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A novel cross-talk between CXCR4 and PI4KIII α in prostate cancer cells.

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Abstract

Chemokine signaling regulates cell migration and tumor metastasis. CXCL12, a member of the chemokine family, and its receptor, CXCR4, a G protein coupled receptor (GPCR), are key mediators of prostate-cancer (PC) bone metastasis. In PC cells androgens activate *CXCR4* gene expression and receptor signaling on lipid rafts, which induces protease expression and cancer cell invasion. To identify novel lipid-raft-associated CXCR4 regulators supporting invasion/metastasis, we performed a SILAC-based quantitative proteomic analysis of lipid-rafts derived from PC3 stable cell lines with overexpression or knockdown of CXCR4. This analysis identified the evolutionarily conserved phosphatidylinositol 4-kinase III α (PI4KIII α), and SAC1 phosphatase that dephosphorylates phosphatidylinositol-4-phosphate as potential candidate CXCR4 regulators. CXCR4 interacted with PI4KIII α membrane targeting machinery recruiting them to the plasma membrane for PI4P production. Consistent with this interaction, PI4KIII α was found tightly linked to the CXCR4 induced PC cell invasion. Thus, ablation of PI4KIII α in CXCR4-expressing PC3 cells reduced cellular invasion in response to a variety of chemokines. Immunofluorescence microscopy in CXCR4 expressing cells revealed localized production of PI4P on the invasive projections. Human tumor studies documented increased PI4KIII α expression in metastatic

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tumors vs. the primary tumor counterparts, further supporting the PI4KIII α role in tumor metastasis. Furthermore, we also identified an unexpected function of PI4KIII α in GPCR signaling where CXCR4 regulates PI4KIII α activity and mediate tumor metastasis. Together, our study identifies a novel cross-talk between PI4KIII α and CXCR4 in promoting tumor metastasis and suggests that PI4KIII α pharmacological targeting may have therapeutic benefit for advanced prostate cancer patients.

Introduction

CXCR4 is a G-protein coupled chemokine receptor expressed on epithelial, endothelial and mesenchymal cells. Binding to its ligand CXCL12, also known as SDF-1 α leads to activation of intracellular signaling pathways culminating in cellular migration and invasion. Cancer cells co-opt the CXCL12/CXCR4 pathway for various stages of progression, including angiogenesis, tumor inflammation and metastasis (1–3). Transcriptional regulation of the CXCR4 gene is a key determinant of net cell surface expression of CXCR4 and its subsequent function in transformed epithelial cancer cells. We have shown that TMPRSS2-ERG fusions regulate CXCR4 expression in prostate tumors; thus, androgen induced ERG expression transcriptionally regulates CXCR4 expression in prostate cancer (PC) cells (4, 5). In addition, several factors and organ microenvironments have been shown to regulate CXCR4 expression in tumor cells (6–15). In bone metastasis, the CXCL12/CXCR4 axis is critically involved in initial colonization of cancer cells to stem cell niches for establishment of metastasis (16, 17). The CXCR4 antagonist plerixafor is effective in preventing initial establishment of prostate cancer bone metastasis, whereas the drug has no effect on established metastasis (18). To determine the CXCR4 interacting proteins contributing to PC cell invasion and metastasis, we performed SILAC analysis on prostate cancer cells and found PI4KIII α and Sac1 as potential CXCR4 effector proteins which regulate cellular phosphatidylinositol (PtdIns) metabolism.

Phosphatidylinositol (PtdIns) phosphates (PIPs), or phosphoinositides (PIs), are cellular phospholipids phosphorylated at 3, 4 and/or 5 positions on the myo-inositol ring by several types of kinases specific to each position. PIs species are important regulatory lipid messengers involved in recruitment of several effector proteins to activate signaling pathways involved in cellular vesicular traffic (19–21). Phosphatidylinositol 4 kinases phosphorylate position 4 on PtdIns to generate PtdIns4P that is an essential precursor for PtdIns(4, 5)P₂ and PtdIns(3,4,5)P₃ production. There are four members of PI4K kinases: two type II (α and β) and two type III (α and β), each one specifically localized to distinct cellular compartments. Between these two families, the PI4KIII α members are essential for life (22), suggesting their critical role in PIP metabolism. PI4KIII α is predominantly localized to ER/Golgi and plasma membrane (PM) where it is primarily involved in the production of PtdIns4P (21, 23). The PI4KIII α has dual functions: to regulate the PIP binding proteins involved in intracellular vesicular traffic, and to generate the abundant PtdIns(4)P and PtdIns(4,5)P₂ in PM. In addition to the catalytic function of these kinases, the structural domains also modulate activity of vesicular trafficking proteins (24). A complex regulation of accessory proteins, which are evolutionary conserved mediates PI4KIII α localization to PM, where it participates in generation of PtdIns4P (25–27). The

