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Special Section: From Insight to Modulation of CXCR4 and ACKR3 (CXCR7) Function – Minireview

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

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ABSTRACT

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

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

Introduction

G protein-coupled receptor (GPCR) signaling involves numerous factors that influence cellular functions. These include: 1) the variety of ligands binding to the receptor, 2) the

kinetics of the processes, 3) the location of the GPCR, and 4) the available interactome or cellular context:

1. Different ligands can induce a variety of conformational changes in a receptor and, therefore, adopt several conformations (Kim et al., 2013; Manglik et al., 2015; Masureel et al., 2018). These conformations could preferentially activate different pathways, which is known as biased agonism (Vaidehi and Kenakin, 2010; Lane et al., 2017).
2. GPCR activation is also influenced by the kinetics of both ligand binding and receptor signaling, which can

¹J.H., C.P.V., and A.I. contributed equally to this work.

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

possibly lead to the observation of bias profiles, such as in the case of the dopamine D₂ receptor (Klein Herebrink et al., 2016).

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

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

Similar to most chemokine receptors, CXCR4 and ACKR3 are important therapeutic targets due to their involvement in immune-related diseases and cancer. The CXCL12/CXCR4 axis is involved in over 23 types of cancer, including breast, lung, colon, and ovary cancer (Guo et al., 2014; Panneerselvam et al., 2015; Zheng et al., 2017; Raschioni et al., 2018), and acts as a coreceptor for the human immunodeficiency virus (HIV) to enter host T cells (Feng et al., 1996). The discovery of ACKR3 as another CXCL12 receptor added complexity to the understanding of the CXCR4/CXCL12 signaling axis (Balabanian et al., 2005a). ACKR3 is also overexpressed in many cancer types, playing an important role in tumor development and metastasis by promoting cell survival and adhesion (Burns et al., 2006; Miao et al., 2007; Wang et al., 2008). Importantly, ACKR3 has a functional cross-talk with CXCR4, and they are proposed to heteromerize (Balabanian et al., 2005a; Burns et al., 2006; Levoye et al., 2009; Decailiot

et al., 2011). Several other receptors can also alter the function of CXCR4 and ACKR3, either through a functional cross-talk or as a consequence of heteromerization (Contento et al., 2008; Martínez-Muñoz et al., 2014; Becker et al., 2017; Dinkel et al., 2018).

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

CXCR4 and ACKR3 Signaling

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

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

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

In addition to its coupling to the $G_{\alpha_{i/o}}$ subfamily, CXCR4 can also signal through other G proteins. Studies using a more endogenous-like setting suggested that CXCR4 mediates some of its functions through $G_{\alpha_{i3}}$. For example, migration of Jurkat T cells in response to CXCL12 is controlled not only by G_{α_i} through the activation of Rac but also by $G_{\alpha_{i3}}$ through the activation of Rho (Tan et al., 2006). Importantly, it seems that the coordinated activation of these two pathways is also essential for the CXCR4-induced migration of metastatic basal-like breast cancer cells in vitro and in vivo in response

