Endocytosis is required for C-X-C chemokine receptor type 4 (CXCR4)-mediated Akt activation and anti-apoptotic signaling

Elizabeth J. English, Sarah A. Mahn and Adriano Marchese

From the Department of Biochemistry, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

Running title: Endocytosis regulates CXCR4 signaling

To whom correspondence should be addressed: Prof. Adriano Marchese, Department of Biochemistry, Medical College of Wisconsin, TBRC C3850, 8701 Watertown Plank Rd., Milwaukee, Wisconsin 53226, Telephone: (414) 955-4191; FAX: (414) 955-6510; E-mail: amarchese@mcw.edu

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ABSTRACT

Signaling activated by binding of the C-X-C motif chemokine ligand CXCL12 to its cognate G protein-coupled receptor (GPCR), chemokine C-X-C motif receptor 4 (CXCR4), is linked to metastatic disease. Yet the mechanisms governing CXCR4 signaling remain poorly understood. Here we show that endocytosis and early endosome antigen 1 (EEA1), which is part of the endosome fusion machinery, are required for CXCL12-mediated AKT Ser/Thr kinase (Akt) signaling selective for certain Akt substrates. Pharmacological inhibition of endocytosis partially attenuated CXCL12-induced phosphorylation of Akt, but not phosphorylation of ERK-1/2. Similarly, phosphorylation of Akt, but not ERK-1/2, stimulated by CXCL13, the cognate ligand for the chemokine receptor CXCR5, was also attenuated by inhibited endocytosis. Further, siRNA-mediated depletion of the Rab5-effector EEA1, but not of adaptor protein, phosphotyrosine-interacting with PH domain and leucine zipper 1 (APPL1), partially attenuated Akt, but not ERK-1/2, phosphorylation promoted by CXCR4. Attenuation of Akt phosphorylation through inhibition of endocytosis or EEA1 depletion was associated with reduced signaling to Akt substrates forkhead box O1/3a, but not the Akt substrates TSC complex subunit 2 or glycogen synthase kinase 3β. This suggested that endocytosis and endosomes govern discrete aspects of CXCR4- or CXCR5-mediated Akt signaling. Consistent with this hypothesis, depletion of EEA1 reduced the ability of CXCL12 to attenuate apoptosis in suspended, but not adherent, HeLa cells. Our results suggest a mechanism whereby compartmentalized chemokine-mediated Akt signaling from endosomes suppresses the cancer related process known as anoikis. Targeting this signaling pathway may help inhibit metastatic cancer involving receptors such as CXCR4.

Signaling by the chemokine receptor C-X-C motif receptor 4 (CXCR4) instigated by binding to its cognate ligand CXCL12 plays an important role in cancer progression (1-3). Upon activation by CXCL12, CXCR4 signals to a variety of intracellular signaling pathways, including the Akt signaling pathway (3,4). This pathway has been linked to CXCR4-mediated cell survival, especially in the context of metastatic breast cancer (5). Akt is a serine/threonine protein kinase that phosphorylates diverse proteins to regulate many cellular functions (6,7). Akt acts on several factors relevant to anti-apoptosis and on transcription factors that regulate gene expression that promotes a cell survival phenotype (7). Yet the mechanisms by which Akt signaling is activated by cell signaling receptors remains poorly understood.

CXCR4 couples with heterotrimeric guanine nucleotide binding protein Goζi and the associated Gβγ heterodimer to initiate Akt
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Role of endocytosis on CXCR4-mediated Akt activation.

CXCL12 binding to its cognate receptor CXCR4 activates many signaling pathways, including the ERK-1/2 (extracellular-signal-regulated kinase 1 and 2) and Akt signaling pathways (1,2,4). Previously, we reported that CXCR4-mediated activation of ERK-1/2 does not require endocytosis (17). However, whether endocytosis is required for activation of Akt remains an open question. To address this, HeLa cells were treated with dynasore, an inhibitor of dynamin, a GTPase that is required for endocytosis (18) and is often used to inhibit GPCR endocytosis (19,20). Dynasore treatment of HeLa cells attenuated CXCL12 internalization of CXCR4 (Fig. 1a). To examine whether dynasore treatment impacted CXCR4-mediated activation of Akt, HeLa cells were serum starved for 3 hr and then treated with 10 nM CXCL12 or vehicle for 5 min. Akt activation was assessed by immunoblotting to detect phosphorylation of Akt at serine residue 473 using a phospho-specific antibody, as we have previously described (8). Dynasore treatment significantly attenuated CXCL12-instigated phosphorylation of Akt at Ser-473 (pAkt-S473) by approximately 50% compared to control (Fig. 1b and quantified in 1c). Dynasore treatment did not impact ERK-1/2 phosphorylation (Fig. 1b and quantified in 1d), indicating that dynasore does not globally impact signaling and confirming our previous results that endocytosis is not required for CXCR4-mediated ERK-1/2 activation (17). These data suggest that CXCR4-mediated activation of Akt, in part, requires endocytosis.

