functions as a scavenger for CXCL12 and CXCL11. *PLoS One* 5(2): 1-11.

Nguyen DH and Taub D (2002) CXCR4 Function Requires Membrane Cholesterol: Implications for HIV Infection. *J Immunol* 168(8): 4121-4126.

Nijmeijer S, Leurs R, Smit MJ, and Vischer HF (2010) The Epstein-Barr virus-encoded G protein-coupled receptor BILF1 hetero-oligomerizes with human CXCR4, scavenges Galphai proteins, and constitutively impairs CXCR4 functioning. *J Biol Chem* 285(38): 29632-29641.

Ödemis V, Lipfert J, Kraft R, Hajek P, Abraham G, Hattermann K, Mentlein R, and Engele J (2012) The presumed atypical chemokine receptor CXCR7 signals through G(i/o) proteins in primary rodent astrocytes and human glioma cells. *Glia* 60(3): 372-381.

Pándy-Szekeres G, Munk C, Tsonkov TM, Mordalski S, Harpsøe K, Hauser AS, Bojarski AJ, and Gloriam DE (2017) GPCRdb in 2018: adding GPCR structure models and ligands. *Nucleic Acids Res* 46(D1): D440-D446.

Panneerselvam J, Jin J, Shanker M, Lauderdale J, Bates J, Wang Q, Zhao YD, Archibald SJ, Hubin TJ, and Ramesh R (2015) IL-24 Inhibits Lung Cancer Cell Migration and Invasion by Disrupting the SDF-1/CXCR4 Signaling Axis. *PLoS One* 10(3): e0122439.

Pello OM, Martínez-Muñoz L, Parrillas V, Serrano A, Rodríguez-Frade JM, Toro MJ, Lucas P, Monterrubio M, Martínez-A C, and Mellado M (2008) Ligand stabilization of CXCR4/delta-opioid receptor heterodimers reveals a mechanism for immune response regulation. *Eur J Immunol* 38(2): 537-549.

Percherancier Y, Berchiche YA, Slight I, Volkmer-Engert R, Tamamura H, Fujii N, Bouvier M, and Heveker N (2005) Bioluminescence resonance energy transfer reveals ligand-induced conformational changes in CXCR4 homo- and heterodimers. *J Biol Chem* 280(11): 9895-9903.

Qin L, Kufavera I, Holden LG, Wang C, Zheng Y, Zhao C, Fenalti G, Wu H, Han GW, Cherezov V, Abagyan R, Stevens RC, and Handel TM (2015) Structural biology. Crystal structure of the chemokine receptor CXCR4 in complex with a viral chemokine. *Science* 347(6226): 1117-1122.

Quoyer J, Janz JM, Luo J, Ren Y, Armando S, Lukashova V, Benovic JL, Carlson KE, Hunt SW, and Bouvier M (2013) Pepducin targeting the C-X-C chemokine receptor type 4 acts as a biased agonist favoring activation of the inhibitory G protein. *Proc Natl Acad Sci USA* 110(52): E5088-5097.

Rajagopal S, Kim J, Ahn S, Craig S, Lam CM, Gerard NP, Gerard C, and Lefkowitz RJ (2010) Beta-arrestin- but not G protein-mediated signaling by the "decoy" receptor CXCR7. *Proc Natl Acad Sci USA* 107(2): 628-632.

Rapp C, Snow S, Laufer T, and McClendon CL (2013) The role of tyrosine sulfation in the dimerization of the CXCR4:SDF-1 complex. *Protein Sci* 22(8): 1025-1036.

Raschioni C, Bottai G, Sagona A, Errico V, Testori A, Gatzemeier W, Corsi F, Tinterri C, Roncalli M, Santarpia L, and Di Tommaso L (2018) CXCR4/CXCL12 Signaling and Protumor Macrophages in Primary Tumors and Sentinel Lymph Nodes Are Involved in Luminal B Breast Cancer Progression. *Dis Markers* 2018: 1-9.

