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CXCR4/ACKR3 phosphorylation and recruitment of interacting proteins: key mechanisms regulating their functional status

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Phosphorylation/interactome regulate CXCR4/ACKR3 functions

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ACACA, acetyl-CoA carboxylase 1

ACKR3, atypical chemokine receptor 3

AIP4, E3 ubiquitin ligase atrophin Interacting protein 4;

AMFR, E3 ubiquitin-protein ligase AMFR

AP-MS, affinity purification coupled with mass spectrometry

ARIH1, E3 ubiquitin-protein ligase ARIH1

ATP13A2, cation-transporting ATPase 13A2

ATP5A1/ATP5B/ATP5H/ATP5O, ATP synthase subunit alpha/beta/d/o, mitochondrial

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AUP1, ancient ubiquitous protein 1

BiFC, bimolecular fluorescent complementation assay

BioID, proximity-dependent biotin identification

BIRC6, baculoviral IAP repeat-containing protein 6

BRET, bioluminescence resonance energy transfer

CD11B, cyclin-dependent kinase 11B

CD164, endolyn

CD74, HLA class II histocompatibility antigen gamma chain

CDC73, parafibromin

CH2K, checkpoint kinase 2

CHAPSO, 3-([3-Cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate

CHEK2, Serine/threonine-protein kinase Chk2

Co-IP, co-immunoprecipitation

CTR9, SH2 domain binding protein

CXCL12, C-X-C motif chemokine 12

CXCR4, C-X-C motif chemokine receptor 4

DDM, n-Dodecyl-β-D-Maltopyranoside

EFNB1, ephrin-B1

EGF, epidermal growth factor

EGFR, epidermal growth factor receptor

eIF2B, eukaryotic translation initiation factor 2B

ERK, extracellular signal–regulated kinases

ESPL1, HECTD2, probable E3 ubiquitin-protein ligase

ETFA, electron transfer flavoprotein subunit alpha

ETFB, electron transfer flavoprotein subunit beta

FRET, fluorescence resonance energy transfer

GET4, Golgi to ER traffic protein 4 homolog

GIP, G protein-coupled receptor interacting protein

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GJB2, gap junction beta-2 protein

GLRX3, glutaredoxin-3

GNAI, guanine nucleotide-binding protein G(i) subunit alpha-1

GOLT1B, vesicle transport protein GOT1B

GPCR, G protein-coupled receptor

GRK, G protein-coupled receptor kinase

HA, hemagglutinin

HEK293, human embryonic kidney 293 cells

Herg, heregulin

HM13, minor histocompatibility antigen H13

HUWE1, E3 ubiquitin-protein ligase HUWE1

iBAQ, intensity-based absolute quantification

IP, immunoprecipitation

KCNK1, potassium channel subfamily K member 1

KISS, kinase substrate sensor

LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry

LGALS8, galectin

LYN, tyrosine-protein kinase Lyn

MaMTH, mammalian membrane two-hybrid assay

MAP2K2, dual specificity mitogen-activated protein kinase kinase 2

mDIA2, diaphanous-related formin-2

MIF, macrophage migration inhibitory factor

MRPL4, 54S ribosomal protein L4, mitochondrial

MYBL2, myb-related protein B

MYTH, membrane yeast two-hybrid assay

NMMHC, motor protein non-muscle myosin H chain

NPM, nucleophosmin

NR2F2, COUP transcription factor 2

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PAF1, hypothetical protein PD2

PECAM-1, platelet endothelial cell adhesion molecule

PI3Kγ, PI3-kinase isoform p110γ

PKC, protein kinase C

PLA, proximity ligation assay

POLD1, DNA polymerase delta catalytic subunit

PPP6R1, protein phosphatase 6 regulatory subunit 1

PTPRS, receptor-type tyrosine-protein phosphatase S

RAC, RAC-alpha serine/threonine-protein kinase

RHO, Rho family of GTPases

RIC-8A, guanine nucleotide exchange factor synembryn-A

RNF5, E3 ubiquitin-protein ligase RNF5

RTN3, reticulon-3

STAT, signal transducer and activator of transcription

TLR2, toll-like receptor 2

TMEM63B, CSC1-like protein 2

UBE2K, ubiquitin-conjugating enzyme E2 K

UBR7, Putative E3 ubiquitin-protein ligase

WHIM, warts, hypogammaglobulinemia, infections, and myelokathexis

Y2H, yeast two-hybrid assay

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Abstract

The C-X-C chemokine receptor type 4 (CXCR4) and the atypical chemokine receptor 3 (ACKR3/CXCR7) are class A G protein-coupled receptors (GPCRs). Accumulating evidence indicates that GPCR sub-cellular localization, trafficking, transduction properties and, ultimately, their pathophysiological functions are regulated by both interacting proteins and post-translational modifications. This has encouraged the development of novel techniques to characterize the GPCR interactome and to identify residues subjected to post-translational modifications, with a special focus on phosphorylation. This review first describes state-of-the-art methods for the identification of GPCR-interacting proteins and GPCR phosphorylated sites. In addition, we provide an overview of the current knowledge of CXCR4 and ACKR3 post-translational modifications and an exhaustive list of previously identified CXCR4 or ACKR3 interacting proteins. We then describe studies highlighting the importance of the reciprocal influence of CXCR4/ACKR3 interactomes and phosphorylation state. We also discuss their impact on the functional status of each receptor. These studies suggest that deeper knowledge of the CXCR4/ACKR3 interactomes along with their phosphorylation and ubiquitination status would shed new lights on their regulation and pathophysiological functions.

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Introduction

The C-X-C chemokine receptor type 4 (CXCR4) and the atypical chemokine receptor 3 (ACKR3), earlier referred to as CXCR7, are class A G protein-coupled receptors (GPCRs). Stromal cell-derived factor-1/ C-X-C motif chemokine 12 (CXCL12) binds to both CXCR4 and ACKR3 receptors, whereas C-X-C motif chemokine 11 (CXCL11) binds only to the latter and to the C-X-C chemokine receptor type 3. CXCR4 and ACKR3 are co-expressed in various cell types (e.g. endothelial cells (Volin *et al.*, 1998; Berahovich *et al.*, 2014), neurons (Banisadr *et al.*, 2002; Sánchez-Alcañiz *et al.*, 2011) and glial cells (Banisadr *et al.*, 2002, 2016; Odemis *et al.*, 2010)) where they play a pivotal role in migration, proliferation and differentiation. They are also over-expressed in various tumours and control invasion and metastasis (Sun *et al.*, 2010; Zhao *et al.*, 2015; Nazari *et al.*, 2017).

There is now considerable evidence indicating that GPCRs do not operate as isolated proteins within the plasma membrane. Instead, they physically interact with numerous proteins that influence their activity, trafficking, and signal transduction properties (Bockaert *et al.*, 2004; Ritter and Hall, 2009; Magalhaes *et al.*, 2012). These include proteins canonically associated with most GPCRs such as G proteins, G protein-coupled receptor kinases (GRKs) and β-arrestins, specific partner proteins and even GPCRs themselves. In fact, in comparison to monomers, GPCRs can form homo and heteromers with specific pharmacological and signal transduction properties (Ferré *et al.*, 2014).

Phosphorylation is another key mechanism contributing to the regulation of GPCR functional status and signal transduction (Tobin, 2008). Both canonical GRKs and other specific protein kinases are able to phosphorylate GPCRs at multiple sites (Luo *et al.*, 2017), generating the so-called GPCR phosphorylation barcode that determines β-arrestin recruitment, receptor intracellular fate and signalling outcomes (Reiter *et al.*, 2012).

This review will describe recent data highlighting the influence of the CXCR4 and ACKR3 interactome on their functional activity and signal transduction properties. A special focus will be paid to the reciprocal influence of the interactome on CXCR4/ACKR3 phosphorylation and their impact on the functional status and pathophysiological functions of each receptor.