phosphatases, which dephosphorylate PIPs are fairly specific to each phosphate in the myo-inositol ring, and often localize to subcellular locations where their substrates are produced. In addition, they form a complex with kinases to tightly regulate the kinase activity (28, 29). PI4P phosphatase Sac1 is expressed in ER and a critical determinant of PM associated PI4P levels.

In the present study we investigate the relationship between CXCR4 and PI4KIII α activity in terms of biochemical characterization and consequences to cancer cell invasion and metastasis.

Results

A novel association of PI4KIII α with CXCR4 in lipid rafts of CXCR4-expressing prostate cancer cells:

We have previously shown that CXCR4 localizes to the lipid rafts in prostate cancer cells, and that its cellular functions in this cell type are dependent on its localization to lipid rafts (17, 18, 30). To identify CXCR4 interacting proteins in lipid rafts, CXCR4-overexpressing (CXCR4) and CXCR4-knockdown (shCXCR4) PC3 stable cell lines were generated by lentiviral transduction. FACS analysis of CXCR4 and shCXCR4 cell lines showed large positive shifts in median fluorescence intensity (MFI) for CXCR4-1 and CXCR4-2, indicative of large CXCR4 overexpression, while the shCXCR4 cell line shCXCR4-1 showed a large negative shift consistent with CXCR4 knockdown (Figure 1A and B). The CXCR4 overexpression and knockdown in these cell lines were confirmed by quantitative RT-PCR (Figure 1C) and Western blot analysis. A comparative SILAC-based proteomics analysis of lipid-raft microdomains purified from CXCR4-2 and shCXCR4-1 cell lines by sucrose-gradient buoyant-density ultracentrifugation was performed (Supplementary figure 1 for experimental flow). Among the 277 proteins that were identified, 126 proteins showed a >1.5-fold change either above (79 proteins) or below (47 proteins) unity, being either more or less abundant in CXCR4-overexpressing vs. shCXCR4 knockdown cells, respectively. As expected G-protein coupled receptor signaling components (e.g., gamma-2, -5, -10 and -12, beta-1, -2 and -5, and G(i) alpha-2 and -3, G(s) alpha, and G(q) alpha-11) and Src kinase family members (Src and Fyn) are overexpressed in CXCR4 cells (Supplementary figure 2). Unexpectedly, we found phosphatidylinositol 4-kinase type III α (PI4KA) isoform overexpressed in CXCR4 cells by SILAC analysis (Figure 1D). Its counterpart the Sac1 phosphatase was 11.0% more abundant in CXCR4 cells. Likewise, Western blot analysis of lipid rafts showed higher expression of PI4KIII α and Sac1 in CXCR4 overexpressing cells (Figure 1E). Proteomic data of CXCR4 cells suggested a potential functional association between CXCR4 and PI4KIII α /Sac1 in prostate cancer cells.

CXCR4 regulates protein expression and lipid kinase activity of PI4KIII α in prostate cancer cells.

To determine the significance of CXCR4 and PI4KIII α association in lipid rafts, we first assessed whether CXCR4 regulates PI4KIII α expression in prostate cancer cells. Data from Western-blotting experiments in CXCR4-overexpressing cells showed approximately a 2-fold increase in PI4KIII α levels vs. the control PC3 parent cell line (Figure 2A,B).