To determine how broadly applicable the requirement for endocytosis is in chemokine receptor-instigated Akt activation we examined CXCL13, the sole cognate ligand for the chemokine receptor CXCR5(3). This receptor is expressed endogenously in HeLa cells (21) and treatment of HeLa cells with increasing doses of CXCL13 robustly promoted phosphorylation of Akt and ERK-1/2 (Fig. 2a and quantified in 2b). Similar to CXCR4, CXCR5 couples with a pertussis toxin-sensitive heterotrimeric G protein to phosphorylate Akt or ERK-1/2 (Fig. 2c and quantified in 2d and 2e, respectively). HeLa cells also express the C-X-C chemokine receptors CXCR7 (a.k.a. ACKR3) and CXCR3 (21), however treatment of HeLa cells with CXCL11 or CXCL10, ligands for these
receptors, respectively, did not significantly promote phosphorylation of Akt (Fig. 2f and quantified in 2g).

We focused on CXCL13 signaling via CXCR5 for further experiments. Similar to CXCL12 (Fig. 1a), dynasore treatment of HeLa cells significantly attenuated CXCL13 internalization of CXCR5 (Fig. 3a) and phosphorylation of Akt, but not ERK-1/2, by CXCL13 (Fig. 3b and quantified in 3c and 3d, respectively). To determine whether the requirement for endocytosis was restricted to HeLa cells, we examined chemokine-mediated phosphorylation of Akt in WEHI-231 cells, a B lymphoma cell line that expresses CXCR4 and CXCR5 (22). Dynasore treatment of WEHI-231 cells significantly attenuated CXCL12- or CXCL13-instigated phosphorylation of Akt (Fig. 4a and quantified in 4b). These data suggest that endocytosis-mediated Akt activation may be broadly applicable to chemokine receptors and cell types.

Chemokine receptor signaling to FoxO1/3a is impaired by dynasore treatment.

Once activated, Akt can phosphorylate many proteins involved in discrete processes (6,7). To examine whether endocytosis impacts Akt signaling we examined the phosphorylation status of Akt substrates forkhead box O transcription factors (FoxO1/3a), glycogen synthase kinase 3β (GSK3β) and tuberous sclerosis complex 2 (TSC2) (7). Akt phosphorylates FoxO1/3a at Thr-24/32, GSK3β at Ser-9 and TSC2 at Thr-1462 (7). Dynasore treatment significantly attenuated CXCL12- or CXCL13-instigated phosphorylation of FoxO1/3a, but not TSC2 or GSK3β (Fig. 3b and quantified in 3e, 3f and 3g, respectively). Taken together, these data suggest that endocytosis specifies Akt substrate phosphorylation downstream of chemokine receptor signaling.

Role of APPL1 and EEA1 on CXCR4-mediated Akt signaling.

Given that endocytosis is required for CXCR4-mediated Akt signaling we next addressed whether endosomes are also required. Rab5-positive endosomes containing the Rab5-effector protein adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1 (APPL1) have been linked to Akt signaling (23-25). APPL1 interacts with certain signaling receptors suggesting that APPL1 endosomes may provide a platform for the assembly of signaling complexes (23-27). Depletion of APPL1 with two discrete siRNA significantly attenuated insulin-instigated phosphorylation of Akt at Ser 473 (Fig. 5a and quantified in 5b), consistent for a role of APPL1 in Akt signaling (25). In contrast, depletion of APPL1 did not impact CXCL12- or EGF-instigated phosphorylation of Akt (Fig. 5a and quantified in 5b). These results reveal that APPL1 is not required for CXCR4-mediated Akt activation.

A second class of Rab5-positive endosomes contains the Rab5-effector EEA1 (early endosome antigen 1) (28). EEA1-positive endosomes have been previously linked to GPCR-mediated Akt signaling (15). Depletion of EEA1 with two discrete siRNA significantly attenuated CXCL12-instigated phosphorylation of Akt at Ser-473 (Fig. 6a and quantified in 6b). Phosphorylation of ERK-1/2 was not impacted by EEA1 depletion (Fig. 6a and quantified in 6d), indicating that there was not a global defect in signaling. Further, depleting EEA1 attenuated CXCL12-instigated signaling to Akt substrate FoxO1/3a, but not GSK3β or TSC2 (Fig. 6a and quantified in 6c and 6d, respectively). Together these data suggest that EEA1-positive, but not APPL1-positive endosomes specify discrete Akt substrate phosphorylation downstream of CXCR4 signaling.

EEA1 is required for CXCR4-mediated suppression of apoptosis in detached, but not adherent cells.