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

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

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

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

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

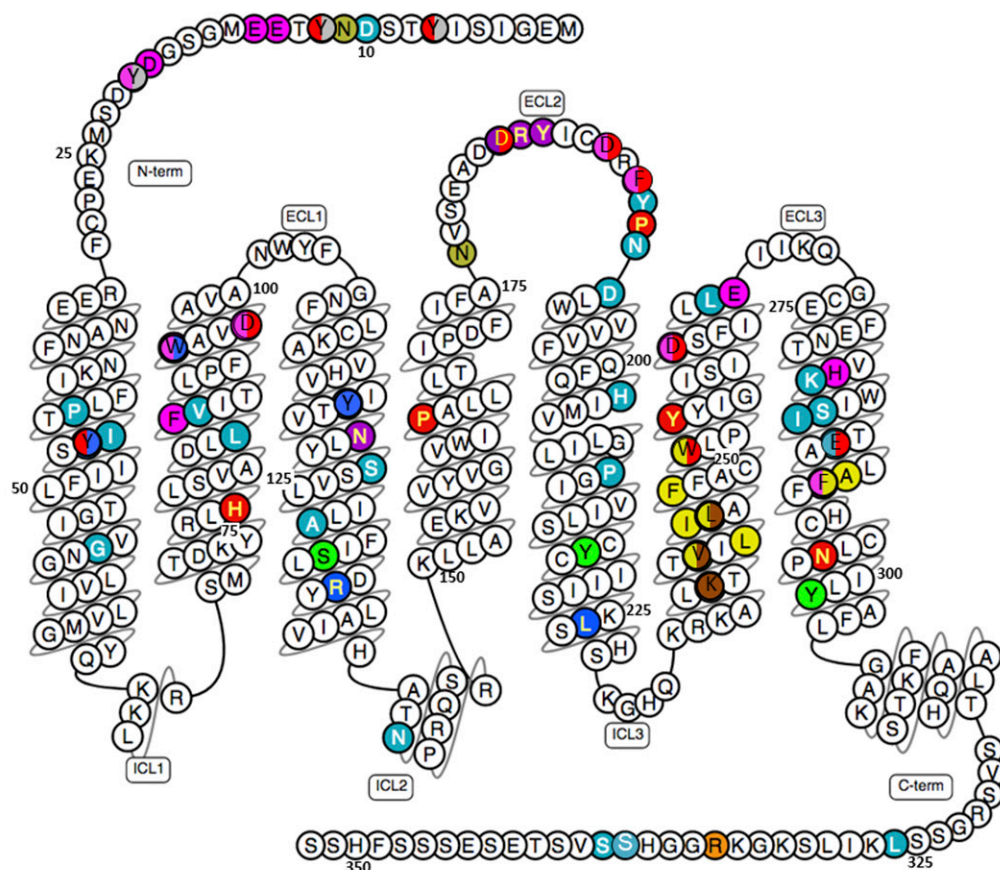
hypogammaglobulinemia, immunodeficiency, and myelokathexis syndrome (Hernandez et al., 2003; Balabanian et al., 2005b; Luo et al., 2017).

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

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

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

G Protein-Dependent Signaling through ACKR3. Many studies have shown that ligand binding to ACKR3 does not result in either coupling to or activation of G proteins, or the triggering of signaling pathways typical of G proteins, in contrast to CXCR4. In fact, ACKR3 lacks the specific 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 signaling, such as G protein activation, intracellular Ca^{2+} mobilization, G protein-mediated ERK phosphorylation, or chemotaxis



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 ^{4,7} , D187 ^{4,7} , F189 ³ , 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

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

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

Nonetheless, the interaction of ACKR3 with G proteins has been proposed in two studies. In the first case, a specific BRET signal was detected between ACKR3–yellow fluorescent protein (YFP) and α_{i1} –Renilla luciferase (RLuc), which decreased

upon treatment with guanosine 5'-3-O-(thio)triphosphate, suggesting that ACKR3 can interact with G proteins in the absence of an agonist but fails to activate them (Levoye et al., 2009). In the second case, CXCL12 was still able to promote $G_{i/o}$ protein activation in primary astrocytes after CXCR4 depletion but not after ACKR3 depletion. In addition, ACKR3-only-expressing astrocytes also led to ERK and Akt activation in response to both CXCL12 and CXCL11, although only the former appeared to be G protein dependent (Ödemis et al., 2012). Both $G_{i/o}$ and ACKR3 are highly abundant in astrocytes and glioma cells (Schönemeier et al., 2008; Tiveron et al., 2010; Ödemis et al., 2012; Banisadr et al., 2016), and therefore, a hypothesis is that ACKR3 might be able to activate G proteins specifically in these cell types, indicating once again how important the interactome might be for a given GPCR.

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

G Protein-Independent Signaling through ACKR3. Many studies have shown that ACKR3 can act as a “decoy” or “scavenging” receptor, since it can efficiently internalize its chemokine ligands CXCL11 and CXCL12 (Naumann et al., 2010). By internalizing CXCL12, ACKR3 finely tunes the CXCL12 gradient necessary for the CXCR4-mediated migration (Dambly-Chaudière et al., 2007; Boldajipour et al., 2008; Donà et al., 2013). Nevertheless, ACKR3 is not only a “decoy” receptor, it can also activate downstream pathways via β -arrestins, in response to both CXCL11 and CXCL12, directly promoting Akt and mitogen-activated protein kinase activity, ERK phosphorylation (Hattermann et al., 2010; Rajagopal et al., 2010; Decaillet et al., 2011; Ödemis et al., 2012; Torossian et al., 2014), and activation of the Janus kinase 2/STAT3 pathway (Hao et al., 2012). CXCL11-dependent ERK phosphorylation could be seen in ACKR3-overexpressing HEK293 cells but not in rat vascular smooth muscle cells (VSMCs) that endogenously express ACKR3, again demonstrating the importance of the cellular context (Rajagopal et al., 2010). Interestingly, AMD3100, an antagonistic small-molecule against CXCR4, can have an agonistic effect on ACKR3. In high concentrations, this molecule can induce β -arrestin recruitment to ACKR3 and increase CXCL12 binding to the receptor (Kalatskaya et al., 2009). A similar scenario was observed with the CXCR4 inverse agonist TC14012, which acts as an agonist on ACKR3 (Gravel et al., 2010). Therefore, when considering CXCR4 as a therapeutic target, it should be taken into account that a molecule can have unexpected effects via ACKR3 and vice versa.