Concordantly, CXCR4 knockdown cells had reduced expression of both PI4KIII α and Sac1 expression. QPCR analysis of PI4KIII α and Sac1 did not show changes in gene expression between parental PC3 cells and CXCR4 manipulated cells (Supplementary figure 3) suggesting a post-transcriptional mode of PI4KIII α protein elevation in CXCR4 overexpressing PC3 cells. Cycloheximide treatment demonstrated that both CXCR4 and PI4KIII α protein expression were decreased in PC3-CXCR4 cells compared to control PC3 cell which have lower levels of both proteins; thus the overexpressed CXCR4 stabilizes PI4KIII α in PC3 CXCR4 cells. The levels of both CXCR4 and PI4KIII α in these cells were downregulated upon cycloheximide treatment in a similar fashion, further suggesting a post-transcriptional mode of their cellular regulation (Supplementary figure 4). Recent studies have indicated that CXCR4 signaling regulates the translational machinery involved in mTORC1 signaling (31), suggesting that PI4KIII α overexpression could be due to a translational mechanism involving CXCR4/mTORC1 signaling. To identify whether the greater protein levels of PI4KIII α is accompanied by elevated lipid kinase activity, we immunoprecipitated PI4KIII α from prostate cancer cells with anti-PI4KIII α antibody and subjected the immunoprecipitates to in vitro lipid kinase activity using PtdIns as a substrate. In vitro lipid kinase assays also confirmed that PI4KIII α kinase activity was similarly elevated ~ 2 fold in the CXCR4-overexpressing cells and reduced in CXCR4 knockdown cells (Figure 2C and H). SiRNA mediated knockdown of PI4KIII α resulted in a large downregulation of PI4KIII α and small downregulation of Sac1 (Figure 2D) without a change in PI4KIII β expression (Supplementary figure 5) in PC3 cells. PI4KIII α knock down experiments resulted in loss of PI4KIII α lipid kinase activity (Figure 2E). Comparison of PI4KIII α and Sac1 protein expression among three PC cell lines, namely, LNCaP, C42B and VCaP, by WB showed higher expression in VCaP cells compared to LNCaP cells (Figure 2F) and in vitro lipid kinase activity is also similarly higher in VCaP cells (Figure 2G). Comparison of PI4KIII α expression and in vitro lipid kinase activity between the prostate cancer cells (LNCaP and C4-2B) and PC3 scr show that the latter have higher PI4KIII α expression and in vitro lipid kinase activity (Figure 2A, F and H). VCaP cells which are TMPRSS2-ERG fusion positive, where ERG factor was shown to regulate CXCR4 expression (17), had 3-fold higher PI4KIII α lipid kinase activity vs. the other two PC cell lines (Figure 2G and H). The higher lipid kinase activity may be reflective of the more active state of the PI4KIII α in VCaP cells, which may be dependent on the expression of accessory proteins which bind and translocate PI4KIII α to the membrane.

To further determine the role of PI4KIII α activity in maintaining cellular PI4P levels, and the requirement of PI4P for the conversion to downstream PtdIns species, PI(4,5)P₂ and PI(3,4,5)P₃, we monitored cellular PI profiles using our previously established procedures (32, 33). PC3 CXCR4 cells transfected with scrambled (Scr) siRNA show that PI4P is the major constituent of PtdIns species followed by PI(4,5)P₂ in prostate cancer cells (Table 1). PI4KIII α knockdown through siRNA significantly decreases the protein along with in vitro lipid kinase activity (Figure 2D and E). PtdIns profiles were significantly altered upon PI4KIII α knockdown; as expected total PI4P levels were reduced suggesting the major role of PI4KIII α in maintaining PI4P levels, whereas there is an unexpected increase in total PI(4,5)P₂ levels. The minor PtdIns species PI(3,4)P₂ was decreased whereas PI(3,4,5)P₃ was increased (Table 1). These data suggest that PI4P synthetic processes in prostate cancer

cells were complexly related with steady state levels of other PtdIns species, and knockdown of PM associated PI4KIII α levels leads to significant alterations in the cellular PtdIns profiles. These results indicated that upregulated PI4KIII α levels are active and positively correlate with CXCR4 expression in prostate cancer cells.