Akt promotes cell survival by suppressing apoptosis via directly phosphorylating and inactivating proapoptotic proteins (7). CXCR4-mediated Akt signaling has been linked to cell survival, which is key to its role in metastatic disease (29). This is especially relevant to when tumor cells detach and transit the blood stream to distant sites. Typically, upon detachment cells undergo anchorage-independent cell death or apoptosis known as anoikis (30). Because EEA1 is required for Akt signaling we tested whether EEA1 is required for suppression of apoptosis by CXCR4 in detached or adherent cells. In control siRNA cells that were maintained in an adherent setting, CXCL12 reduced PARP cleavage, a hallmark of apoptosis. In detached cells, cleaved PARP was
somewhat elevated compared to adherent cells, suggesting greater apoptosis. Importantly, the ability of CXCL12 to reduce PARP cleavage was similar or not statistically significant between adherent and detached cells (Fig. 7a and quantified in 7b). In EEA1 depleted cells, basal levels of cleaved PARP were higher than in control cells, suggesting active apoptosis. Importantly, CXCL12 signficantly reduced PARP cleavage in adherent cells, but not in detached cells (Fig. 7a and quantified in 7b). To further confirm these findings, in parallel samples we quantified caspase-3/7 activity using a luminescence assay. In control siRNA cells, the ability of CXCL12 to reduce caspase-3/7 activity was not significantly different between adherent and detached cells (Fig. 7c). In contrast, EEA1 depletion significantly attenuated the ability of CXCL12 to reduce caspase-3/7 activity in detached cells, but not in adherent cells (Fig. 7c). These results are consistent with EEA1 being required for CXCR4-mediated suppression of apoptosis.

**DISCUSSION**

Our study provides mechanistic insight by which chemokine receptors activate Akt signaling. Our data reveal that CXCR4-mediated Akt activation and signaling requires endocytosis and the Rab5-effector EEA1, but not APPL1, suggesting a role for EEA1-positive endosomes in Akt signaling. This is likely broadly applicable to other chemokine receptors because CXCL13, the cognate ligand for the chemokine receptor CXCR5, also requires endocytosis for Akt signaling. This is not cell-type dependent because in addition to HeLa cells, endocytosis is also required for CXCR4- or CXCR5-mediated Akt activation in WEHI-231 cells. CXCL12 time-dependent stimulation indicates that endocytosis impacts the efficiency of Akt phosphorylation, rather than the kinetics, while not impacting ERK-1/2 phosphorylation (Supporting Figure S1). Further, our data suggest that Akt signaling from EEA1-positive endosomes specifies Akt substrate specificity. Furthermore, and importantly, EEA1-positive endosomes are likely required for CXCL12-instigated suppression of anoikis, a type of anchorage-independent cell death. Our study provides evidence that GPCRs can drive Akt signaling with qualitatively distinct functional consequences depending on the compartment from which Akt signaling occurs.

Our results extend the concept that GPCR signaling requires endocytosis. Blocking endocytosis pharmacologically attenuates CXCR4- and CXCR5-mediated Akt activation and signaling (Figs. 1 & 3). Dynasore attenuated Akt phosphorylation by approximately 50%, suggesting that canonical Akt activation at the plasma membrane remains intact when endocytosis is blocked. Although dynasore is known to have off-target effects (31), it efficiently blocks endocytosis and it was only acutely applied in our experiments (Figs. 1a & 3a), thus likely avoiding any cellular changes that may occur when using RNAi or dominant-negative approaches. Dynasore did not globally impact signaling as ERK-1/2 phosphorylation by CXCR4 or CXCR5 remained intact (Figs. 1 & 3). We have previously shown that endocytosis is not required for ERK-1/2 phosphorylation by CXCR4; but requires the protein caveolin-1, a major component of caveolae or lipid-rafts at the plasma membrane (17). Therefore, CXCR4 signaling is highly compartmentalized, whereby discrete receptor and G protein pools likely mediate Akt or ERK-1/2 signaling.

Endosomes are required for Akt activation and signaling. EEA1 is a Rab5-effector and a key player of the endosome fusion machinery, therefore its depletion likely disrupts endosome dynamics (28,32), suggesting that an intact endosomal network, rather than a direct effect of EEA1, is required for Akt activation and signaling (Fig. 6). EEA1 depletion did not impact ERK-1/2 phosphorylation (Fig. 6), indicating that there is not a global impact on signaling when EEA1 is depleted and that EEA1-positive endosomes are not required for ERK-1/2 signaling by CXCR4. A requirement for EEA1 in Akt signaling by GPCRs is in line with previous studies. EEA1-positive endosomes have been linked to Akt activation by angiotensin II in vascular smooth muscle cells (15). Another Rab5-effector APPL1, which is mainly localized to a subpopulation of peripheral endosomes devoid of EEA1 (33), is not involved in CXCR4-mediated Akt activation or signaling (Fig. 5). APPL1-positive endosomes have been implicated in Akt and ERK-1/2 signaling mediated by several cell signaling receptors, such as the insulin receptor (Fig. 5) and the GPCR that binds to LPA (34). It is likely that APPL1-positive endosomes may be required for Akt signaling of a
subset of cell signaling receptors. APPL1 has a related family member called APPL2 (25), which we have not examined in this study and therefore cannot rule it out. Our results are consistent with EE1-positive endosomes being required for Akt activation and signaling mediated by a subset of GPCRs.