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Methods for the identification of GPCR-interacting proteins

Considerable evidence suggests that GPCRs recruit GPCR-interacting proteins (GIPs) (Maurice *et al.*, 2011). This prompted investigations aimed at identifying GIPs and at characterising GPCR-GIP interactions, using either unbiased or targeted approaches. In unbiased methods, no knowledge of the GIPs is required beforehand and the GPCR of interest is used as bait to purify unknown GIPs. Meanwhile, targeted methods are devoted to the validation and characterisation of previously identified GPCR-GIP interactions. Methods for identifying GIPs or characterising GPCR-GIP interactions include genetic, biophysical, biochemical or proteomic approaches and are summarised in **Table 1**.

Genetic methods. The first method belonging to this class is the yeast two-hybrid assay (Fields and Song, 1989). In this method, the protein of interest (the bait protein) is expressed in yeast as a fusion to the DNA-binding domain of a transcription factor lacking the transcription activation domain. To identify partners of this bait, a plasmid library that expresses cDNA-encoded proteins fused to a transcription activation domain is introduced into the yeast strain. Interaction of a cDNA-encoded protein with the bait protein results in the activation of the transcription factor and expression of a reporter gene, enabling growth on specific media or a colour reaction and the identification of the cDNA encoding the target proteins. A first disadvantage is the loss of spatial-temporal localisation of the interaction; in fact, the yeast two-hybrid assay only captures a snapshot of potential interactions in an artificial biological system. A second disadvantage is that it is not possible to investigate membrane-anchored proteins since the two proteins must cross the nuclear membrane to carry the reconstituted transcription factor to the DNA. To overcome this issue, the membrane yeast two-hybrid assay (Stagljar *et al.*, 1998) was developed. In this assay, the ubiquitin protein is split into two fragments, which are fused to the two proteins of interest. The ubiquitin C-terminal fragment is then conjugated to a transcription factor that is released when the interaction occurs, and ubiquitin protein is reformed. However, as in the yeast two-hybrid assay, the spatial-temporal localisation of the interaction is lost. A second limitation is that the ubiquitin C-terminus carrying the transcription factor cannot be fused to soluble proteins because they could diffuse into the nucleus. Thereafter, a mammalian version of the assay called mammalian membrane two-hybrid (Petschnigg *et al.*,

2014) has been developed. The kinase substrate sensor assay (Lievens, 2014), using the signal transducer and activator of transcription 3 (STAT3) as transcription factor, can also be used for investigating protein-protein interactions including those involving cytosolic and membrane proteins in mammalian cells. However, the kinase substrate sensor assay cannot be used for studying GPCR interaction with proteins involved in the STAT3 cascade.

Biophysical methods. Energy transfer-based methods, such as bioluminescence and fluorescent resonance energy transfer (BRET (Xu, 1999) and FRET (Clegg, 1995)) assays, are targeted methods that are generally used to investigate previously reported interactions. Both are based on the transfer of energy from a donor to a nearby acceptor (<100 Å). Their high sensitivity allows the study of weak and transient interactions. The high spatial-temporal resolution permits accurate kinetic studies for investigating interaction dynamics.

Another biophysical method, based on FRET and employed for the study of protein-protein interaction, is fluorescent lifetime imaging microscopy (Sun *et al.*, 2012). The fluorescence lifetime is the average time that a molecule spends in the excited state before returning to the ground state. Since in FRET the energy transfer from the acceptor to the donor depopulates the excited state energy of the latter, it also shortens its lifetime. Fluorescent lifetime imaging microscopy can accurately measure the shorter donor lifetime that results from FRET, thus allows mapping of the spatial distribution of protein-protein interactions in living cells (Sun *et al.*, 2011). Its main advantage over intensity-based FRET is a more accurate measurement of FRET, because only donor signals are measured eliminating the corrections for spectral bleed through (Sun *et al.*, 2011). Its main disadvantages are the necessity of live specimen and the complexity of data recording and analysis.

Biochemical methods. The proximity ligation assay (Fredriksson *et al.*, 2002) is another powerful targeted method. In the direct version of the technique, two DNA oligonucleotide-conjugated antibodies are used against the proteins of interest. In the indirect version, secondary DNA-conjugated antibodies are used after targeting the proteins of interest with an appropriate primary antibody. If the two conjugated antibodies are close enough (30-40nm), they can bind together. The DNA connecting the two probes is then amplified and hybridised with fluorophores. This allows the visualisation of the interaction in the place where it occurs, at a single molecule

resolution. The main disadvantages of the approach are the high costs and the necessity for specific antibodies that are not always available.

In the bimolecular fluorescent complementation assay (Hu *et al.*, 2002; Hu and Kerppola, 2003),a fluorescent protein is divided into two non-fluorescent fragments that are fused to the proteins of interest. Interaction reconstitutes the entire fluorescent protein. This method allows the direct visualisation of the interaction and can be used for soluble or membrane-bound proteins. In addition, several interactions can be investigated in parallel using different fluorescent proteins. Since there is a delay in fluorescence formation upon reconstitution of the fluorescent proteins, and the fluorophore formation is irreversible, these methods are usually not well suited for kinetic studies. In order to overcome these limitations a novel assay called NanoBiT was developed. In this assay the nanoluciferase enzyme is divided in two subunits (LgBiT and SmBiT), with low affinity for each other, that can be brought together by the two interacting proteins. The low affinity makes the interaction reversible and therefore suited for the investigation of kinetics(Duellman *et al.*, 2017).

Proteomics methods. Proteomic methods aim to identify GIPs of a receptor of interest *via* the use of affinity purification combined with mass spectrometry (AP-MS). This approach is usually employed as an unbiased method for screening virtually all the GIPs of a GPCR of interest. Targeted versions of the method also exist and rely on GIP identification by Western blotting. However, the requirement for specific antibodies seriously limits its application. Several strategies can be used for the affinity purification step. In co-immunoprecipitation (Co-IP), specific antibodies against the protein of interest are used for precipitating the bait from a protein lysate. As specific GPCR antibodies providing high immunoprecipitation (IP) yields are rarely available, epitope-tagged versions of the receptor of interest are often expressed in the cell type or the organism of interest and precipitated using antibodies against the tag. The main advantages of Co-IP are the purification of proteins interacting with the entire receptor (whenever possible the native receptor) in living cells or tissues and its ability to purify the entire associated protein complex. The main limitations are the necessity for specific antibodies to precipitate GPCRs, the loss of spatial-temporal information and the use of detergents for cell lysis that might denaturate the GPCR of interest and, accordingly, disrupt interactions with their protein partners. For this reason, special attention must be paid to lysis conditions that efficiently solubilize the

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receptor while conserving the receptor's native conformation and its interactions with GIPs. For instance, detergents such as Triton and NP-40 completely denaturate CXCR4 (Palmesino *et al.*, 2016), whereas 3-([3-Cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate, also called CHAPSO (Babcock *et al.*, 2001), and n-Dodecyl-β-D-Maltopyranoside, also called DDM (Palmesino *et al.*, 2016), yield the highest proportion of receptor in the native conformation. Despite this limitation, several CXCR4 interacting proteins have been identified using co-immunoprecipitation approaches (see **Table 2**).

Alternatively, pull-down assays can be performed to purify GPCR partners from a cell or tissue lysate. This approach uses the receptor (or one of its domains) fused with an affinity tag (e.g. glutathione S-transferase) and immobilized on beads as bait. Such *in vitro* binding assays can also be used to prove direct physical interaction between two protein partners. In this case, the bait is incubated with a purified protein instead of a cell or tissue lysate. In all methods, affinity purified proteins are systematically identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A two-step version, named tandem affinity purification (Puig *et al.*, 2001), has also been reported (Daulat *et al.*, 2007) and applies to both Co-IPs or pull-downs. Although tandem affinity purification methods drastically reduce the number of false-positive identifications, they require larger amounts of starting material.