Rasheed SA, Teo CR, Beillard EJ, Voorhoeve PM, Zhou W, Ghosh S, and Casey PJ (2015) MicroRNA-31 controls G protein alpha-13 (GNA13) expression and cell invasion in breast cancer cells. *Mol cancer* 14: 67.

Ray P, Mihalko LA, Coggins NL, Moudgil P, Ehrlich A, Luker KE, and Luker GD (2012) Carboxy-terminus of CXCR7 regulates receptor localization and function. *Int J Biochem Cell Biol* 44(4): 669-678.

Rebois RV, Robitaille M, Galés C, Dupré DJ, Baragli A, Trieu P, Ethier N, Bouvier M, and Hébert TE (2006) Heterotrimeric G proteins form stable complexes with adenylyl cyclase and Kir3.1 channels in living cells. *J Cell Sci* 119(13): 2807-2818.

Salazar N, Muñoz D, Kallifatidis G, Singh RK, Jordà M, and Lokeshwar BL (2014) The chemokine receptor CXCR7 interacts with EGFR to promote breast cancer cell proliferation. *Mol Cancer* 13: 198.

Salcedo R and Oppenheim JJ (2003) Role of chemokines in angiogenesis: CXCL12/SDF-1 and CXCR4 interaction, a key regulator of endothelial cell responses. *Microcirculation* 10(3-4): 359-370.

Sánchez-Martín L, Estecha A, Samaniego R, Sánchez-Ramón S, Ángel-Vera M, and Sánchez-Mateos P (2011) The chemokine CXCL12 regulates monocyte-macrophage differentiation and RUNX3 expression. *Blood* 117(1): 88-97.

Schönemeier B, Kolodziej A, Schulz S, Jacobs S, Hoellt V, and Stumm R (2008) Regional and Cellular Localization of the CXCL12/SDF-1 Chemokine Receptor CXCR7 in the Developing and Adult Rat Brain. *J Comp Neurol* 510(2): 207-220.

Schutyser E, Su Y, Yu Y, Gouwy M, Zaja-Milatovic S, van Damme J, and Richmond A (2007) Hypoxia enhances CXCR4 expression in human microvascular endothelial cells and human melanoma cells. *Eur Cytokine Netw* 18(2): 59-70.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Shi G, Partida-Sánchez S, Misra RS, Tighe M, Borchers MT, Lee JJ, Simon MI, and Lund FE (2007) Identification of an alternative Gaq-dependent chemokine receptor signal transduction pathway in dendritic cells and granulocytes. *J Exp Med* 204(11): 2705-2718.

Sierro F, Biben C, Martínez-Muñoz L, Mellado M, Ransohoff RM, Li M, Woehl B, Leung H, Groom J, Batten M, Harvey RP, Martínez-A C, Mackay CR, and Mackay F (2007) Disrupted cardiac development but normal hematopoiesis in mice deficient in the second CXCL12/SDF-1 receptor, CXCR7. *Proc Natl Acad Sci USA* 104(37): 14759-14764.

Soede RDM, Zeelenberg IS, Wijnands YM, Kamp M, and Roos E (2001) Stromal Cell-Derived Factor-1-Induced LFA-1 Activation During In Vivo Migration of T Cell Hybridoma Cells Requires Gq/11, RhoA, and Myosin, as well as Gi and Cdc42. *J Immunol* 166(7): 4293-4301.

Sohy D, Parmentier M, and Springael JY (2007) Allosteric Transinhibition by Specific Antagonists in CCR2/CXCR4 Heterodimers. *J Biol Chem* 282(41): 30062-30069.

Stumpf AD and Hoffmann C (2016) Optical probes based on G protein-coupled receptors - added work or added value? *Br J Pharmacol* 173: 255-266.

Sugiyama T, Kohara H, Noda M, and Nagasawa T (2006) Maintenance of the Hematopoietic Stem Cell Pool by CXCL12-CXCR4 Chemokine Signaling in Bone Marrow Stromal Cell Niches. *Immunity* 25(6): 977-988.