EEA1-positive endosomes may serve as an ideal signaling platform for assembling complexes required for activation of Akt signaling. We have yet to directly demonstrate that CXCR4 or CXCR5 located at endosomes directly drive Akt signaling. However, we have previously shown that CXCR4 traffics very efficiently to EEA1-positive endosomes (35-37). Other factors required for Akt phosphorylation have also been found at endosomes. Previously, we have shown that PDK1 localizes to EEA1-positive endosomes (38) and mTORC2 activity and phosphorylation of Akt has been linked to the surface of endosomes (39). We recently reported that ESCRT complexes, which are typically found at the surface of endosomes, are also required for CXCR4-mediated Akt activation and signaling (8). Because heterotrimeric G proteins are required for CXCR4-mediated Akt signaling, they may also be required to be present on endosomes. There is evidence that Gβγ-mediated PI3-K signaling may occur from Rab11-positive recycling endosomes via the LPA receptor (16), although it is unclear how this localization of PI3-K relates to the site of relevant phosphoinositide production. S1P-mediated Goa signaling has been linked to the Golgi, not endosomes, and instigated by an unnatural ligand (40). Whether heterotrimeric G protein Goa localizes to EEA1-positive endosomes remains unclear. β-arrestins have also been linked to GPCR-mediated Akt activation from endosomes (41), however considering that CXCR4- or CXCR5-mediated Akt activation is completely G protein dependent (Fig. 2c) it is difficult to envision a role for β-arrestins. However, β-arrestin-dependent signaling is complex and its relationship to G protein signaling may be more intertwined than previously appreciated (42), therefore β-arrestins cannot be completely ruled out at this time.

We provide evidence that EEA1-positive endosomes specify Akt substrate specificity. Pharmacological inhibition of endocytosis (Figs. 1 & 3) or depletion of EEA1 (Fig. 6) attenuates CXCR4- or CXCR5-mediated phosphorylation of Akt substrates FoxO1/3a, but not TSC2 or GSK3β. Notably, ESCRTs are also required for CXCR4-mediated Akt signaling to FoxO1/3a, but not GSK3β or TSC2 (8). As mentioned above, this signaling specificity could relate to localization of Akt activation, but it could also relate to localization of its substrates. GSK3β or TSC2 are likely localized to compartments other than EEA1-positive endosomes (7,25). FoxO1/3a are members of the forne box O (FoxO) family of transcription factors (43). Akt phosphorylation of FoxO1/3a promotes their association with 14-3-3 phospho-binding proteins leading to their nuclear export and retention in the cytoplasm (44). How endosomes relate to this canonical manner by which Akt regulates FoxO phosphorylation and localization remains unclear and requires further investigation.

Akt signaling is typically anti-apoptotic by directly inhibiting the activity of pro-apoptotic proteins and also by suppressing the expression of pro-apoptotic genes in part by blocking the function of transcription factors FoxO1 and FoxO3a (7). Our data provide evidence that EEA1 is required for CXCR4-mediated suppression of apoptosis in detached cells, but not adherent cells (Fig. 7). This is consistent with the notion that endosomes are required for CXCR4-mediated suppression of the cancer-related process known as anoikis. Our results are somewhat reminiscent with integrin mediated suppression of anoikis, which requires endocytosis and EEA1-positive endosomes (45). In this example, endosomes provide a platform for non-canonical activation of focal adhesion kinase (FAK) and survival signaling (45). FAK is not required for Akt activation, nor is Akt involved in FAK activation downstream of CXCR4 (unpublished data). Although endosomes may have a general role in cell signaling to suppress anoikis, the mechanisms we describe here may be unique to GPCRs. It is important to note that in siLuc treated cells CXCL12-mediated suppression of PARP cleavage or caspase-3/7 activity was similar, yet only moderate in both the adherent and detached setting (Fig. 7). Additional studies with more robust cellular and physiological model systems are needed to examine this further. Another interesting aspect of our findings is that although EEA1 depletion attenuates CXCR4-mediated
phosphorylation of Akt and FoxO1/3a (Fig. 5), suppression of apoptosis is still intact in adherent cells (Fig. 7). This is likely because other aspects of CXCR4 signaling are involved in suppressing apoptosis in adherent cells (3). However, this signaling redundancy is lost when cells are kept in suspension, consistent with endosomes selectively specifying Akt signaling that suppresses anoikis. How this signaling relates to non-adherent cells such as WEHI-231 cells (Fig. 4) or in cancers in which CXCR4 signaling may have opposite effects on anoikis (46,47) requires further investigation.

Our study provides evidence that chemokine receptor-mediated Akt signaling occurs in a compartmentalized manner to regulate context specific aspects of cell physiology. Our data are consistent with chemokine-receptor mediated Akt signaling occurring from the plasma membrane and from the surface of EEA1-positive endosomes. Signaling from endosomes may be selective for signaling to certain Akt substrates (FoxO1/3a), but not others (GSK3β, TSC2), suggesting that Akt signaling from endosomes may lead to different cellular outcomes than signaling from the plasma membrane. Endosomes may be required for suppression of apoptosis, but only in detached cells, not adherent cells. This is consistent with endosomes sensing and specifying (i.e. decoding) proper context-specific signaling. Although this compartmentalized signaling may be a general property of chemokine receptors, it remains to be determined if it can be broadly applied to other GPCRs. It will be important in the future to further elucidate the mechanisms involved in Akt signaling from endosomes and how it suppresses apoptosis. This may reveal novel aspects of this signaling that can be targeted in cancer linked to GPCRs such as CXCR4.