Direct interaction between CXCR4 and PI4KIII α adaptor proteins:

To further determine the mechanism of CXCR4 regulation of PI4KIII α , first we determined whether the two proteins interact with each other. For this purpose, we performed tagged gene transfection followed by WB analysis; data show no direct interaction between PI4KIII α and CXCR4 (Figure 3A). PI4KIII α is localized to the inner plasma membrane through two adaptor proteins, EFR3B and TTC7B. EFR3B is a membrane bound protein and TTC7B is an adaptor protein which binds with both EFR3B and PI4KIII α . We tested whether EFR3B and TTC7B bind with the chemokine receptor CXCR4. Our data show that chemokine receptors exhibit a specific binding with both EFR3B and TTC7B (Figure 3B and 4A). In addition, we also found other chemokine receptors including CXCR7 (Figure 3C) and CXCR1 (Supplementary figure 6A) to interact with EFR3B, whereas other GPCRs, such as Gprc6a (Figure 3D) and human α 2b adrenergic receptor (ADR2B), did not interact with EFR3B (Supplementary figure 6B). As a negative control myc tagged GDI2 (GDP dissociation inhibitor) did not interact with CXCR4 in our assay, suggesting chemokine family receptor interaction with either EFR3B or TTC7B is highly specific. Interestingly, EFR3B binds with CXCR4 dimers whereas TTC7B binds mostly with CXCR4 monomers (Figure 3B,C and Figure 4A). Immunofluorescence study shows that both CXCR4 and EFR3B give a punctate staining and are highly expressed at cell-cell contacts. Merged images show a co-localization of CXCR4 and EFR3B along cell-cell contacts (Figure 3E). This data supports SILAC studies and concludes that chemokine receptors may activate PI4KIII α kinase by binding with PI4KIII α adaptor proteins and, through this interaction, recruit PI4KIII α to the plasma membrane.

PI4KIII α positively regulates chemokine stimulated invasion:

To determine the functional significance of PI4KIII α mediated CXCR4 regulation and its effect on cellular invasion, we determined PI4P localization in cells and performed in vitro cellular invasion studies. Immunostaining of PI4P (green color for PC3 RFP and red color for PC3 CXCR4-2 cells) shows a uniform punctate staining in PC3 RFP cells, whereas, in PC3 CXCR4 cells, it is localized to invasive fronts (Figure 5A). The quantitation of PI4P signal intensity showed that PC3 CXCR4 cells have significantly higher expression over PC3 RFP cells (Figure 5B). Higher localization of PI4P in invasive projections in PC3 CXCR4 cells and known role of CXCL12/CXCR4 in cellular invasion prompted us to determine the effect of PI4KIII α on cellular invasion. As expected overexpression of CXCR4 enhanced basal and CXCL12 induced cell invasion (Figure 5C) and knockdown of PI4KIII α severely reduced both basal and CXCL12 induced cellular invasion in PC3-CXCR4 cells (Figure 5D). To further determine if the PI4KIII α effect is confined to CXCL12/CXCR4 or is a general effect of chemokine mediated cellular invasion, we tested three other chemokines, CXCL11, CXCL8 and CCL2,1 all of which are known to mediate invasion of prostate cancer cells. PI4KIII α knockdown reduced invasion of basal as well as all of the other tested chemokine induced cell invasions (Figure 5E) without significantly

affecting the cell proliferation (Figure 5F), suggesting that PI4KIII α is a positive regulator of chemokine induced cell invasion.

PI4KIII α expression associates with prostate cancer metastasis.

To determine the clinical significance of PI4KIII α in prostate cancer progression, we extracted expression values for PI4KIII α and Sac1 from NCBI data set GDS3289 of human PC tumor tissues. The analysis shows that PI4KIII α expression is significantly higher and Sac1 is lower in PC metastasis compared to either localized PC or normal adjacent tissue (Figure 6A). No difference in the expression of either PI4KIII α or Sac1 is found between low PIN and high PIN lesions in PC patients (Figure 6A). Similarly, no changes in expression are observed between naïve and hormonal therapies in metastatic patients (Figure 6A). Analysis of PI4KIII β isoform did not show significant changes in expression between PC metastasis compared to localized PC (supplemental figure 7). To confirm protein expression, we performed immunohistochemical analysis of PI4KIII α expression in human PC patient tumor tissues containing matched pairs of primary PC and their corresponding metastatic sites (Figure 6B). IHC analysis shows that PI4KIII α is expressed on both human primary as well as metastatic tissues, but is higher in metastasis tissue compared to primary prostate tumor tissues. Higher expression of PI4KIII α in metastatic tissues implies elevated expression coupled with higher activity can promote the growth of metastasis.