In the proximity-dependent biotin identification method (Roux *et al.*, 2013), the bait protein is fused to a prokaryotic biotin ligase molecule that biotinylates proteins in close proximity once expressed in cells. The method can detect weak and transient interactions occurring in living cells and detergents do not affect the results. Though the fusion of biotin ligase to the bait might alter its targeting or functions, proximity-dependent approaches were recently applied to identify a GPCR-associated protein network with a high temporal resolution. Specifically, engineered ascorbic acid peroxidase was employed in combination with quantitative proteomics to decipher beta-2 adrenergic (Lobingier *et al.*, 2017; Paek *et al.*, 2017) and angiotensin II type 1 (Paek *et al.*, 2017) receptor interacting proteins.

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Methods for the identification of GPCR phosphorylated sites

GPCR phosphorylation is a key regulatory mechanism of receptor function (Lefkowitz *et al.*, 2004). Over the past years, numerous techniques have appeared with increasing resolution to pinpoint phosphorylated residues (summarized in Table 1), which consist of serines, threonines or tyrosines.

Radioactive labelling. The first method that was introduced for deciphering the phosphorylation status of GPCRs is a radioactivity-based technique, consisting of culturing cells in a medium in which phosphate is replaced with its radioactive counterpart ^{32}P, resulting in radioactive phosphorylated residues (Meisenhelder *et al.*, 2001). After culturing, cells are lysed and receptors are immunoprecipitated using specific antibodies and then resolved by SDS-polyacrylamide gel electrophoresis. Receptors can then be digested using an enzyme, such as trypsin, and the resulting peptides are separated by 2D migration using electrophoresis and chromatography. Radioactive peptides are then detected *in-gel* by autoradiography or using a phosphorimager, yielding a phosphorylation map for a given receptor in a given cell line (Chen *et al.*, 2013). This method is very sensitive but does not give precise information on the number of phosphorylated sites nor their position. Radioactive labelling was initially employed to characterize CXCR4 phosphorylation upon agonist stimulation (Haribabu *et al.*, 1997). These studies characterized the C-terminal domain as preferred site for phosphorylation (Haribabu *et al.*, 1997) and identified a Serine cluster present in the C-terminal domain and containing two residues (Ser^{338}, Ser^{339}) phosphorylated following CXCL12 challenge (Orsini *et al.*, 1999).

Liquid chromatography coupled to tandem mass spectrometry. More recently, radioactive labelling-based methods have been progressively supplanted by the identification of phosphorylated residues by LC-MS/MS. In this method, the GPCR of interested is digested enzymatically, using one or several proteases, to generate peptides that cover a large part of the receptor sequence. The resulting peptides are then analysed by LC-MS/MS (Dephoure *et al.*, 2013). Although this approach can pinpoint any phosphorylated residue with high confidence, a few limitations complicate phosphorylated residue identification. Firstly, phosphorylation can be lost during fragmentation. Secondly, since phosphorylation sites have a limited level of phosphorylation, only a small percentage of peptide is actually phosphorylated (Wu *et al.*, 2011). Thirdly, the identification of the phosphorylated residues in peptides with

multiple adjacent phosphorylated residues can be challenging. For each modified site, a phosphorylation index can be estimated by dividing the ion signal intensity corresponding to the phosphorylated peptide by the sum of the ion signals of the phosphorylated peptide and its non-phosphorylated counterpart. Absolute quantification, and thus the stoichiometry of phosphorylation, can also be determined for each modified residue by spiking the sample with a known concentration of high purity heavy isotope-labelled peptides (AQUA peptides) corresponding to the phosphorylated peptide and not phosphorylated one and comparing the respective ion signals of un-labelled and labelled peptides (Gerber *et al.*, 2003). This powerful technology allowed a first comprehensive phosphorylation map of CXCR4 stimulated or not with CXCL12 in human embryonic kidney (HEK)293 cells: LC-MS/MS analyses identified six phosphorylated residues: Ser^{321}, Ser^{324}, Ser^{325}, one between $Ser^{338/339/341}$, one between $Ser^{346/347/348}$, and either Ser^{351} or Ser^{352} (Busillo *et al.*, 2010).

Mutagenesis. Another approach that can be used as a stand-alone technique or in complement with the previously described methods is to mutate potential or predicted phosphorylated residues (into alanine or aspartate to inhibit or mimic their phosphorylation, respectively) and assess functional differences between mutated and wild-type receptor (Okamoto and Shikano, 2017). Nevertheless, introducing those mutations can potentially alter expression of the receptor, its conformation or its cellular localisation. Despite these limitations, mutagenesis approaches have shown unequivocal efficiency to identify or validate several phosphorylated residues on CXCR4 (Orsini *et al.*, 1999; Mueller *et al.*, 2013) in combination with a radioactive labelling method or use of phospho-specific antibodies. Furthermore, mutating all the serine and threonine residues to alanine in the ACKR3 C-terminus abolished β-arrestin recruitment and receptor internalization, suggesting that receptor trafficking depends on the phosphorylation of some of these residues (Canals *et al.*, 2012).

Phospho-specific antibody. To be able to detect and assess phosphorylation of residues in cells or tissues, antibodies that specifically target previously identified phosphorylated residues of GPCRs can be generated by immunising animals with purified synthetic phosphorylated peptides encompassing the phosphorylated residues (Chen *et al.*, 2013). After selection and functional validation, those antibodies can be used in Western Blot or immunohistochemistry experiments.

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Phosphorylation can also be indirectly detected using antibodies specific to the unphosphorylated GPCR, showing decreased binding to the target when residues are phosphorylated, and recovery of the binding by using a protein phosphatase to dephosphorylate the receptor (Hoffmann *et al.*, 2012). Antibodies that recognize several CXCR4 phosphorylated residues ($Ser^{324/325}$, Ser^{330}, Ser^{339}, $Ser^{338/339}$ and $Ser^{346/347}$ (Woerner *et al.*, 2005; Busillo *et al.*, 2010; Mueller *et al.*, 2013)) have been generated and used to investigate the receptor phosphorylation status in various conditions. To our knowledge, such phospho-specific antibodies are still lacking for ACKR3.

Association of CXCR4 and ACKR3 with canonical GPCR interacting proteins

G proteins, GRKs and β-arrestins are the protein families considered as canonical GPCR interacting proteins controlling receptor activity or being involved in signal transduction. GPCR activity is a result of a tightly regulated balance between activation, desensitisation and re-sensitisation events. After receptor activation and interaction with G proteins, several mechanisms integrate to trigger GPCR desensitisation and/or modulate additional signalling cascades including phosphorylation by GRKs and recruitment of β-arrestins (Penela *et al.*, 2010; Nogués *et al.*, 2018).

G proteins. CXCR4 is known to couple to the *pertussis toxin* sensitive $G\alpha_i$ protein family that mediates most of its signalling pathways (Busillo and Benovic, 2007). However, CXCR4 can also couple to other G proteins such as $G\alpha_{12/13}$ (Tan *et al.*, 2006; Kumar *et al.*, 2011) and $G\alpha_q$ (Soede *et al.*, 2001). Tan and colleagues observed that both $G\alpha_i$ and $G\alpha_{13}$ as well as Gβγ subunits are involved in the CXCL12-dependent migration of Jurkat T cells (Tan *et al.*, 2006). Specifically, $G\alpha_i$ proteins promote migration through the activation of Rac, whereas $G\alpha_{12/13}$ proteins activate Rho. Though CXCR4 is coupled to both $G\alpha_{12/13}$ and $G\alpha_i$ proteins in Jurkat cells, such a dual coupling has not been observed in other cell lines where the receptor specifically activates one or the other G protein family (Yagi *et al.*, 2011). In fact, *pertussis toxin* inhibited the migration of non-metastatic breast cancer cells (MCF-7), indicating that $G\alpha_i$ activation is required. However, it did not prevent the migration of metastatic breast cancer cells such as MDA-MB-231 and SUM-159. In

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those cell lines, $G\alpha_{12/13}$ activation mediates CXCL12-induced migration *via* the activation of the Rho signalling axis (Yagi *et al.*, 2011). Therefore, CXCR4 coupling to one or the other G protein family might depend on the abundance of GPCRs, G proteins, and downstream targets.