**EXPERIMENTAL PROCEDURES**

**Cell culture, antibodies and reagents** — HeLa (CCL-2) and WEHI-231 (CRL-1701) cells were from the American Type Culture Collection (Manassas, VA). Cells were maintained in minimum essential medium (MEM; Gibco Thermo Fisher Scientific Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Sigma Aldrich St. Louis, MO). Antibodies against pAkt-S473 (catalog no. 9271), Akt (catalog no. 9272), GSK3β (catalog no. 12456), pGSK3β-Ser9 (catalog no. 5558), pFoxO1/3a-T24/T32 (catalog no. 9464), FoxO3a (catalog no. 2497), FoxO1 (catalog no. 2880), TSC2 (catalog no. 4308), pTSC2-T1462 (catalog no. 3617), PARP (catalog no. 9542), and cleaved caspase-3 (catalog no. 9664) were from Cell Signaling Technologies (Danvers, MA). Antibodies against ERK-1/2 (catalog no. M8159) and pERK-1/2 (catalog no. M8159) were from Sigma. The antibody against APPL1 was from Protein Tech (catalog no. 19885) and the antibody against EEAI was from BD Transduction Laboratories (catalog no. BDB610456). The anti-β-tubulin (catalog no. E7) antibody was from Developmental Studies Hybridoma Bank (Iowa City, IA). Horseradish peroxidase-conjugated secondary antibodies were from Vector Laboratories (Burlingame, CA). CXCL12 (catalog no. PFP001) and CXCL13 (1-87) (catalog no. PFP019) were from Protein Foundry (Milwaukee, WI). Insulin (catalog no. 300-191P) was from Gemini Bio-Products (West Sacramento, CA). Dimethyl sulfoxide (DMSO; catalog no. D8418) and epidermal growth factor (EGF) were from Sigma. Pertussis toxin (catalog no. 3097) and Dynasore (catalog no. 2897) were from Tocris (Minneapolis, MN).

**siRNAs and transient transfection** — siRNAs directed against control luciferase (catalog no. 51-01-08-22), EEAI (catalog no. HSC.RNAI.N003566.12.1); HSC.RNAI.N003566.12.4 or APPL1 (catalog no. HSC.RNAI.N012096.12.1; HSC.RNAI.N012096.12.3) were from Integrated DNA Technologies (Coralville, IA). Lipofectamine 3000 (catalog no. L3000008, Life Technologies) transfection reagent was used for transfection of siRNA. HeLa cells were grown in 10-cm or 6-well dishes with 10 nM final siRNA against EEAI, APPL1, or control luciferase siRNA, similar to what we have previously described (8).

**Signaling Assay** — HeLa cells that were either transfected with siRNA or untransfected were passaged onto 6-well dishes and grown for an additional 24 hr to approximately 90% confluency. Cells were washed once with warm MEM containing 20 mM Hepes, pH 7.4 and incubated in the same medium for 3 h at 37°C. Cells transfected with siRNA were treated with 10 nM CXCL12, 100 nM CXCL13, 100 ng/mL EGF, 50 nM insulin, or...
vehicle (water, 1:1000 dilution) for 5 min at 37°C. To examine chemokine signaling in HeLa cells, cells were treated with 10 nM CXCL12 and 10-1000 nM CXCL13, CXCL11 or CXCL10. To examine the effect of dynasore, untransfected HeLa or WEHI-231 cells grown in 6-well dishes were preincubated with 80 μM dynasore or DMSO (1:1000 dilution) for 30-60 min at 37°C before treatment with CXCL12 or CXCL13 for 5 min. HeLa cells were then washed once with cold PBS and scraped off the dish in 300 μL of 2x sample buffer (8% SDS, 10% glycerol, 5% β-mercaptoethanol, 37.5 mM Tris-HCl, pH 6.5, 0.003% bromophenol blue). WEHI-231 cells were transferred to round bottom centrifuge tubes, centrifuged at 1000 x g for 5 min at 4°C, washed once in PBS and centrifuged to pellet cells. Cell pellets were solubilized in 300 μL 2x sample buffer. Equal amounts were analyzed by 10% SDS-PAGE and immunoblotting with antibodies against phosphorylated and total proteins. The phosphorylation status of various proteins was quantitated by densitometric analysis of similar exposures across multiple experiments using ImageJ software (NIH, Bethesda, MD).