Sun Y, Cheng Z, Ma L, and Pei G (2002) Beta-arrestin2 Is Critically Involved in CXCR4-mediated Chemotaxis, and This Is Mediated by Its Enhancement of p38 MAPK Activation. *J Biol Chem* 277(51): 49212-49219.

Sungkaworn T, Jobin ML, Burnecki K, Weron A, Lohse MJ, and Calebiro D (2017) Single-molecule imaging reveals receptor-G protein interactions at cell surface hot spots. *Nature* 550(7677): 543-547.

Tachibana K, Hirota S, Iizasa H, Yoshida H, Kawabata K, Kataoka Y, Kitamura Y, Matsushisma K, Yoshida N, Nishikawa SI, Kishimoto T, and Nagasawa T (1998) The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature* 393: 591-594.

Tan W, Martin D, and Gutkind JS (2006) The Galpha13-Rho Signaling Axis Is Required for SDF-1-induced Migration through CXCR4. *J Biol Chem* 281(51): 39542-39549.

Tian S, Choi WT, Liu D, Pesavento J, Wang Y, An J, Sodroski JG, and Huang Z (2005) Distinct functional sites for human immunodeficiency virus type 1 and stromal cell-derived factor 1alpha on CXCR4 transmembrane helical domains. *J Virol* 79(20): 12667-12673.

Tiveron MC, Boutin C, Daou P, Moepps B, and Cremer H (2010) Expression and function of CXCR7 in the mouse forebrain. *J Neuroimmunol* 224: 72-79.

Torossian F, Anginot A, Chabanon A, Clay D, Guerton B, Desterke C, Boutin L, Marullo S, Scott MGH, Lataillade JJ, Le bousse-Kerdilès MC (2013) CXCR7 participates in CXCL12-induced CD34+ cell cycling through β-arrestin-dependent Akt activation. *Blood* 123(2): 191-202.

Tripathi A, Vana PG, Chavan TS, Brueggemann LI, Byron KL, Tarasova NI, Volkman BF, Gaponenko V, and Majetschak M (2015) Heteromerization of chemokine (C-X-C motif) receptor 4 with alpha1A/B-adrenergic receptors controls alpha1-adrenergic receptor function. *Proc Natl Acad Sci USA* 112(13): E1659-E1668.

Ulvmar MH, Hub E, and Rot A (2011) Atypical chemokine receptors. *Exp Cell Res* 317(5): 556-568.

Urizar E, Yano H, Kolster R, Galés C, Lambert N, and Javitch JA (2011) CODA-RET reveals functional selectivity as a result of GPCR heteromerization. *Nat Chem Biol* 7: 624-630.

Vaidehi N and Kenakin T (2010) The role of conformational ensembles of seven transmembrane receptors in functional selectivity. *Curr Opin Pharmacol* 10(6): 775-781.

Valentin G, Haas P, and Gilmour D (2007) The Chemokine SDF1a Coordinates Tissue Migration through the Spatially Restricted Activation of Cxcr7 and Cxcr4b. *Curr Biol* 17(12): 1026-1031.

Vila-Coro AJ, Rodríguez-Frade JM, Martín De Ana A, Moreno-Ortíz MC, Martínez-A C, and Mellado M (1999) The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J* 13(13): 1699-1710.

Vilardaga JP, Jean-Alphonse FG, and Gardella TJ (2014) Endosomal generation of cAMP in GPCR signaling. *Nat Chem Biol* 10(9): 700-706.

Wang J, He L, Combs CA, Roderiquez G, and Norcross MA (2006) Dimerization of CXCR4 in living malignant cells: control of cell migration by a synthetic peptide that reduces homologous CXCR4 interactions. *Mol Cancer Ther* 5(10): 2474-2483.

Wang J, Shiozawa Y, Wang J, Wang Y, Jung Y, Pienta KJ, Mehra R, Loberg R, and Taichman RS (2008) The role of CXCR7/RDC1 as a chemokine receptor for CXCL12/SDF-1 in prostate cancer. *J Biol Chem* 283(7): 4283-4294.