As an atypical chemokine receptor, ACKR3 lacks the DRYLAIV (Asp-Arg-Tyr-Leu-Ala-Ile-Val) motif necessary for interaction with G proteins. Nevertheless, using BRET, a study showed interaction of the receptor with G proteins in transfected HEK293 cells (Levoye *et al.*, 2009). Yet, this interaction did not lead to the activation of G proteins (Levoye *et al.*, 2009), reinforcing the common consensus of the inability of ACKR3 to activate G proteins. Consistently, other studies showed that ACKR3 signals independently of G proteins through a mechanism requiring β-arrestins (Rajagopal *et al.*, 2010; Canals *et al.*, 2012).

Although these findings clearly indicate that ACKR3 cannot activate G proteins in most of the cell types, a report suggested that ACKR3 might activate G proteins in two specific cellular contexts, namely primary rodent astrocytes and human glioma cells (Ödemis *et al.*, 2012). Using [^{35}S]GTPγS-binding assay, calcium mobilization and *pertussis toxin*-dependent activation of downstream signalling pathways (ERK1/2 and AKT phosphorylation), this group showed an ACKR3-dependent activation of $G\alpha_{i/o}$ proteins in primary astrocytes (Ödemis *et al.*, 2012). They also reported *pertussis toxin*-dependent migration, proliferation and activation of downstream signalling effectors in two human glioma cell lines (A764 and U343), further suggesting an ACKR3-dependent activation of $G\alpha_{i/o}$. So far, this is the only report suggesting a possible coupling of ACKR3 with G proteins. Though these data must be further confirmed, one possibility is that such a coupling is cell type-specific. Since ACKR3 was shown to form a heterodimer with CXCR4 in transfected cell lines (Levoye *et al.*, 2009) and CXCR4 is well known for its coupling with G proteins (*vide supra*), the authors also investigated the possibility that the ACKR3-dependent activation of $G\alpha_{i/o}$ proteins was mediated by the ACKR3/CXCR4 complex. However, constitutive suppression of CXCR4 expression in primary astrocytes did not influence the ability of ACKR3 to activate G proteins in the [^{35}S]GTPγS-binding assay (Ödemis *et al.*, 2012). Consistently, transient suppression of CXCR4 expression did not influence the ACKR3-dependent calcium mobilization in primary cultures of

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astrocytes. This suggests that ACKR3 coupling to G proteins in astroglial cells, if any, occurs independently of the ACKR3/CXCR4 complex assembly.

Although in that specific case CXCR4 did not influence ACKR3 signalling, accumulating evidence supports the hypothesis that ACKR3 might conversely influence CXCR4 signalling. Specifically, the organisation of CXCR4 and ACKR3 in heterodimers appears to inhibit CXCR4 interaction with G proteins in transfected HEK293T cells, as assessed by saturation BRET (Levoye *et al.*, 2009). In accordance with a possible influence of ACKR3 on CXCR4-dependent $G\alpha_i$ activation, Sierro and colleagues showed that the co-expression of ACKR3 with CXCR4 hindered the fast and G protein-dependent ERK activation triggered by CXCL12 exposure (Sierro *et al.*, 2007). In spite of these observations demonstrating that ACKR3 influences CXCR4-dependent G protein signalling a direct proof of the role of the physical interaction between both receptors is still missing.

GRKs. Agonist-occupied GPCRs are specifically phosphorylated by different GRKs, a family of 7 serine/threonine kinases (Ribas *et al.*, 2007; Petronila Penela, 2010). GRK 2, 3, 5 and 6 phosphorylate CXCR4 in the C-terminus, which contains 15 serine and 3 threonine residues that are potential phosphorylation sites (**Figure 1**). At least six of these residues were shown to be phosphorylated following receptor activation by CXCL12 (Busillo *et al.*, 2010; Barker and Benovic, 2011; Mueller *et al.*, 2013). In HEK293 cells, Ser^{321}, Ser^{324}, Ser^{325}, Ser^{330}, Ser^{339}, and two sites between Ser^{346} and Ser^{352} were shown to be phosphorylated in response to CXCL12 in the CXCR4 C-terminus using LC-MS/MS and phosphosite-specific antibodies (Busillo *et al.*, 2010). GRK6 is able to phosphorylate $Ser^{324/5}$, Ser^{339} and Ser^{330}, the latter with slower kinetics, whereas GRK2 and GRK3 phosphorylate residues between Ser^{346} and Ser^{352} (**Figure 2**) (Busillo *et al.*, 2010), and specifically $Ser^{346/347}$ (Mueller *et al.*, 2013). Interestingly, the latter study suggested a hierarchy in such phosphorylation events, since $Ser^{346/347}$ phosphorylation is achieved faster and is needed for the subsequent phosphorylation of $Ser^{338/339}$ and $Ser^{324/325}$. Notably, ligand washout resulted in rapid $Ser^{324/325}$ and $Ser^{338/339}$ de-phosphorylation, whereas $Ser^{346/347}$ residues did not exhibit major dephosphorylation during the 60-minute period studied (Mueller *et al.*, 2013). Phosphorylation of CXCR4 by different GRKs can elicit several molecular responses, such as fluctuations in intracellular calcium concentration and extracellular signal–regulated kinases (ERK) 1 and 2 phosphorylation, leading to

integrated cellular responses. In HEK293 cells, calcium mobilisation is negatively regulated by GRK2, GRK6, and β-arrestin2. On the other hand, GRK3 and 6 together with β-arrestins act as positive regulators of ERK1/2 (Busillo *et al.*, 2010). Overall, these studies show non-overlapping roles of the different GRKs in the regulation of CXCR4 signalling. These differential roles may explain distinct cell type-dependent responses to CXCL12. However, what dictates the specific GRK subtype recruitment still needs to be investigated. Changes in the normal CXCR4 phosphorylation pattern as a result of receptor mutations or altered GRK activity can lead to abnormal receptor expression and/or responsiveness, promoting aberrant cell signalling and thus can contribute to several pathologies. Deletion of $Ser^{346/347}$ leads to a gain-of-CXCR4-function and decreases receptor internalisation and subsequent desensitisation, indicating that mutations in the far C-terminus affect CXCR4-mediated signalling (Mueller *et al.*, 2013). In this regard, a subpopulation of patients affected by WHIM (warts, hypogammaglobulinemia, infections, and myelokathexis) syndrome, a rare primary immunodeficiency disease, display C-terminally truncated CXCR4, leading to refractoriness to desensitisation and enhanced signalling (Balabanian, 2008). On the contrary, increased CXCR4 phosphorylation at Ser^{339} is associated with poor survival in adults with B-acute lymphoblastic leukaemia and correlates with poor prognosis in acute myeloid leukaemia patients (Konoplev *et al.*, 2011; Brault *et al.*, 2014). Altered GRK expression/activity can also impair CXCR4 phosphorylation patterns. GRK3 suppression may contribute to abnormally sustained CXCR4 signalling in classical types of glioblastomas (Woerner *et al.*, 2012), some WHIM patients (Balabanian *et al.*, 2008) and in triple negative breast cancer, thus potentiating CXCR4-dependent migration, invasion and metastasis (Billard *et al.*, 2016; Nogués *et al.*, 2018). It is interesting to note that, although GRK2 and 3 share a high homology and are able to phosphorylate the same residues in CXCR4 in model cells, their function is not redundant. Whereas both CXCR4 and GRK2 levels are increased in breast cancer patients, GRK3 is decreased, suggesting a differential role for both GRKs in a cancer context (Billard *et al.*, 2016; Nogués *et al.*, 2018). In fact, deregulation of GRK2 potentiates several malignant features of breast cancer cells, and its level positively correlates with tumour growth and increased metastasis occurrence (Nogués *et al.*, 2016), but whether these roles involve changes in CXCR4 modulation is still under investigation. On the other hand, impaired chemotaxis of T and B cells towards CXCL12 is noted in the absence of GRK6, whilst GRK6

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deficiency potentiates neutrophil chemotactic response to CXCL12 (Fong *et al.*, 2002; Vroon *et al.*, 2004)**,** suggesting that the occurrence of highly cell-type specific mechanisms in the control of the CXCL12-CXCR4-GRK6 axis. Overall, these data indicate the complexity of CXCR4 modulation by GRKs and support the need for a better characterization of cell type or disease-specific CXCR4-GRKs interactions.