Discussion

In an effort to identify CXCR4 effector proteins involved in cellular migration and invasion, we performed SILAC proteomics analysis and identified PI4KIII α as one of the proteins whose expression is tightly linked to CXCR4 in the prostate cancer cell line PC3. Here we have characterized the novel cross-talk between CXCR4 and PI4KIII α , and revealed that both proteins localize to lipid rafts together, participating in cancer cell invasion. Our studies also conclude that CXCR4 does not directly bind PI4KIII α , but interacts through PI4KIII α adaptor proteins, EFR3 and TTC7. CXCR7, which is closely related to CXCR4, and another chemokine receptor, CXCR1, also interact with EFR3 similar to CXCR4. EFR3 and TTC7 interaction with PI4KIII α is evolutionarily conserved (34, 35), where EFR3B is a peripheral membrane protein targeted to PM through a palmitoylated anchor (36), which interacts with soluble adaptor protein TTC7 bound PI4KIII α . Structural studies demonstrate that TTC7 binds with both EFR3 and PI4KIII α (26), and membrane anchored EFR3 serves as a docking site for this complex in PM. Binding studies demonstrate highly specific interaction between CXCR4 and both EFR3B and TTC7B (Figure 3 and 4). EFR3B binds exclusively with CXCR4 dimers, whereas TTC7B binds predominantly with CXCR4 monomer. CXCR4 has been shown to homodimerize as well as heterodimerize with other GPCRs, including members of chemokine receptors (37, 38), and CXCR4 dimers plays a key role in cell migration and metastasis (30, 39). Our data imply that EFR3B interaction with CXCR4 dimers may recruit TTC7B-PI4KIII α to PM where TTC7B may also involve CXCR4 binding. Alternatively, CXCR4/EFR3B/PI4KIII α complexes can be formed which involve dimeric CXCR4 and thus promote a signaling active state in cells. In support of this complex, a previous report demonstrates that PI4KIII α can participate in complex formation on cell membrane devoid of TTC7 (25). Whereas TTC7B binding with CXCR4 monomer

may not possess productive signaling competence, this issue deserves merit for further investigation. Nevertheless, our study demonstrated a novel interaction between chemokine receptors and members of PI4KIII α adaptor proteins and this interaction can target PI4KIII α to PM in prostate cancer cells (Figure 7). Chemokine receptors in prostate cancer cells can fulfill a role similar to FAM126 (27) and TMEM150 (25) which have been shown to participate in recruiting PI4KIII α to PM through both TTC7 and EFR3 interactions. Thus, this novel interaction between chemokine receptors and PI4KIII α complexes may contribute to prostate cancer progression.

Among phosphatidylinositol 4 kinases, PI4KIII α has been demonstrated to be critical for maintenance of PI4P pools in PM (23, 40), where it serves as a major precursor for the bulk of PI(4,5)P₂ and, subsequently, for PI(3,4,5)P₃. In addition, PI4P also has major roles on localization of other lipids such as cholesterol through oxysterol binding protein (Osh4) and sphingolipids. Profiling different members of the PI family in the CXCR4 overexpressing prostate cancer cell line PC3 showed that PI4P is the abundant PtdIns species (Table 1). PI4KIII α knockdown through siPI4KIII α RNA in PC3-CXCR4 cells indicated a significant reduction in steady-state levels of PtdIns4P, suggesting the key role of PI4KIII α in maintenance of cellular PI4P in prostate cancer cells similarly to non-cancer cells as shown previously. Our studies also demonstrated that steady-state levels of PI(4,5)P₂ are increased upon PI4KIII α knockdown in PC3-CXCR4 cells, suggesting that, in the absence of PI4KIII α activity, other lipid kinases enhance their activities to produce higher levels of cellular PI(4,5)P₂ levels. Previous studies also proposed that PI4KIII α knockdown in cells leads to compensatory upregulation of PI4P kinases, whereby normal PI(4,5)P₂ levels are maintained at low PI4P through the activation of PI4P kinases (35), though the molecular details of this regulation still need investigation. This homeostatic compensatory regulation appears to be specific for PI4KIII α , owing to the fact that it is a critical regulator of PM identity (35). Knockdown of other intracellular PI4K isoforms (PI4KII α and PI4KIII β) leads to reduction in PI(4,5)P₂ levels (41). Interestingly, CXCR4 knockdown cells have lower PI4KIII α expression and activity but somewhat higher expression of PIP5K1 α (supplementary fig 8), which can explain at least in part the higher PI(4,5)P₂ in PI4KIII α knockdown cells. What is the intracellular distribution and functional importance of PI(4,5)P₂ in cancer cells remain to be determined in future work.