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

Ubiquitination, a constitutive modification on ACKR3, is the key modification responsible for the correct trafficking of the receptor from and to the plasma membrane (Canals et al., 2012). Also, the phosphorylation of serine and threonine residues at the cytoplasmic C-terminal tail of

ACKR3 has been implicated in ACKR3 internalization, chemokine scavenging, and receptor-arrestin interactions (Ray et al., 2012).

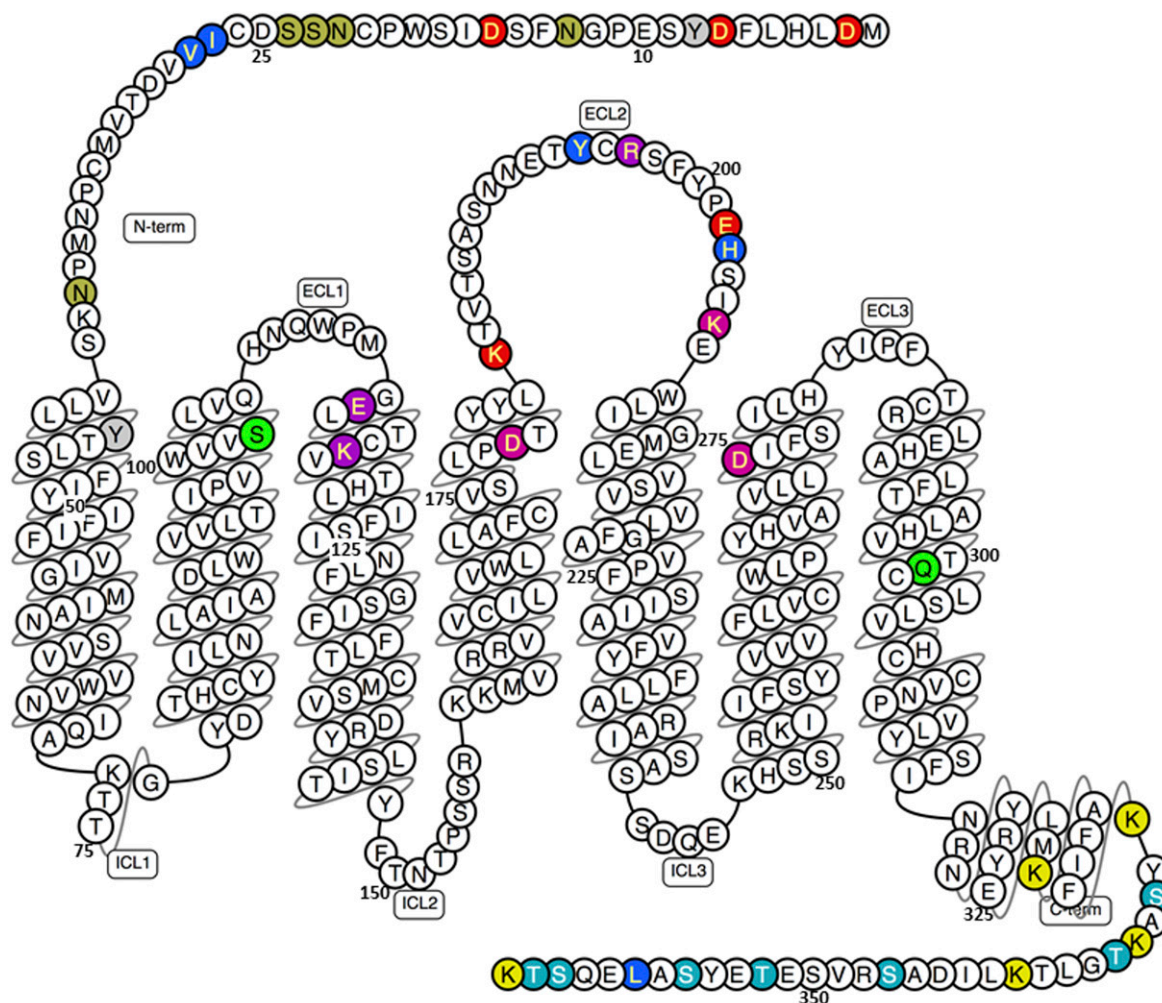
There are some controversies regarding the involvement of ACKR3 in chemotaxis. Some reports suggest that ACKR3 induces migration of different cell types via ACKR3 exclusively (Rajagopal et al., 2010; Chen et al., 2015), while others report a role in migration by only modulating the CXCR4 function (Abe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