ACKR3 has lately been the focus of many studies, in particular because of its role in cancer progression and metastasis. However, the mechanisms underlying its regulation are still not well understood, although this receptor has been shown to interact with GRKs and arrestins and to associate with other partners. The C-terminus of ACKR3 contains five serine and four threonine residues that can potentially be phosphorylated (**Figure 2**). Contrary to CXCR4, little is known about their actual phosphorylation status during the activation of the receptor, as no mass spectrometry data is available to date and only few mutational studies have been conducted (Canals, 2012 *et al.*; Hoffmann *et al.*, 2012). In fact, only one study conducted in astrocytes showed that ACKR3 is phosphorylated by GRK2, but not other GRKs, and that this phosphorylation is essential for subsequent ACKR3-operated activation of ERK1/2 and AKT pathways (Lipfert *et al.*, 2013). This study suggests that ACKR3 is indeed phosphorylated by GRKs, but the isoform(s) involved and subsequent responses are likely cell type-dependent and remain to be investigated in detail.

Arrestins. A study revealed that site-specific phosphorylation of CXCR4 by GRK isoforms has contrasting effects upon β-arrestin recruitment: while receptor phosphorylation at its extreme C-terminus (two residues between Ser^{346} and Ser^{352} (Busillo *et al.*, 2010), and specifically $Ser^{346/347}$ (Mueller *et al.*, 2013)) by GRK2/3 is a necessary step in β-arrestin binding, its phosphorylation by GRK6 at upstream residues ($Ser^{324/5}$, Ser^{330}, and Ser^{339} (Busillo *et al.*, 2010)) appears to inhibit arrestin recruitment to CXCR4 or results in a receptor/arrestin complex that adopts a conformation that is distinct from that induced by phosphorylation of extreme C-terminal residues (Oakley *et al.*, 2000; Busillo *et al.*, 2010; Mueller *et al.*, 2013). Further supporting the importance of $Ser^{324/5}$ and Ser^{339} phosphorylation in β-arrestin recruitment, CXCR4 truncation mutants showing impaired phosphorylation at $Ser^{324/325}$ and $Ser^{338/339}$ also exhibit reduced CXCL12-induced receptor internalization (Mueller *et al.*, 2013)

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β-arrestins are also scaffold proteins for several signalling molecules, thus eliciting additional β-arrestin-dependent signalling pathways (Shenoy and Lefkowitz, 2011; Peterson and Luttrell, 2017). Following the recognition of β-arrestin-dependent signalling, the notion of biased ligands that preferentially induce G protein-dependent or independent signalling has emerged (Reiter *et al.*, 2012). Biased signalling at chemokine receptors has been exhaustively reviewed elsewhere (Steen *et al.*, 2014). For instance, a CXCR4-derived pepducin, ATI-2341, acts as a biased CXCR4 agonist that promotes $G\alpha_i$ signalling but not β-arrestin signalling, in contrast to CXCL12 which activates both G protein-dependent and independent pathways (Quoyer *et al.*, 2013; Steen *et al.*, 2014).

Upon activation by its cognate ligands, CXCL11 and CXCL12, ACKR3 recruits β-arrestin2 both *in vitro* (Rajagopal *et al.*, 2010; Benredjem *et al.*, 2016) and *in vivo* (Luker *et al.*, 2009), a process leading to receptor internalisation (Canals *et al.*, 2012), transport to lysosomes and degradation of the receptor-bound chemokine (Luker *et al.*, 2010; Naumann *et al.*, 2010; Hoffmann *et al.*, 2012). The receptor is then mainly recycled back to the plasma membrane (Luker *et al.*, 2010) even if a partial degradation of ACKR3 can be observed (Hoffmann *et al.*, 2012). Interestingly, the rate of receptor internalization is faster and recycling is lower in presence of CXCL11, compared to CXCL12 (Montpas *et al.*, 2018).

As previously mentioned, systematic mutation of C-terminal serine/threonine residues to alanine abolished ligand-induced β-arrestin2 recruitment to ACKR3, as monitored by BRET (Canals *et al.*, 2012) and decreased ACKR3 internalisation and subsequent degradation of radiolabelled CXCL12 in HEK293 cells (Hoffmann *et al.*, 2012). Selective mutations of the two C-terminal serine/threonine clusters to alanine revealed differences in their functional properties. Mutating Ser^{335}, Thr^{338} and Thr^{341} (first cluster) or Ser^{350}, Thr^{352} and Ser^{355} (second cluster) to alanine decreased CXCL12 internalization only after a 5-min challenge but not following longer agonist receptor stimulation. Yet, only mutation of the second cluster prevented CXCL12 degradation. Furthermore, ACKR3 appears to undergo ligand-independent internalisation to a much greater extent than CXCR4 (Ray *et al.*, 2012), and residues 339-362 (the two serine/threonine clusters) are essential for this peculiar cell fate in HEK293 cells. Although numerous studies showed that ACKR3 internalization and

the resulting chemokine degradation are dependent on β-arrestin, recent findings have been challenging this consensus (Montpas *et al.*, 2018). Specifically, this study shows that β-arrestins are dispensable to chemokine degradation, suggesting that other scaffold proteins might be involved in this process.

Association of CXCR4 with non-canonical GPCR interacting proteins

Functional interaction of CXCR4 with second messenger-dependent kinases and receptor tyrosine kinases. Accumulating evidence indicates that phosphorylation of GPCRs by second messenger-dependent kinases such as protein kinase A and protein kinase C (PKC) (Lefkowitz, 1993; Ferguson *et al.*, 1996; Krupnick and Benovic, 1998) as well as members of the receptor tyrosine kinase family (Delcourt *et al.*, 2007) participate in the regulation of GPCR signalling. CXCR4 is phosphorylated by PKC at $Ser^{324/5}$ upon CXCL12 stimulation (Busillo *et al.*, 2010), and this kinase has also been involved in $Ser^{346/7}$ phosphorylation (Luo *et al.*, 2017), even though these results are not entirely consistent with a previous study using different PKC inhibitors (Mueller *et al.*, 2013). In some glioblastoma cell types, CXCR4 is phosphorylated at Ser^{339} in response to the PKC activator Phorbol myristate acetate (Woerner *et al.*, 2005). This suggests that Ser^{339} is also a PKC phosphorylation site and that this phosphorylation event may serve as cross-talk mechanism between CXCR4 and GPCRs that activate $G\alpha_q$-PKC signalling Sphingosine 1-phosphate receptors, neurokinin-1 and lysophosphatidic acid receptors may be possibly involved in glioblastoma progression via this means (Cherry and Stella, 2014). Nevertheless, the functional impact of Ser^{339} phosphorylation in glioblastoma remains to be established. Likewise, epidermal growth factor (EGF) through activation of its receptor can also promote CXCR4 phosphorylation at Ser^{339} in glioblastoma cells (Woerner *et al.*, 2005), and both EGF and heregulin trigger $Ser^{324/325}$ and Ser^{330} phosphorylation in the breast cancer T47D cell line (Sosa *et al.*, 2010). Interestingly, in MCF7 breast cancer cells, heregulin also promotes CXCR4 phosphorylation on tyrosine residues via Epidermal Growth Factor Receptor (EGFR), leading to β□arrestin2 association with CXCR4 and downstream activation of the PRex1/Rac1 axis. However, it is still unclear whether the EGFR-CXCR4 functional interaction is direct or depends on other kinases (Sosa *et al.*,

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2010). In another breast cancer line, BT-474, CXCR4 is phosphorylated on tyrosine residues in response to CXCL12 and through activation of ErbB2/ErbB3 and EGFR (Sosa *et al.*, 2010). Although the specific Tyr residue(s) phosphorylated were not identified, it is worth noting that CXCR4 displays four intracellular Tyr residues (Ahr, 2005). Tyr^{157} in the third intracellular loop has been be involved in CXCR4-dependent STAT3 signalling (Ahr *et al.*, 2005), whereas Tyr^{135}, within the conserved DRY motif, might be involved in receptor coupling to G proteins (Rovati *et al.*, 2007). Consistent with this hypothesis, EGFR-mediated phosphorylation of the equivalent Tyr in another GPCR (the mu-opioid receptor) has been reported to reduce coupling to G proteins (Clayton *et al.*, 2009). Therefore, identification of tyrosine residues phosphorylated in CXCR4 might add some insight into the mechanisms by which growth factor-receptor tyrosine kinases modulate CXCR4 activity. The crosstalk between CXCR4 and ErbB2/ErbB3 and EGFR remains an interesting avenue for future research, given the involvement of both receptors in cancer.