Wang L, Wang Z, Yang B, Yang Q, Wang L, and Sun Y (2009) CXCR4 nuclear localization follows binding of its ligand SDF-1 and occurs in metastatic but not primary renal cell carcinoma. *Oncol Rep* 22: 1333-1339.

Wang Y, Li G, Stanco A, Long JE, Crawford D, Potter GB, Pleasure SJ, Behrens T, and Rubenstein JL (2011) CXCR4 and CXCR7 Have Distinct Functions in Regulating Interneuron Migration. *Neuron* 69(1): 61-76.

Watts AO, van Lipzig MM, Jaeger WC, Seeber RM, van Zwam M, Vinet J, van der Lee MM, Siderius M, Zaman GJ, Boddeke HW, Smit MJ, Pfleger KD, Leurs R, and Vischer HF (2013) Identification and profiling of CXCR3-CXCR4 chemokine receptor heteromer complexes. *Br J Pharmacol* 168(7): 1662-1674.

Wescott MP, Kufareva I, Paes C, Goodman JR, Thaker Y, Puffer BA, Berdougo E, Rucker JB, Handel TM, and Doranz BJ (2016) Signal transmission through the CXC chemokine receptor 4 (CXCR4) transmembrane helices. *Proc Natl Acad Sci USA,* 113(35): 9928-9933.

Whorton MR, Bokoch MP, Rasmussen SG, Huang B, Zare RN, Kobilka B, and Sunahara RK (2007) A monomeric G protein-coupled receptor isolated in a high-density lipoprotein particle efficiently activates its G protein. *Proc Natl Acad Sci USA* 104(18): 7682-7687.

Wu G, Krupnick JG, Benovic JL, and Lanier SM (1997) Interaction of arrestins with intracellular domains of muscarinic and alpha2-adrenergic receptors. *J Biol Chem* 272(28): 17836-17842.

Wu B, Chien EYT, Mol CD, Fenalti G, Liu W, Katritch V, Abagyan R, Brooun A, Wells P, Bi FC, Hamel DJ, Kuhn P, Handel TM, Cherezov V, and Stevens RC (2010) Structures of the CXCR4 chemokine GPCR with small-molecule and cyclic peptide antagonists. *Science* 330(6007): 1066-1071.

Yagi HT, Tan W, Dillenburg-Pilla P, Armando S, Amornphimoltham P, Simaan M, Weigert R, Molinolo AA, Bouvier M, and Gutkind JS (2011) A Synthetic Biology Approach Reveals a

CXCR4 G13-Rho Signaling Axis Driving Transendothelial Migration of Metastatic Breast Cancer Cells. *Sci Signal* 4(191): 1-13.

Zhang M, Qiu L, Zhang Y, Xu D, Zheng JC, and Jiang L (2017) CXCL12 enhances angiogenesis through CXCR7 activation in human umbilical vein endothelial cells. *Sci Rep* 7(1): 8289.

Zheng F, Zhang Z, Flamini V, Jiang WG, and Cui Y (2017) The Axis of CXCR4/SDF-1 Plays a Role in Colon Cancer Cell Adhesion Through Regulation of the AKT and IGF1R Signalling Pathways. *Anticancer Res* 37(8): 4361-4369.

Zhou N, Luo Z, Luo J, Liu D, Hall JW, Pomerantz RJ, and Huang Z (2001) Structural and Functional Characterization of Human CXCR4 as a Chemokine Receptor and HIV-1 Co-receptor by Mutagenesis and Molecular Modeling Studies. *J Biol Chem* 276(46): 42826-42833.

Zhu B, Xu D, Deng X, Chen Q, Huang Y, Peng H, Li Y, Jia B, Thoreson WB, Ding W, Ding J, Zhao L, Wang Y, Wavrin KL, Duan S, and Zheng J (2012) CXCL12 enhances human neural progenitor cell survival through a CXCR7- and CXCR4-mediated endocytotic signaling pathway. *Stem cells* 30(11): 2571-2583.