**Anoikis Assay** – Cells were plated onto 6-well dishes 24 h after transfection. Twenty-four h later cells were analyzed in adhesion and detached settings. Cells were washed once with warm MEM containing 20 mM HEPES, pH 7.4 and incubated in the same medium for 1 h at 37°C. For the adhesion setting, adherent cells were then treated with 10 nM CXCL12 or vehicle and incubated at 37°C for 2-5 h. For the detached setting, cells were detached from the plate with HyQtase [Hyclone Laboratories (Logan, UT)], centrifuged, washed in PBS and resuspended in MEM containing 20 mM HEPES, pH 7.4 and seeded onto 6-well plates pretreated with 10 mg/mL poly(2-hydroxyethyl methacrylate; polyHEMA) (catalog no. P3932, Sigma) in 100% ethanol to prevent adhesion. Cells were then treated with 10 nM CXCL12 or vehicle and incubated at 37°C for 2 - 5 h. Adherent cells were washed once with cold PBS and harvested in 300 μL cold RIPA buffer [50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, 10 mM NaF, 10 mM sodium orthovanadate, protease inhibitors (10 μg/mL each of aprotinin, pepstatin A, and leupeptin; Roche Applied Science)]. Detached cells were collected into microcentrifuge tubes and centrifuged at 4°C at 1000xg for 5 m. Supernatant was aspirated and cells were washed twice with cold PBS. Cells were lysed in 300 μL RIPA buffer. Lysates were cleared by centrifugation at 14,000 rpm for 20 min at 4°C using a 5417r-Eppendorf microcentrifuge. Protein concentration of cleared lysates was determined using the BCA protein assay kit (Pierce Thermo Fisher). A small aliquot of sample (40 μL) was saved in 5x sample buffer for immunoblotting. PARP, cleaved caspase-3 and EEA1. The remaining sample with protein amount ranging from 10 μg to 58 μg across multiple experiments was used in accordance with Caspase-Glo 3/7 Assay kit (catalog no. G8091; Promega, Madison, WI) to measure the luminescence associated with the amount of caspase activity per sample.

**Internalization Assay** – Whole cell ELISA was used to measure CXCR4 and CXCR5 internalization, essentially as we have previously described (36,48). HeLa cells grown on 10-cm dishes were transiently transfected with HA-CXCR4 (10 μg) or HA-CXCR5 (10 μg) using polyethyleneimine (PEI). The next day, ~200,000 cells were seeded onto poly-l-lysine–coated 24-well plates. Cells were serum starved for 1 h; pretreated with DMSO or 80 μM dynasore for 30 min, followed by stimulation with 10 nM CXCL12 or 100 nM CXCL13 for 5 min. After fixation with 3.7% paraformaldehyde for 5 min and incubation with Tris-buffered saline (TBS) supplemented with 1% bovine serum albumin (BSA)/TBS for 45 min, cells were incubated with anti-HA monoclonal antibody (catalog no. 901513, Biolegend) for 1 h at room temperature. Cells were washed and incubated with alkaline phosphatase conjugated anti-Mouse IgG (Sigma; catalog no. A5153) in 1% BSA/TBS for 1 h at room temperature. Cells were washed and incubated in p-nitrophenyl phosphate diluted in diethanolamine buffer (catalog no. 9701861, Bio-Rad Laboratories). Reactions were stopped by adding 0.4 M NaOH and an aliquot was used to measure the absorbance at 405 nm. Percent receptor internalization was calculated by subtracting the fraction of absorbance after agonist treatment and vehicle treatment (following background subtraction) from 1 and multiplied by 100.

**Statistical Analysis** — Data are represented as the mean ± standard deviation of at least three
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independent experiments or determinations. All statistical tests were done using GraphPad Prism 7.0c for Mac OS X (GraphPad Software, San Diego). Student’s t test was used to compare the difference between two groups; one-way analysis of variance (ANOVA) was used to compare the difference between three or more groups, and two-way ANOVA was used to compare the difference between different groups under different treatment conditions. ANOVA was followed by Tukey’s or Newman-Kuel’s post hoc test. A probability (p) value of 0.05 was considered significant. Specific values are provided in the figure panels or in the figure legends.

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Conflict of interest
Authors declare they do not have any conflict of interests. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contributions
EJE and SAM performed all the experiments, analyzed data and helped write manuscript; AM conceived study, directed research, analyzed data and wrote manuscript.
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Figure Legends

Figure 1. Role of endocytosis on CXCL12-instigated Akt phosphorylation.
A, CXCR4 internalization was quantified by ELISA. HeLa cells transfected with HA-CXCR4 were treated with 80 μM dynasore or DMSO for 30 min followed by 10 nM CXCL12 or vehicle for 5 min. Bars represent the average percentage of CXCL12-induced receptor internalization from 3 independent experiments. Error bars represent the standard deviation. Data were analyzed by a Student’s unpaired t-test. p value is indicated.
B, Immunoblot analysis of Akt and ERK-1/2 phosphorylation. Serum starved HeLa cells were treated with 80 μM dynasore or DMSO for 30-60 min, followed by 10 nM CXCL12 or vehicle for 5 min. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. Representative immunoblots from four independent experiments are shown.
C,D, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 (C) or pERK-1/2 (D) to DMSO and CXCL12 treated samples. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test. p value is indicated.