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

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

Oligomerization of CXCR4 and ACKR3 Influences Signaling

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



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

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

oligomers. This could suggest that higher-order oligomers might be present in cancer cells, where CXCR4 is expressed abundantly (Lao et al., 2017), which is consistent with the involvement of dimers in migration (Wang et al., 2006). Recently, nanoclusters of CXCR4 were also observed in

Jurkat T cells using single-molecule tracking and super-resolution microscopy (Martínez-Muñoz et al., 2018). CXCL12 promoted the formation of these nanoclusters by decreasing the amount of monomers and dimers. The disruption of these nanoclusters using a TM6 analog strongly

impaired CXCR4 functioning, suggesting that not only dimers but also bigger clusters of CXCR4 might be involved in signaling. Coexpression of cluster of differentiation 4 (CD4) or inhibition of the actin cytoskeleton reduced the size of CXCR4 nanoclusters and hence reduced the Ca^{2+} flux (Martínez-Muñoz et al., 2018). So, the presence of CD4 in the cellular system seems to be important when interpreting the signaling outcome mediated via CXCR4.

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

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

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

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

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

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

Another example of how such cross-talk can affect currently used treatments is the cross-talk between CXCR4 and the opioid receptors. In mice studies, CXCR4 activation by CXCL12 decreased the effect of antinociceptive drugs on the μ - and δ -opioid receptors, but activation of these opioid receptors did not desensitize CXCR4 (Chen et al., 2007). A cross-desensitization in both directions could be detected only

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

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

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

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

Cross-Talk between CXCR4 and ACKR3. Upon the discovery of ACKR3 as a receptor that can also bind CXCL12, which was previously known as a CXCR4-exclusive chemokine (Balabanian et al., 2005a), several studies focused on coexpression of CXCR4 and ACKR3 in diverse cell types and the influence of a possible CXCR4:ACKR3 interaction and/or cross-talk on the signaling properties. CXCR4 and ACKR3 are coexpressed in diverse cell types. These include human T and B lymphocytes (Balabanian et al., 2005a), dendritic cells (Infantino et al., 2006), monocytes (Sánchez-Martín et al., 2011), renal progenitor cells (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 hypothesized that ACKR3 might regulate CXCR4 activity by scavenging or segregating CXCL12. ACKR3 generates a gradient of available ligand for CXCR4, thus finely tuning CXCR4-mediated cellular signaling and hence controlling, for example, primordial germ cell migration in zebrafish (Boldajipour et al., 2008; Naumann et al., 2010). The work of Naumann et al. (2010) suggested that

the modulation of CXCR4 activation via ACKR3 is achieved by the scavenging activity of ACKR3, rather than heterodimerization, as they did not observe any cointernalization of these receptors.

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

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

Another study also linked direct interactions of CXCR4/ACKR3 with oligomerization-specific functional outcomes (Decaillot et al., 2011). In this case, the evidence of CXCR4/ACKR3 hetero-oligomerization comes from the coimmunoprecipitation of overexpressed CXCR4-C9 and ACKR3-FLAG in HEK293 cells. In the same study, coexpression of ACKR3 with CXCR4 inhibited CXCR4/ $\text{G}\alpha_i$ -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 signaling, since their endogenous expression patterns can differ in different cell types and might influence the outcome of the experiments. Regarding drug development, one must acknowledge the complexity of targeting CXCR4 in different diseases and tissues, since heteromerization or cross-talk with other receptors can strongly impact its signaling.