Footnotes

a) This research was funded by European Union's Horizon2020 MSCA Program under grant agreement 641833 (ONCORNET). This mini review is part of the mini review series 'From insight to modulation of CXCR4 and ACKR3 (CXCR7) function'.

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Legends to the figures

Figure 1: Snake plot of human CXCR4 with highlighted residues important for receptor function as determined in the following studies: [1](Wescott et al., 2016), [2](Berson et al., 1996), [3](Zhou et al., 2001), [4](Cronshaw et al., 2010), [5](Rapp et al., 2013), [6](Doranz et al., 1999), [7](Brelot et al., 2000), [8](Tian et al., 2005), [9](Armando et al., 2014), [10](Ballester et al., 2016), [11](Martínez-Muñoz et al., 2018). Snake plot adapted from GPCRdb (Pándy-Szekeres et al., 2017).

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

Table 1. Summary of CXCR4 signalling

CXCR4				
G protein-dependent signalling				
Primary	**Secondary**	**Function**	**Studied system**	**Reference**
$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$	PI3K/Akt, inhibition of cAMP production	Cell survival and migration	HEK293 cells	Quoyer et al., 2013
			Metastatic basal-like breast cancer cells	Yagi et al., 2011
			T- and B-cells	Shi et al., 2007
$G\alpha_{i1}$, $G\alpha_{i2}$, $G\alpha_{i3}$, $G\alpha_{o}$	CXCR4 couples more effectively to $G\alpha_{i1}$ and $G\alpha_{i2}$ than to $G\alpha_{i3}$ and $G\alpha_{o}$		SF9 cells	Kleemann et al., 2008
$G\alpha_{13}$	Activation of Rho	Migration	Jurkat T-cells	Tan et al., 2006
			Metastatic basal-like breast cancer cells	Yagi et al., 2011
		Trafficking of CXCR4 into endosomes	Human PBMC T-cells	Kumar et al., 2011
$G\alpha_{q}$	Activation of PLC-β subfamily		Activation in dendritic cells and granulocytes	Soede et al., 2001
			No activation in T- and B-cells	Shi et al., 2007
G protein-independent signalling				
Primary	**Secondary**	**Function**	**Studied system**	**Reference**
β-arrestin-1	Attenuation of G protein signalling	Reduced inhibition of cAMP production	HEK293 cells	Cheng et al., 2000
	Increased internalisation of CXCR4 (only with GRK2)		HEK293 cells	Cheng et al., 2000
β-arrestin-2	Attenuation of G protein signalling	Reduced inhibition of cAMP production	HEK293 cells	Cheng et al., 2000
	Increased internalisation of CXCR4		HEK293 cells	Cheng et al., 2000
	Desensitisation	Decreased G protein signalling	Lymphocytes	Fong et al., 2002
	p38-MAPK	Chemotaxis	HeLa cells	Sun et al., 2002