Figure 2. Chemokine-mediated signaling in HeLa cells.
A, Immunoblot analysis of CXCL13-induced phosphorylation of Akt and ERK-1/2 in HeLa cells. Serum starved cells were treated with increasing dose of CXCL13 (10 – 1000 nM), 10 nM CXCL12 or vehicle for 5 min. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. Representative immunoblots from three independent experiments are shown.
B, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 or pERK-1/2 to CXCL12 treated samples. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test.
C, CXCL13-instigated phosphorylation of Akt and ERK-1/2 is pertussis toxin sensitive. HeLa cells were serum starved and treated with or without 50 ng/mL pertussis toxin for 7 h, followed by 10 nM CXCL12 or 100 nM CXCL13. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. Representative immunoblots from three independent experiments are shown.
D, E, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 (D) or pERK-1/2 (E) to CXCL12 treated samples. Error bars represent the standard deviation.
F, The effect of chemokines CXCL11 and CXCL10 in HeLa cells on phosphorylation of Akt or ERK-1/2. Serum starved cells were treated with vehicle, increasing doses (10 – 1000 nM) of CXCL11, CXCL10 or 10 nM CXCL12 for 5 min. Whole cell lysates were analyzed by immunoblotting for the indicated proteins. Representative immunoblots from three independent experiments are shown. Panels are separated from each other to remove irrelevant intervening bands, but are from the same exposure.
G, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 to CXCL12 treated samples. Error bars represent the standard deviation.

Figure 3. Role of endocytosis on CXCL12- or CXCL13-instigated Akt signaling.
A, CXCR5 internalization was quantified by ELISA. HeLa cells transfected with HA-CXCR5 were treated with 80 μM dynasore or DMSO for 30 min followed by 100 nM CXCL13 or vehicle for 5 min. Bars represent the average percentage of CXCL13-induced receptor internalization from
Endocytosis regulates CXCR4 signaling

3 independent experiments. Error bars represent the standard deviation. Data were analyzed by a Student’s unpaired t-test. p value is indicated.

B, CXCL12- or CXCL13-instigated Akt signaling was analyzed by immunoblotting. HeLa cells were treated with 80 µM dynasore or DMSO for 30 min followed by stimulation with 10 nM CXCL12 or 100 nM CXCL13 for 5 min at 37˚C. Equal amounts of whole cell lysates were analyzed by immunoblotting for the phosphorylated or total forms of the indicated proteins.

C-G, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 (C), pERK-1/2 (D), pFOXO1/3a (E), pGSK3β (F) or pTSC2 (G) to DMSO and CXCL12 treated samples. Error bars represent the standard deviation. Significance was determined by two-way ANOVA, followed by Newman-Kuel’s (B) or Tukey’s (D) multiple comparison test. Asterisks in C indicate significance between DMSO-CXCL12 vs. Dynasore-CXCL12 (*) and DMSO-CXCL13 vs. Dynasore-CXCL13 (**). p values are indicated in D.

Figure 4. Role of endocytosis on CXCL12- or CXCL13-instigated Akt phosphorylation in WEHI-231 cells.

A, CXCL12- or CXCL13-instigated Akt phosphorylation in WEHI-231 cells. Serum starved WEHI-231 cells were treated with 80 µM dynasore (DYN) or DMSO for 30 min and then 10 nM CXCL12, 100 nM CXCL13 or vehicle for 5 min at 37˚C. Whole cell lysates were analyzed by immunoblotting for pAkt-S473 and Akt.

B, Immunoblots were analyzed by densitometry. Bars represent the mean from three independent experiments relative to cells treated with DMSO and CXCL12. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test. p value is indicated.

Figure 5. Role of APPL1 on CXCL12-instigated Akt phosphorylation.

A, CXCL12-, insulin- and EGF-instigated Akt phosphorylation was examined in cells depleted of APPL1. Serum starved HeLa cells transfected with control siRNA (siLuc) or two distinct siRNA against APPL1 (siAPPL1-1 and siAPPL1-2) were treated with CXCL12 (10 nM), insulin (50 nM) or EGF (100 ng/mL) for 5 min at 37˚C. Whole cell lysates were analyzed by immunoblotting for pAkt-S473, total Akt-1/2 and APPL1.

B, Immunoblots were analyzed by densitometry. Bars represent the mean of the relative levels of pAkt-S473 to siLuc and CXCL12 treated cells. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test. The p values for insulin treated cells are indicated. CXCL12 or EGF treated samples were not significant.

Figure 6. Role of EEA1 on CXCL12-instigated Akt phosphorylation.

A, CXCL12-instigated Akt phosphorylation was examined in cells depleted of EEA1. Serum starved HeLa cells transfected with control siRNA (siLuc) or two distinct siRNA against EEA1 (siEEA1-1 and siEEA1-2) were treated with 10 nM CXCL12 for 5 min at 37˚C. Whole cell lysates were analyzed by immunoblotting for the phosphorylated or total forms of the indicated proteins.