Location of CXCR4 and ACKR3 Can Influence Receptor Signaling

Signaling of CXCR4 in Microdomains. As CXCR4 is expressed in diverse tissues, different microenvironments within different cell types play an important role in the manner of CXCR4 signaling. CXCR4 localizes to membrane

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

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

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

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

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

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

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

Discussion

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

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

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

TABLE 1

Summary of CXCR4 signaling

Primary	Secondary	Function	Studied System	Reference
G protein-dependent signaling G α_{11} , G α_{12} , G α_{13}	PI3K/Akt, inhibition of cAMP production	Cell survival and migration	HEK293 cells	Quoyer et al., 2013
G α_{11} , G α_{12} , G α_{13} , G α_6	CXCR4 couples more effectively to G α_{11} and G α_{12} than to G α_{13} and G α_6		Metastatic basal-like breast cancer cells	Yagi et al., 2011
G α_{13}	Activation of Rho	Migration	T and B cells SF9 cells	Shi et al., 2007 Kleemann et al., 2008
G α_q	Activation of PLC- β subfamily	Trafficking of CXCR4 into endosomes	Jurkat T cells Metastatic basal-like breast cancer cells Human PBMC T cells	Tan et al., 2006 Yagi et al., 2011 Kumar et al., 2011
G protein-independent signaling β -arrestin-1	Attenuation of G protein signaling	Reduced inhibition of cAMP production	Activation in dendritic cells and granulocytes No activation in T and B cells	Soede et al., 2001 Shi et al., 2007
β -arrestin-2	Increased internalization of CXCR4 (only with GRK2) Attenuation of G protein signaling	Reduced inhibition of cAMP production	HEK293 cells	Cheng et al., 2000
	Increased internalization of CXCR4	Decreased G protein signaling Chemotaxis	HEK293 cells	Cheng et al., 2000
	Desensitization p38-MAPK		HEK293 cells	Cheng et al., 2000
Influence on signaling due to coexpression/oligomerization Influence on/oligomer with... CXCR4 (homomer)	Involved in CXCL12-mediated migration	Secondary effect HEK293 cells, HeLa cells, human nonsmall lung carcinoma cell line (NCI-H2126)	HEK293 cells	Cheng et al., 2000
	CXCL12-mediated G protein signaling Dimers internalize more than monomers Nanoclusters implicated in signaling G α_z activation \rightarrow Ca $^{2+}$ mobilization Negative binding cooperativity between CXCR4 and CCR2 Facilitates HIV infection Heteromer loses interaction with NHERF1 Heteromer recruits β -arrestin-2 Negative binding cooperativity of CXCR3 onto CXCR4 β -arrestin-2 recruitment to CXCR4 Internalization of CXCR4		Lymphocytes HeLa cells	Fong et al., 2002 Sun et al., 2002
CCR2			T-Rex 293 cells	Wang et al., 2006
CCR7			T-Rex 293 cells	Ge et al., 2017
CCR5			Human T-cells	Ge et al., 2017
CXCR3			HEK293 cells	Martínez-Muñoz et al., 2018 Armando et al., 2014
α_1 -AR			CHO-K1 cells, primary leukocytes	Sohy et al., 2007
			CD4 T-cells HEK293 cells	Hayasaka et al., 2015 Hammad et al., 2010
			HEK293 cells HEK293 cells	Watts et al., 2013 Watts et al., 2013
			HEK293 cells Human vascular smooth muscle cells	Gao et al., 2018 Gao et al., 2018

(continued)

TABLE 1—Continued

Primary	Secondary	Function	Studied System	Reference
	Reduced migration of CXCR4 toward 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 antinociceptive drugs		In vivo (mice)	Chen et al., 2007
δ -OR	CXCL12 decreases effect of antinociceptive drugs		In vivo (mice)	Chen et al., 2007
			MM-1, IM-9, HEK293, Jurkat, T-cell leukemia cell lines,	Pello et al., 2008
κ -OR	Cross-desensitization between CXCR4 and κ -OR		Human primary monocytes	Finley et al., 2008
BILF1 (viral)	Impairs G protein signaling by CXCR4		Jurkat T cells, primary human neutrophils, murine B cells, mice	Nijmeijer et al., 2010
ACKR3	ACKR3 scavenges CXCL12 from CXCR4	Fine tuning of primordial germ cell migration	Zebrafish	Boldajipour et al., 2008
	ACKR3 defines directionality of migration	Creating chemokine gradient for migration	Daudi cells, MDCK, HeLa cells, Raji B cells, HUVECs, zebrafish, mouse hearts, human umbilical cords	Naumann et al., 2010 Donà et al., 2013
	ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness		Zebrafish lateral line primordium	Dambly-Chaudière et al., 2011
	Potentiation of Ca^{2+} flux ^a		T lymphocytes, CD34+ cells	Hartmann et al., 2008
	Negative modulation of Ca^{2+} flux ^a		HEK293 cells	Sierro et al., 2007
	Delay of ERK phosphorylation		HEK293 cells	Levoye et al., 2009
	Enhancement of p38 MAPK and SAPK pathways		HEK293 cells	Sierro et al., 2007 Decaillet et al., 2011
	Heteromer constitutively recruits β -arrestin-2 ^a	Enhanced cell migration ^a	HEK293 cells, MDA-MB-231, U87 cells	Decaillet et al., 2011
	Reduced inhibition of cAMP production		HEK293 cells	Decaillet et al., 2011
	Decreased potency for ³⁵ GTP- γ S binding after CXCR4 activation		HEK293 cells	Levoye et al., 2009
	ACKR3 inhibits migration by CXCR4 for low CXCL12 dose ^a		T lymphocytes	Levoye et al., 2009