Influence on signalling due to co-expression/oligomerisation				
Oligomer with…	**Effect**	**Secondary effect**	**Studied system**	**Reference**
CXCR4 (homomer)	Involved in CXCL12-mediated migration		HEK293 cells, HeLa cells, human non-small lung carcinoma cell line (NCI-H2126)	Wang et al., 2006
	CXCL12-mediated G protein signalling		T-Rex 293 cells	Ge et al., 2017
	Dimers internalise more than monomers		T-Rex 293 cells	Ge et al., 2017
	Nanoclusters implicated in signalling		Human T-cells	Martínez-Muñoz et al., 2018
CCR2	$G\alpha_z$ activation ➔ Ca^{2+} mobilisation		HEK293 cells	Armando et al., 2014
	Negative binding cooperativity between CXCR4 and CCR2		CHO-K1 cells, primary leukocytes	Sohy et al., 2007
CCR7	Facilitates HIV infection		CD4 T-cells	Hayasaka et al., 2015
CCR5	Heteromer loses interaction with NHERF1		HEK293 cells	Hammad et al., 2010
CXCR3	Heteromer recruits β-arrestin-2		HEK293 cells	Watts et al., 2013
	Negative binding cooperativity of CXCR3 onto CXCR4		HEK293 cells	Watts et al., 2013
α_1-AR	β-arrestin-2 recruitment to CXCR4		HEK293 cells	Gao et al., 2018
	Internalisation of CXCR4		Human Vascular Smooth Muscle Cells	Gao et al., 2018
	Reduced migration of CXCR4 towards CXCL12		Human Vascular Smooth Muscle Cells	Gao et al., 2018
	Activating CXCR4 potentiates effect of α_1-AR agonists		Rat and human Vascular Smooth Muscle Cells	Tripathi et al., 2015
μ-OR	CXCL12 decreases effect of antinoceptive drugs		*In vivo* (mice)	Chen et al., 2007
δ-OR	CXCL12 decreases effect of antinoceptive drugs		*In vivo* (mice)	Chen et al., 2007
			MM-1, IM-9, HEK293, Jurkat, T-cell leukemia cell lines, Human primary monocytes	Pello et al., 2008
κ-OR	Cross-desensitization between CXCR4 and κ-OR		Jurkat T-cells, primary human neutrophils, murine B-cells, mice	Finley et al., 2008
BILF1 (viral)	Impairs G protein signalling by CXCR4		HEK293 cells	Nijmeijer et al., 2010
ACKR3	ACKR3 scavenges CXCL12 from CXCR4	Finetuning of primordial germ cell migration	Zebrafish	Boldagipour et al., 2008
			Daudi cells, MDCK, HeLa cells, Raji B-cells, HUVECs, zebrafish, mouse hearts, human umbilical cords	Naumann et al., 2010
	ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness		T lymphocytes, CD34+ cells	Hartmann et al., 2008
	Potentiation of Ca^{2+} flux*		HEK293 cells	Sierro et al., 2007
	Negative modulation of Ca^{2+} flux*		HEK293 cells	Levoye et al., 2009
	Delayment of ERK phosphorylation		HEK293 cells	Sierro et al., 2007
	Enhancement of p38 MAPK and SAPK pathways		HEK293 cells	Decaillot et al., 2011
	Heteromer consitutively recruits β-arrestin-2*	Enhanced cell migration*	HEK293 cells, MDA-MB-231, U87 cells	Decaillot et al., 2011
	Reduced inhibition of cAMP production		HEK293 cells	Decaillot et al., 2011
	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*		T lymphocytes	Levoye et al., 2009