PI4KIII α activity has been shown to be a critical determinant of plasma membrane identity by maintaining the levels of PI4P and PI(4,5)P₂. Embryonic fibroblasts lacking PI4KIII α showed impairment of PI4P and PI(4,5)P₂ production at plasma membrane and relocation of several of plasma membrane PI4P/PI(4,5)P₂-binding proteins to internal compartments (35). Our data indicate that PI4KIII α activity has additional functions in prostate cancer cells, where PI4P highly localizes to invasive projections in CXCR4 overexpressing cells thereby contributing to the invasiveness of cancer cells (Figure 5). In support of this hypothesis, we show that PI4KIII α knockdown causes a severe defect in in vitro invasion without a significant alteration in cell proliferation. Our data from the cellular invasion assay also demonstrate that PI4KIII α has a broader role on cellular invasion as its knockdown significantly inhibited multiple members of chemokine mediated PC3 cell invasion (Figure 7). Chemokine signaling has been shown to promote metastasis, where circulating tumor cells arrested at metastatic sites use CXCL12/CXCR4 signaling to colonize metastatic sites

(1, 2). The biological function of CXCL12/CXCR4 is established in bone metastasis, where this activated signaling pathway in PC cells promotes metastasis by direct competition of PC cells with osteoblastic niche in the bone microenvironment (16). In support of this view we have recently shown that CXCR4 antagonists inhibit initial establishment of experimental prostate cancer bone metastasis (18). PI4KIII α signaling may contribute to homing of cells to metastatic sites by promoting attachment, invasion at bone and proliferation at bone.

In conclusion, in this study we assign a novel role of the chemokine receptors in recruiting PI4KIII α activity for cancer cell invasion (Figure 7). By doing so, CXCR4 regulates PI4P production on the plasma membrane via interacting with cellular PI4KIII α machinery. Thus, our data reveal that GPCRs, in addition to their well-known function to utilize PI(4,5)P₂, can also modulate PI4P levels. Our studies form a basis for future research in characterizing the detailed mechanism underlying the chemokine receptor interactions with PI4KIII α in cancer cells and the impact of this regulation on tumor metastasis.

Materials and Methods:

Cell culture

LNCaP and PC3 stable, lentiviral-generated cell lines were maintained in RPMI-1640 with 10% heat-inactivated FBS, 1% P/S (50 units/ml penicillin, 50 μ g/ml streptomycin) and appropriate selection antibiotics (40 μ g/ml blasticidin S for PC3 RFP and PC3 CXCR4 overexpressing cells; puromycin at 3 μ g/ml for PC3 scr-shRNA, or 24 μ g/ml for PC3 CXCR4-shRNA knockdown cells). VCaP cells were maintained in DMEM) and C42B, in T-medium. All cell lines were authenticated with STR analysis (Genomics core at Michigan State University, East Lansing, MI) and shown to have markers respective for each cell line as established by ATCC, and were also tested for mycoplasma contamination prior to use with Venor-GeM mycoplasma detection kit (Sigma Biochemicals, St. Louis, MO).

Stable Isotope Labeling with Amino Acids in Cell Culture (SILAC).

The PC3 CXCR4 shRNA and PC3 CXCR4 cell lines were then maintained in RPMI 1640 media for SILAC as described previously (42).

Lentiviral generation of stable cell Lines.

Stably transduced PC3 cells with a knocked-down (GIPZ shRNA-CXCR4 lentiviral construct) or overexpressed (pLOC-CXCR4 lentiviral construct) CXCR4 gene were produced using a Trans-Lentiviral Packaging Kit (Thermo-Fisher Scientific) according to manufacturer's protocols. Briefly, pLOC-CXCR4 was generated by PCR cloning of CXCR4 gene from a pCDNA3-CXCR4 construct (30) as template. Transfection into HEK293T cells generated infectious, non-replicating pseudoviral particles used to stably transduce PC3 cells and isolate stable clones selected with blasticidin S. GIPZshRNA-CXCR4 lentiviral construct targeting the 5'-UTR of CXCR4 mRNA (mature antisense sequence: 5'-ACAGCAACTAAGAACTTGG-3') was purchased/obtained through GE Dharmacon (Lafayette, CO 80026)/Wayne State University Biobank Core Facility and used in a similar manner to transduce PC3 cells with infectious, replication incompetent lentiviral particles to generate stable CXCR4-knockdown cells using puromycin for selection of stable clones. For

selecting stable clones, lentivirus transduced cells were seeded in 96 well plates at single cell density, monitored for GFP/RFP fluorescence and treated with either blasticidin S or puromycin. Two clones were further characterized for CXCR4 overexpression and knockdown and used in subsequent experiments.