Physical interaction with non-canonical GPCR interacting proteins. Beside canonical GIPs, CXCR4 has been shown to interact with additional proteins that modulate CXCR4 trafficking, subcellular localisation and signalling and proteins whose functions are still unknown. CXCR4 interacting proteins, the methods used for the identification of these proteins, the site of their interaction in the receptor sequence and their functional impact are summarised in **Table 2**.

a) Proteins controlling CXCR4 localization or trafficking

Filamin A directly interacts with CXCR4 and stabilises the receptor at the plasma membrane by blocking its endocytosis (Gómez-Moutón *et al.*, 2015). CXCR4 association with the E3 ubiquitin ligase atrophin interacting protein 4 (AIP4) has opposite consequences: ubiquitination of CXCR4 by AIP4 targets the receptor to multi-vesicular bodies, which is followed by receptor degradation. In addition, agonist treatment increases CXCR4/AIP4 interaction, as assessed by Co-IP and FRET experiments (Bhandari *et al.*, 2009), indicating that this interaction is dynamically regulated by a receptor conformational state. In addition, the authors identified Ser^{324} and Ser^{325} as critical sites for the formation of the CXCR4/AIP4 complex upon CXCL12 exposure. The mutation of both residues to alanine drastically reduces association of AIP4 with CXCR4, whereas their mutation to aspartic acid increases this interaction. Since $Ser^{324/325}$ are phosphorylated by GRK6 (Busillo *et al.*, 2010),

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these results suggest that CXCR4 activation by CXCL12 triggers recruitment of GRK6, which in turn phosphorylates the receptor at $Ser^{324/325}$ to promote its interaction with AIP4. AIP4 then ubiquitinates CXCR4 and mediates its degradation (Bhandari *et al.*, 2009). Reticulon-3 is another CXCR4 interacting protein that promotes its translocation to the cytoplasm (Li *et al.*, 2016).

b) Proteins modulating CXCR4 signalling and functions

CD74, a single-pass type II membrane protein that shares with CXCR4 the ability to bind to the macrophage migration inhibitory factor (MIF), was also shown to interact with CXCR4. The CXCR4/CD74 complex is involved in AKT activation (Schwartz *et al.*, 2009). In fact, blocking either CXCR4 or CD74 inhibits MIF-induced AKT activation. Using FRET, an interaction between CXCR4 and the toll like receptor 2 was observed in human monocytes upon activation by Pg-fimbria (fimbriae produced by the major pathogen associated with periodontitis named *Porphyromonas gingivalis*). Analysis of a possible crosstalk between the two receptors showed that Pg-fimbria, directly binds to CXCR4 and inhibits toll like receptor 2-induced NF-κB activation by *P. gingivali* (Hajishengallis *et al.*, 2008; Triantafilou *et al.*, 2008). In Jurkat cells, CD164 co-precipitates with CXCR4 in the presence of CXCL12 presented on fibronectin (Forde *et al.*, 2007). CXCR4-CD164 interaction participates in CXCL12-induced activation of AKT and protein kinase C zeta *(*PKCζ*).* In fact, the down-regulation of CD164 reduces the activation of both kinases measured upon exposure of Jurkat cells to CXCL12. CXCR4/CD164 interaction has been detected in additional cell lines, such as primary human ovarian surface epithelial cells stably expressing CD164 (Huang *et al.*, 2013).

The ability of CXCR4 to promote cell migration requires deep cytoskeletal rearrangements that can be modulated by CXCR4 interacting proteins. In Jurkat J77 cells, CXCR4 constitutively associates with drebrin (Pérez-Martínez *et al.*, 2010), a protein known to bind to F-actin and stabilise actin filaments. Drebrin is also involved in CXCR4- and CD4-dependent HIV cellular penetration (Gordón-Alonso *et al.*, 2013). CXCR4 interacts with diaphanous-related formin-2 (mDIA2). This interaction induces cytoskeletal rearrangements that lead to non-apoptotic blebbing. mDIA2-CXCR4 interaction is only detected during non-apoptotic amoeboid blebbing and is confined to non-apoptotic blebs upon CXCL12 stimulation (Wyse *et al.*, 2017), suggesting a fine spatio-temporal regulation of the interaction. CXCR4 also

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constitutively associates with the motor protein non-muscle myosin H chain (NMMHC) *via* its C-terminus (Rey *et al.*, 2002). The authors showed that NMMHC and CXCR4 are co-localised in the leading edge of migrating lymphocytes, suggesting that this association might have a role in lymphocyte migration. The PI3-kinase isoform p110γ co-precipitates with CXCR4 in CXCL12-stimulated human myeloid cells. This interaction contributes to receptor-operated integrin activation and chemotaxis of myeloid cells (Schmid *et al.*, 2011). Finally, CXCR4 was found to be part of a junctional mechano-sensitive complex through its interaction with the platelet endothelial cell adhesion molecule (PECAM-1) (Dela Paz *et al.*, 2014).

c) Proteins with unknown functions

Other potential CXCR4-interacting proteins have been identified using unbiased methods. These include the lysosomal protein ATP13A2 (Usenovic *et al.*, 2012) and the nuclear protein Myb-related protein B that is involved in cell cycle progression (Wang *et al.*, 2014). In a study aimed at characterising the human interactome by Co-IP of 1,125 GFP-tagged proteins and LC-MS/MS analysis, CXCR4 was found to co-precipitate with the potassium channel subfamily K member 1, the CSC1-like protein 2 and the vesicle transport protein GOT1B (Hein *et al.*, 2015). In another study, CXCR4 was found to interact with the eukaryotic translation initiation factor 2B complex in an acute lymphoblastic leukemia cell line (pre-B NALM-6 cells) but not in primary lymphocytes (Palmesino *et al.*, 2016). The interaction was negatively regulated by CXCL12 exposure and confirmed by co-localisation analysis. The same study showed that CXCR4 recruits parafibromin, SH2 domain binding protein, hypothetical protein PD2, nucleophosmin, cyclin-dependent kinase 11B, receptor-type tyrosine-protein phosphatase S and galectin (Palmesino *et al.*, 2016).

Association of ACKR3 with non-canonical GPCR interacting proteins

Contrary to CXCR4, only few proteins are described as ACKR3 interacting proteins. Given the described role of ACKR3 in cancer, several studies have addressed ACKR3 crosstalk with well-known pro-oncogenic growth factor receptors. ACKR3 co-localises with and phosphorylates EGFR in breast and prostate cancer cells (Singh and Lokeshwar, 2011; Salazar *et al.*, 2014; Kallifatidis *et al.*, 2016), *via* cell-type specific mechanisms. However, a potential role of EGFR in ACKR3 cross-activation