* controversies between studies

Table 2. Summary of ACKR3 signalling

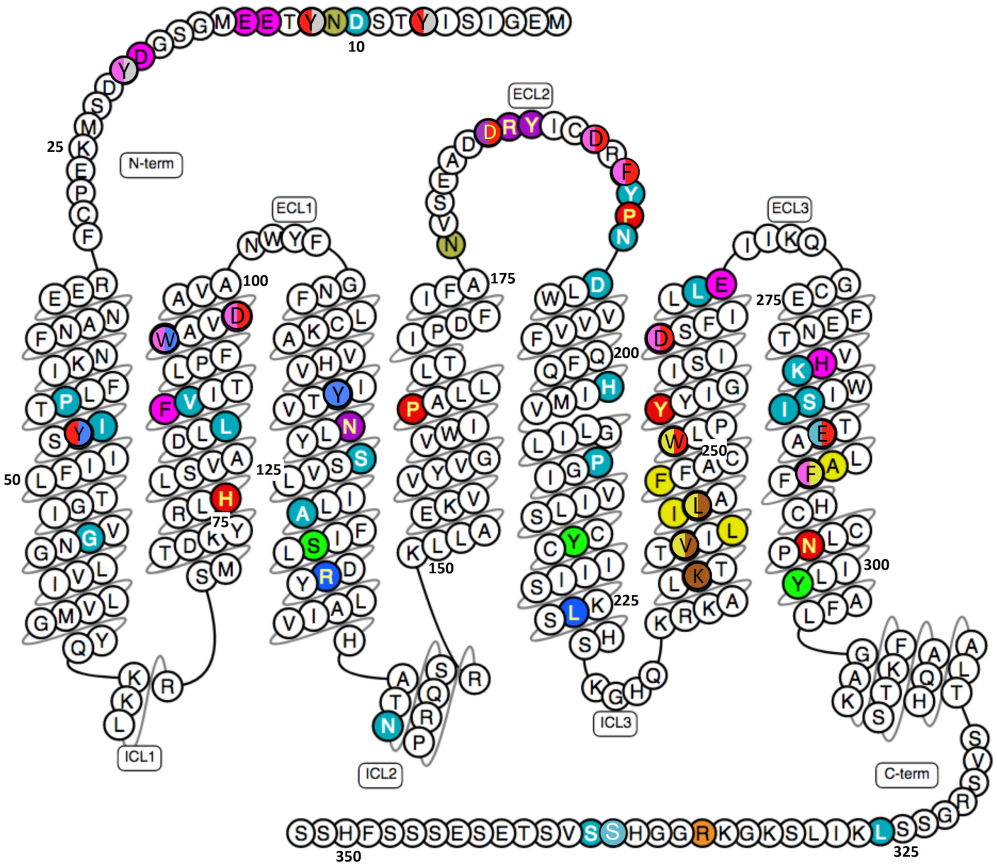
ACKR3				
G protein-dependent signalling				
Primary	**Secondary**		**Studied system**	**Reference**
$G\alpha_{i1}$	No activation, but a constitutive recruitment		HEK293 cells	Levoye et al., 2009
$G\alpha_{i/o}$	ERK/Akt activation		Primary rodent astrocytes	Ödemis et al., 2012
G protein-independent signalling				
Primary	**Secondary**		**Studied system**	**Reference**
β-arrestin-1/2	Akt and MAPK activation		Primary rodent astrocytes	Ödemis et al., 2012
	JAK2/STAT3 pathway		Human bladder cancer cells	Hao et al., 2012
	ERK1/2 phosphorylation		Glioma cells	Hatterman et al., 2010
			HEK293 cells	Rajagopal et al., 2010
	No ERK1/2 phosphorylation		Rat VSMCs	Rajagopal et al., 2010
	Internalisation		HEK293 cells	Canals et al., 2012
			HEK293 cells	Ray et al., 2012
			Breast cancer cells	Luker et al., 2010
Influence on signalling due to co-expression/oligomerisation				
Oligomer with…	**Effect**	**Secondary effect**	**Studied system**	**Reference**
ACKR3 (homomer)	Unknown		HEK293 cells	Levoye et al., 2009
α_1-AR	Negative regulation of α_1-AR		Human VSMCs	Albee et al., 2017
EGFR	CXCR7 involved in phosphorylation of EGFR		Breast cancer cells	Salazar et al., 2014
	Mitosis of breast cancer cells		Breast cancer cells	Salazar et al., 2014
CXCR4	ACKR3 scavenges CXCL12 from CXCR4	Fine tuning of primordial germ cell migration	Zebrafish	Boldagipour et al., 2008
			Daudi cells, MDCK, HeLa cells, Raji B cells, HUVECs, zebrafish, mouse hearts, human umbilical cords	Naumann et al., 2010
	ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness		T lymphocytes, CD34+ cells	Hartmann et al., 2008
	Potentiation of Ca^{2+} flux*		HEK293 cells	Sierro et al., 2007
	Negative modulation of Ca^{2+} flux*		HEK293 cells	Levoye et al., 2009
	Delayment of ERK phosphorylation		HEK293 cells	Sierro et al., 2007
	Enhancement of p38 MAPK and SAPK pathways		HEK293 cells	Decaillot et al., 2011
	Heteromer consitutively recruits β-arrestin-2*	Enhanced cell migration*	HEK293 cells, MDA-MB-231, U87 cells	Decaillot et al., 2011
	Reduced inhibition of cAMP production		HEK293 cells	Decaillot et al., 2011
	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*		T lymphocytes	Levoye et al., 2009