B-D, Immunoblots were analyzed by densitometry. Bars represent the mean relative levels of pAkt-S473 (B), pFoxO1/3a (C) or pTSC2, pGSK3β and pERK-1/2 (D) to siLuc and CXCL12 treated samples. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test. p value is indicated.
Figure 7. Role of EEA1 on CXCR4-mediated suppression of apoptosis.
A, Examination of CXCL12 promoted suppression of apoptosis in HeLa cells depleted of EEA1. Cells transfected with control siRNA (siLuc) or siRNA against EEA1 (siEEA1-1 or siEEA1-2) were left adherent on the culture dish or left detached by seeding onto polyHEMA dishes whereby cells were unable to adhere. Adherent (Ad) or detached (Det) cells were treated with 10 nM CXCL12 for 2-5 h at 37°C. Equal amounts of cleared lysates were analyzed by immunoblotting for PARP. Representative immunoblots are shown from 4 independent experiments.
B, Immunoblots were analyzed by densitometry. Bars represent the mean of cleaved PARP levels relative to siLuc and vehicle treated sample. Error bars represent the standard deviation. Data were analyzed by two-way ANOVA, followed by Tukey’s multiple comparison test. p values are indicated.
C, Parallel samples from A were examined for caspase-3/7 activity, as described in Experimental Procedures. Bars represent the mean fold change in caspase-3/7 activity with siLuc transfected cells that were left adherent to the culture dish and treated with vehicle. Error bars represent the standard deviation. Single points were removed from the analysis if greater than one standard deviation from the mean. Data were analyzed by one-way ANOVA, followed by Tukey’s multiple comparison test. The p values are indicated. n.s., not significant.
Figure 1.

A

% Internalization

DMSO DYN

B

CXCL12: DMSO DYNASORE

pAkt-S473
Akt

pERK-1/2
ERK-1/2

DMSO Dynasore

C

Relative pAkt-S473 Levels

D

Relative pERK-1/2 Levels

p = 0.0005

p = 0.0281
Figure 2.

A

B

C

D

E

F

G

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Figure 3.

A

Percent Internalization

DMSO | DYN

p = 0.0015

B

CXCL12: - - + -
CXCL13: - - + -

DMSO | DYN

p = 0.0316

C

Relative pAkt-S473 Levels

CXCL12 | CXCL13

DMSO | Dynasore

p = 0.0380

D

Relative pERK-1/2 Levels

CXCL12 | CXCL13

DMSO | Dynasore

E

Relative pFoxO Levels

CXCL12 | CXCL13

DMSO | Dynasore

p = 0.0380

F

Relative pGSK3β Levels

CXCL12 | CXCL13

DMSO | Dynasore

G

Relative pTSC2 Levels

CXCL12 | CXCL13

DMSO | Dynasore

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Figure 4.
Figure 5.

A

siLuc | siAPPL1-1 | siAPPL1-2
---|---|---
Veh | CXCL12 | Insulin | EGF | Veh | CXCL12 | Insulin | EGF | Veh | CXCL12 | Insulin | EGF

pAkt-S473
Akt1/2
APPL1

B

Relative pAkt-S473 Levels

p = 0.0058

p = 0.0058

(Mr)
**Figure 6.**

(A) Relative pAkt-S473 Levels

(B) Relative pFoxO1/3a Phosphorylation

(C) Relative pTSC2/TSC2, pGSK3β/GSK3β, pERK-1/2/ERK-1/2

CXCL12: - + - + - +

pAkt-S473, Akt1/2, EEA1, pFoxO1/3a-T24/T32, FoxO1, FoxO3a, pGSK-3β-S9, GSK-3β, pTSC2-T1426, TSC2, pERK-1/2, ERK-1/2

(Mr)

p < 0.0001

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Figure 7.

A

| siLuc | siEEA1-1 | siEEA1-2 |
|-------|---------|---------|
| Ad    | Det     | Ad      | Det     | Ad      | Det     |

- CXCL12:
- PARP
- EEA1
- Tubulin

+ - + - + -

Ad Det Ad Det Ad Det

siLuc siEEA1-1 siEEA1-2

Cleaved PARP

Full-length PARP

B

Fold change in PARP Cleavage

C

Fold change in Caspase-3/7 Activity

n.s. p = 0.0261 p = 0.0019

p = 0.0039 p = 0.0047

Fold change in PARP Cleavage

0 1 2 3 4

75 50 150 150

-+-+-+-+

n.s. p = 0.0047 p = 0.0039

B

Fold change in PARP Cleavage

C

Fold change in Caspase-3/7 Activity

n.s. p = 0.0261 p = 0.0019

-+-+-+-+

B

Fold change in PARP Cleavage

C

Fold change in Caspase-3/7 Activity

n.s. p = 0.0261 p = 0.0019

-+-+-+-+
Endocytosis is required for C-X-C chemokine receptor type 4 (CXCR4)-mediated Akt activation and anti-apoptotic signaling
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