was not assessed in these studies. Some reports also suggest a possible functional interaction between ACKR3 and Transforming growth factor beta (TGF-β) (Rath *et al.*, 2015) or Vascular endothelial growth factor (VEGF) (Singh and Lokeshwar, 2011) receptors, but whether they involve physical interaction with ACKR3 and/or ACKR3 phosphorylation and activation was not assessed. ACKR3 weakly interacts with the MIF receptor CD74 (Alampour-Rajabi *et al.*, 2015). Moreover, ACKR3 co-localizes with PECAM-1, the cell adhesion molecule required for leukocyte transendothelial migration in human coronary artery endothelial cells (Dela Paz *et al.*, 2014). Using a Membrane Yeast two Hybrid assay screen, ATP13A2 was identified as a putative ACKR3 interacting protein (Usenovic *et al.*, 2012). In the study aimed at characterizing the human interactome of 1,125 GFP-tagged proteins, ACKR3 was found to interact with the gap junction beta-2 protein (GJB2), the 54S ribosomal protein L4, mitochondrial MRPL4, different ATP synthases (ATP5H, ATP5B, ATP5A1, ATP50), ACKR3 itself, the caspase Separin ESPL1, the probable E3 ubiquitin-protein ligase HECTD2 and the Putative E3 ubiquitin-protein ligase UBR7 (Hein *et al.*, 2015). Ubiquitination is an essential mechanism of receptor regulation (Marchese and Benovic, 2001; Shenoy, 2007). ACKR3 can undergo ubiquitination in an agonist-dependent and independent manner, regulating receptor trafficking. Ubiquitination is promoted by three enzymes, E1 E2 and E3 that ubiquitinate proteins on lysine residues (Dores and Trejo, 2012; Alonso and Friedman, 2013). Unexpectedly, ACKR3 is ubiquitinated by E3-ubiquitin ligase (E3) in the absence of an agonist and undergoes deubiquitination upon CXCL12 activation (Canals *et al.*, 2012). Mutation of the five lysines in the receptor C-terminus to alanine, to prevent ubiquitination, impaired ACKR3 cell trafficking and decreased ACKR3-mediated CXCL12 degradation (Hoffmann *et al.*, 2012).

Conclusions

The identification of GPCR-interacting proteins and residues subjected to post-translational modification is of utmost importance. Several techniques are nowadays available to decipher GPCR interactome and phosphorylation profile. These techniques have been successfully applied to CXCR4 revealing important interacting proteins as well as key residues involved in the regulation of receptor-mediated signal transduction. In contrast, ACKR3 interactome and phosphorylation sites have not been systematically investigated. Unbiased studies of the ACKR3 interactome

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and its phosphorylated residues and their control by ACKR3 ligands should open new avenues in the understanding of ACKR3 pathophysiological functions and the underlying signalling mechanisms.

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Author contributions

Wrote or contributed to the writing of the manuscript: Fumagalli, Zarca, Neves, Caspar, Hill, Mayor, Smit, Marin.

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Legends for figure:

Figure 1. CXCR4 and ACKR3 residues potentially subjected to post-translational modifications. Schematic representation of the C-terminal tail of CXCR4 and ACKR3 where serine/threonine (red), tyrosine (green) and lysine (blue) residues potentially subjected to post-translational modifications are highlighted.

Figure 2. CXCR4 C-terminus phosphosites. Schematic representation of the C-terminal tail of CXCR4 where serine residues known to be phosphorylated are highlighted in light blue. The kinases or the extracellular stimuli responsible for the phosphorylation are also specified. GRK, G protein-coupled receptor kinase; PKC, protein kinase C; EGF, epidermal growth factor receptor; Hrg, heregulin.

Table 1. Principal methods used to identify GPCR-interacting proteins and phosphorylated residues. Y2H, yeast two-hybrid assay; MYTH, membrane yeast two-hybrid assay; MaMTH, mammalian membrane two-hybrid assay; KISS, kinase substrate sensor; PLA, proximity ligation assay; BiFC, bimolecular fluorescent complementation assay; BioID, proximity-dependent biotin identification.

Methods for the identification of GPCR-interacting proteins (1 to 4) and phosphorylated residues (5)				
Classification	**Method**	**Screening**	**Advantages**	**Disadvantages**
1.Genetic	**Y2H**	Highly suitable	Easy to perform Inexpensive	Loss of spatial-temporal information Membrane anchored proteins cannot be investigated Performed in yeast
	MYTH	Highly suitable	Easy to perform Membrane anchored proteins can be investigated	Loss of spatial-temporal information Soluble proteins cannot be investigated Performed in yeast
	MaMTH	Highly suitable	Easy to perform Membrane anchored proteins can be investigated Performed in mammalian cells	Loss of spatial-temporal information Soluble proteins cannot be investigated

Methods for the identification of GPCR-interacting proteins (1 to 4) and phosphorylated residues (5)

Classification	Method	Screening	Advantages	Disadvantages
1.Genetic	**KISS**	Possible	Sensitive enough for studying interaction dynamic Both membrane and cytosolic proteins can be investigated	Loss of spatial-temporal information Proteins involved in the STAT3 cascade cannot be investigated
2.Biophysical	**BRET/FRET**	Not suitable.	Precise spatial-temporal information. High sensitivity Possibility to study interactions in living cells	Generation of fusion proteins Relies on the proximity and relative orientation between donor and acceptor
	Fluorescent lifetime microscopy	Suitable	More accurate than intensity based FRET	Data analysis more laborious than intensity based FRET
3.Biochemical	**PLA**	Not suitable.	Precise spatial information (single molecule resolution) Possibility to perform in *ex-vivo* models	Relies on antibodies High cost Not easy to scale up in large studies

Methods for the identification of GPCR-interacting proteins (1 to 4) and phosphorylated residues (5)

Classification	Method	Screening	Advantages	Disadvantages
3.Biochemical	**BioID**	Suitable	Precise spatial information Several interactions in parallel. Possibility to perform in living cells	Not well suited for studying interaction dynamic (fluorescent signal is delayed)
	NanoBit	Suitable	Precise spatial information Several interactions in parallel. Possibility to perform in living cells	Suited for studying interaction dynamic
4.Proteomic	**Co-IP**	Highly suitable	Purification of protein complexes in living cells and tissues	Rely on antibodies Loss of spatial-temporal information Lysis conditions might influence results
	Pull-down	Highly suitable	Can prove direct interaction	Loss of spatial-temporal information *In vitro* binding assays Fusion of the receptor on the beads might alter receptor conformation

Methods for the identification of GPCR-interacting proteins (1 to 4) and phosphorylated residues (5)

Classification	Method	Screening	Advantages	Disadvantages
4.Proteomic	**BioID**	Highly suitable	Can detect weak and transient interactions in living cells	Fusion of the biotin to the receptor might alter its targeting or functions
5.Phosphorylation	**[^{32}P]**	Suitable	Very sensitive	Radioactive method Cannot give information on the number of phosphorylated residues nor their position
	LC-MS	Highly suitable	Can pinpoint phosphorylated residues	Can yield false negatives Not quantitative unless combined with very expensive isotope tags

Methods for the identification of GPCR-interacting proteins (1 to 4) and phosphorylated residues (5)

Classification	Method	Screening	Advantages	Disadvantages
5.Phosphorylation	**Mutagenesis**	Suitable	Cheap and easy Based on functional data in living cells Can pinpoint phosphorylated residues	Indirect method Mutagenesis of the C-terminus can impair expression and/or localization of the receptor Labour intensive in case of multiple phosphosites Not quantitative
	Phospho-antibodies	Suitable	Direct and indirect Can be used in any cell line Semi-quantitative and qualitative	Time consuming and expensive for the generation of the antibodies Useless with low affinity antibodies Cannot give information on contiguous phosphorylated residues

Table 2. CXCR4 and ACKR3 interacting proteins described in the literature. TLR2, toll-like receptor 2; AIP4, E3 ubiquitin ligase atrophin Interacting protein 4; RTN3, reticulon3, NMMHC, motor protein non-muscle myosin H chain; CD164, endolyn; mDIA2, diaphanous-related formin-2; PI3Kγ, PI3-kinase isoform p110γ; PECAM-1, platelet endothelial cell adhesion molecule; MYBL2, Myb-related protein B; KCNK1, potassium channel subfamily K member 1; TMEM63B, CSC1-like protein 2; GOLT1B, vesicle transport protein GOT1B; eIF2B, eukaryotic translation initiation factor 2B; CDC73, parafibromin; CTR9, SH2 domain binding protein; PAF1, hypothetical protein PD2; NPM, nucleophosmin; CD11B, cyclin-dependent kinase 11B; PTPRS, receptor-type tyrosine-protein phosphatase S; LGALS8, galectin; EGFR, epidermal growth factor receptor; CD74, HLA class II histocompatibility antigen gamma chain; MIF, macrophage migration-inhibitory factor; PECAM-1, platelet endothelial cell adhesion molecule; ATP13A2, cation-transporting ATPase 13A2; GJB2, gap junction beta-2 protein; MRPL4, 54S ribosomal protein L4, mitochondrial; ATP5H/ATP5B/ATP5A1/ATP5O, ATP synthase subunit d/beta/alpha/O, mitochondrial; ESPL1, HECTD2, probable E3 ubiquitin-protein ligase; UBR7, Putative E3 ubiquitin-protein ligase.