* controversies between studies

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Figures

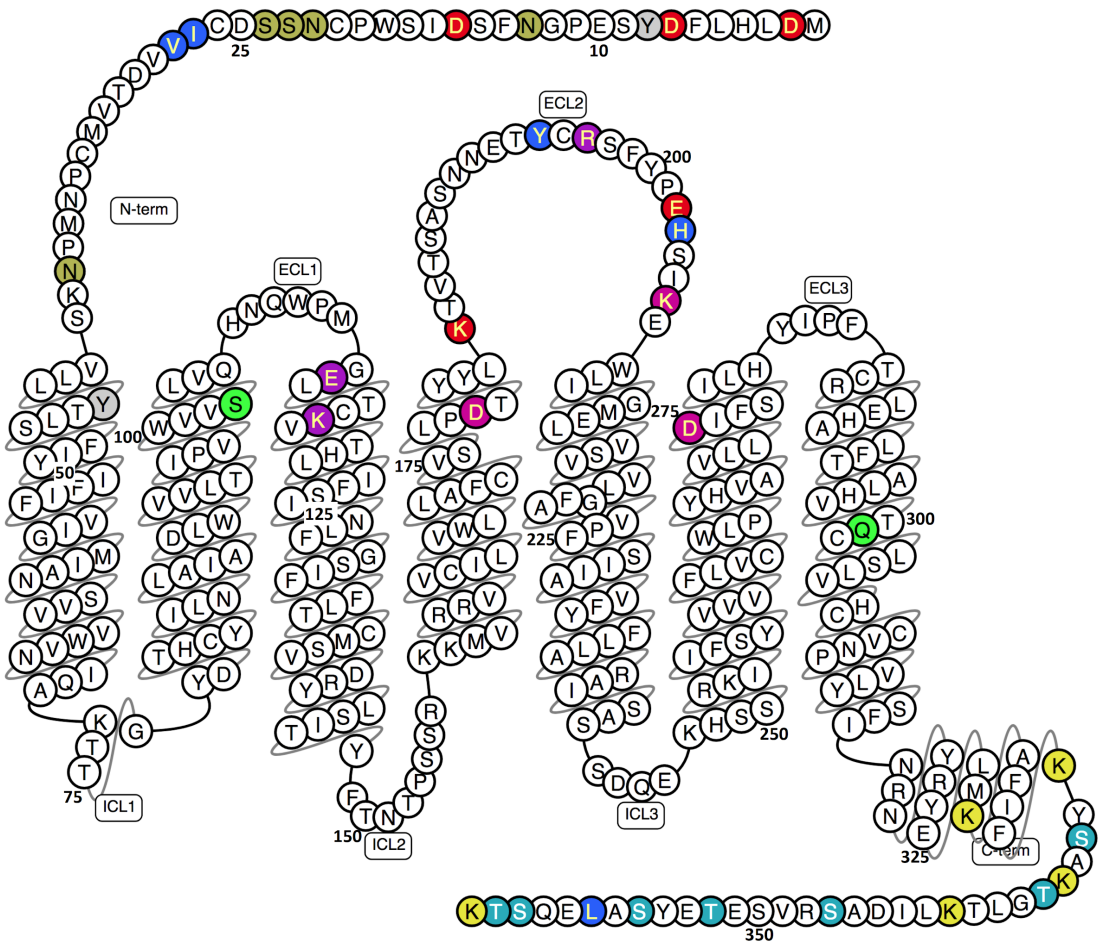
Figure 1



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[1], D97[1,7], D187[1,7], F189[1], D262[1], E268[3], 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[6], R183[6], Y184[6]
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]

Molecular Pharmacology Fast Forward. Published on May 15, 2019 as DOI: 10.1124/mol.118.115477
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2



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]