³⁵GTP- γ S, guanosine 5'-O-(3-{³⁵S}thio)triphosphate; MAPK, mitogen-activated protein kinase; OR, opioid receptor; PBMC, peripheral blood mononuclear cell; PI3K, phosphoinositide 3-kinase; PLC- β , phospholipase C- β ; SAPK, stress-activated protein kinase.

^aControversies between studies.

TABLE 2
Summary of ACKR3 signaling

Primary	Secondary	Function	Studied System	Reference
G protein-dependent signaling				
Primary	No activation, but a constitutive recruitment		HEK293 cells	Levoye et al., 2009
Gα ₁₁	No activation ERK/Akt activation		HEK293 cells Primary rodent astrocytes	Hoffmann et al., 2012 Ödemis et al., 2012
G protein-independent signaling				
Primary	Akt and MAPK activation JAK2/STAT3 pathway ERK1/2 phosphorylation		Primary rodent astrocytes Human bladder cancer cells Glioma cells HEK293 cells Rat VSMCs	Ödemis et al., 2012 Hao et al., 2012 Hattermann et al., 2010 Rajagopal et al., 2010 Rajagopal et al., 2010 Canals et al., 2012
β-arrestin-1/2	No ERK1/2 phosphorylation Internalization		HEK293 cells Breast cancer cells PB CD34+ cells	Ray et al., 2012 Luker et al., 2010 Torossian et al., 2014
	Akt phosphorylation			
Influence on signaling due to coexpression/oligomerization		Secondary Effect		
Influence on oligomer with... ACKR3 (homomer)	Unknown		HEK293 cells HEK293T cells Human VSMCs Breast cancer cells	Levoye et al., 2009 Kalatskaya et al., 2009 Albee et al., 2017 Salazar et al., 2014
α ₁ -AR EGFR	Negative regulation of α ₁ -AR CXCR7 involved in phosphorylation of EGFR Mitosis of breast cancer cells ACKR3 scavenges CXCL12 from CXCR4		Breast cancer cells Zebrafish	Salazar et al., 2014 Boldajipour et al., 2008
CXCR4		Fine tuning of primordial germ cell migration	Daudi cells, MDCK, HeLa cells, Raji B-cells, HUVECs, zebrafish, mouse hearts, human umbilical cords Zebrafish lateral line primordium Zebrafish lateral line primordium T lymphocytes, CD34+ cells	Naumann et al., 2010 Donà et al., 2013 Dambly-Chaudière et al., 2009 Hartmann et al., 2008
	ACKR3 defines directionality of migration ACKR3 negatively modulates CXCR4-mediated lymphocyte integrin adhesiveness	Creating chemokine gradient for migration		
	Potentiation of Ca ²⁺ flux ^a Negative modulation of Ca ²⁺ flux ^a Delay of ERK phosphorylation Enhancement of p38 MAPK and SAPK pathways		HEK293 cells HEK293 cells HEK293 cells HEK293 cells	Sierro et al., 2007 Levoye et al., 2009 Sierro et al., 2007 Decallot et al., 2011
	Heteromer constitutively recruits β-arrestin-2 ^a Reduced inhibition of cAMP production	Enhanced cell migration ^a	HEK293 cells, MDA-MB-231, U87 cells HEK293 cells	Decallot et al., 2011 Decallot et al., 2011
	Decreased potency for ³⁵ GTPγS binding after CXCR4 activation ACKR3 inhibits migration by CXCR4 for low CXCL12 dose ^a		HEK293 cells HEK293 cells T lymphocytes	Levoye et al., 2009 Levoye et al., 2009

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

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

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

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

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

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

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

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