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
			CXCR4-interacting proteins				
Filamin A	Pull-Down Co-IP	HEK293 cells Recombinant protein	Yes	Constitutive and CXCL12-induced. The ROCK inhibitor Y27632, reverses CXCL12-induced increased interaction	C-terminal tail and third loop of CXCR4	Stabilise CXCR4 at the surface	(Gómez-Moutón, 2015)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
AIP4	Pull Down Co-IP FRET	HEK293 cells	Yes	Constitutive and CXCL12-induced	CXCR4 C-tail serines and WW domains of AIP4. Serine 324 and 325 when phosphorylated increase interaction	Increase CXCR4 degradation	(Bhandari, 2009)
RTN3	Y2H Co-IP	HEK293 cells	NA	Constitutive, induction not tested	Carboxyl terminal of RTN3	Increase cytoplasmic localisation of CXCR4	(Li, 2016)
CD74	Co-IP Co-localisation	HEK293 and MonoMac6 cells	NA	Constitutive, induction not tested	NA	Phosphorylation of AKT	(Schwartz, 2009)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
TLR2	FRET Co-IP	Human monocyte and HEK293 cells	NA	Induced by Pg-fimbria	NA	CXCR4 inhibits TLR2-induced NF-κB activation. In addition, CXCR4 found to be receptor of the pattern-recognition receptor complex	(Hajishengallis, 2008; Triantafilou, 2008)
NMMHC	Pull-Down Co-IP Co-localisation	Jurkat T and Peer T cells lymphocytes	NA	Constitutive and not induced by CXCL12	CXCR4 C-terminus	Lymphocytes migration	(Rey, 2002)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
Drebrin	Pull Down Co-IP FRET	J77 T, HEK293T and HIV-infected T cells	YES	Constitutive and induced by superantigen E which also re-localise the interaction to the leading edge of migrating lymphocytes	Drebrin N-terminus positively regulates interaction whereas the C-terminus seems to negatively regulate it	Drebrin affects key physiological processes during antigen presentation in HIV entry	(Pérez-Martínez, 2010; Gordón-Alonso, 2013)
CD164	Co-IP Co-localisation	Jurkat and Ovarian surface epithelial cells	NA	Only induced when CXCL12 is presented on fibronectin	NA	CD164 participates to the CXCL12 mediated AKT and PKC-ζ phosphorylation	(Forde, 2007; Huang, 2013)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
mDIA2	Co-IP Co-localisation	MDA-MB-231 cells	NA	Constitutive (very weak) and CXCL12 induced	NA	Cytoskeletal rearrangement necessary for non-apoptotic blebbing	(Wyse, 2017)
ATP13A2	MYTH	Yeast	YES	Constitutive	NA	NA	(Usenovic, 2012)
PI3Kγ	Co-IP	Human myeloid cells	NA	Only CXCL12 induced	NA	Integrin activation and chemotaxis	(Schmid, 2011)
PECAM-1	PLA	Human Coronary Artery Endothelial Cells	NO	Constitutive. Induction not studied	NA	CXCR4 part of a junctional meccano-sensitive complex	(Dela Paz, 2014)
MYBL2	2HY	Yeast	Yes	NA	NA	NA	(Wang, 2014).
KCNK1	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
TMEM63B	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
GOLT1B	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
eIFB2	Co-IP Co-localization	Pre-B NALM6 cells	NA	Constitutive and negatively regulated by CXCL12	NA	NA	(Palmesino, 2016)
CDC73	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)
CTR9	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)
PAF1	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
NPM	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)
CD11B	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)
PTPRS	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)
LGALS8	Co-IP	Pre-B NALM6 cells	NA	Constitutive. Induction not studied	NA	NA	(Palmesino, 2016)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
			ACKR3-interacting proteins				
EGFR	PLA Co-localization Co-IP	MCF7 cells, breast cancer tissues, CaP cells	Mediated by β-arrestin 2	Interaction constitutive and induced by the epidermal growth factor.	NA	ACKR3 mediates phosphorylation of EGFR at Tyrosine1110 after EGF-stimulation and phosphorylation of ERK1/2 with consequences on tumour proliferation.	(Singh and Lokeshwar, 2011; Salazar, 2014; Kallifatidis, 2016)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
CD74	Co-IP Co-localization PLA	NIH/3T3 cells and human B cells	NA	Constitutive, induction not investigated.	NA	ACKR3 is involved in MIF-mediated ERK-1/2 and zeta-chain-associated protein kinase activation in addition to MIF-mediated chemotaxis.	(Alampour-Rajabi, 2015)
PECAM-1	PLA	HCAECs cells	NA	Constitutive	NA	NA	(Dela Paz, 2014)
ATP13A2	MYTH	Yeast	Yes	NA	NA	NA	(Usenovic, 2012)
GJB2	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
MRPL4	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
ATP5H	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)

Protein	Method of identification	Cellular context	Direct	Constitutive / induced	Site of interaction	Role	Ref
ATP5B	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
ATP5A1	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
ATP5O	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
ACKR3	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
ESPL1	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
HECTD2	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)
UBR7	Co-IP	HeLa cells	NA	NA	NA	NA	(Hein, 2015)

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Figure 1

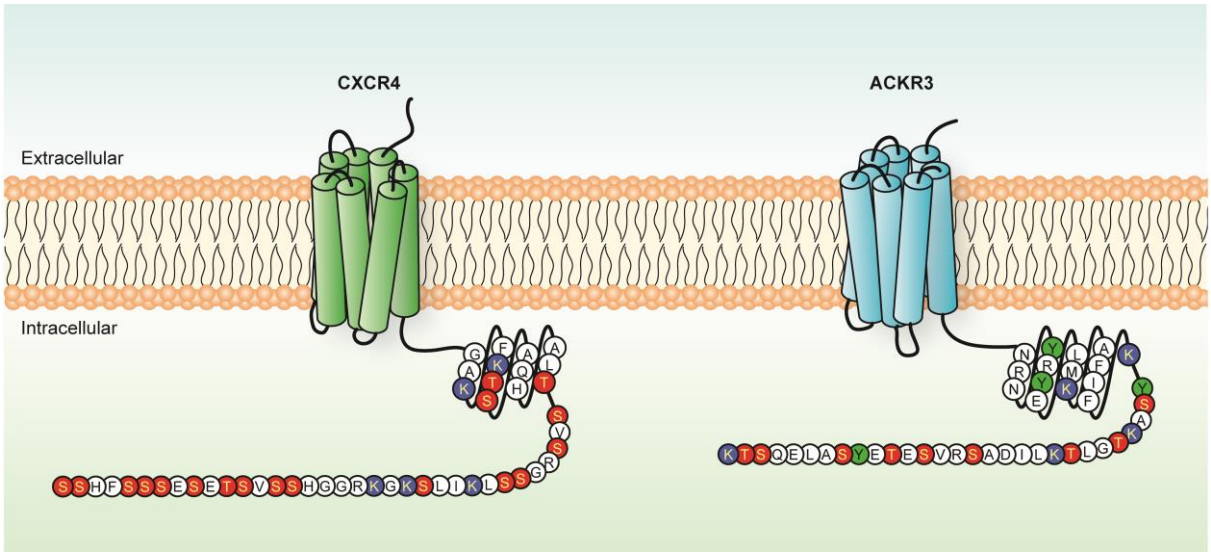

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Figure 2.