Lipid raft preparation.

Lipid rafts were prepared by buoyant density ultracentrifugation as described (30) with the following modification. PC3 shCXCR4 and PC3 CXCR4 cells were cultured in SILAC media for 7 passages as described above and cell lysates were prepared in detergent free buffers and subjected to discontinuous sucrose gradient centrifugation. The fractions tested for the lipid raft marker flotillin (anti-flotillin antibody cat # 610383, lot #52886 from BD Transduction laboratories) showed it to be enriched in #5 fraction.

Western blotting and immunoprecipitation (IP).

Western blot analysis and immunoprecipitation were performed as previously described (18). For immunoprecipitations samples of 100–200 µg cell lysate protein in a 200–300 µl final volume of RIPA⁺⁺ lysis buffer were incubated with appropriate antibodies (anti-HA cat # SC57592, lot #D1515 from Santa Cruz Biotechnology, anti-GFP cat #A11122, lot #1828014 from Invitrogen and anti-Myc Cat # 71D10 from Cell Signaling) overnight at 4 °C on an end-over-end mixer. Following 1 wash in RIPA⁺⁺ buffer, they were used for lipid kinase activity assay or washed 3 more times prior to SDS PAGE.

PI4KIII α lipid kinase assay.

In vitro PI4KIII α lipid kinase assays were performed as described earlier (22). Post kinase assay the chloroform-extracted PI(4)P product was separated by thin-layer chromatography (TLC) in n-propanol-2M acetic acid (65:35 v/v). PtdIns was visualized with I₂ vapor following PI(4)P detection through autoradiography. PI4KIII α activity was set as one fold in control PC3 cells (PC3 scr and PC3 RFP) and compared with CXCR4 manipulated cells. In other prostate cancer cells PI4KIII α activity was set as one fold in LNCaP cells and compared with activity of C4-2B and VCaP cells.

Cellular PtdIns quantitation using high performance liquid chromatography.

Previously described procedures for PtdIns profiling in cells were employed. Briefly, scr and PI4KIII α siRNA transfected PC3 CXCR4 cells were cultured overnight in inositol-free cell type-specific medium and labeled for 24–48 h with 25 µCi/ml of myo-[2-³H] inositol in an inositol free medium following previously published protocols (32). Cellular lipids are extracted with acidified chloroform-methanol, deacylated with methylamine reagent, and the deacylated GroPIInsP products are analyzed by HPLC (Waters 5215) on a 5-micron Partisphere SAX (strong anion exchange) column (Whatman) under a shallow water-ammonium phosphate gradient (33).

Plasma membrane PtdIns 4P immunofluorescence analysis.

For plasma membrane PI4P staining, the protocol of Hammond et al. was followed (43). PC3 RFP and PC3 CXCR4-2 cells grown on coverslips were blocked and permeablized with

0.5% (w/v) saponin and incubated with mouse monoclonal anti-PI4P (Cat # Z-P004 from Echelon Biosciences); subsequently, Alexa Fluor 488 (for PC3 RFP cells) or 594 (for the PC3 CXCR4-2 or M-Luc2 cells) goat anti-mouse IgM(heavy chain) secondary antibody (Invitrogen) was used and were post-fixed with 2% formaldehyde and mounted on glass slide with antifade reagent containing DAPI. The cells were visualized with a Leica DMI3000 B wide-field fluorescence microscope (Leica Microsystems Inc., Buffalo Grove, IL) and images, captured with a SPOT™ Pursuit Slider USB 1.4 Mp color and high sensitivity monochrome CCD digital camera, coupled to Windows SPOT 5.1 Basic imaging software. Image analysis was performed with ImageJ 1.48v software after conversion of monochrome images to grayscale (0–255) and using the line profile tool to plot an intensity profile graph of the intensities of pixels along a line intersecting a given point light source and then measuring the area under the peak using the wand tool and calculating the mean of several of these points of light. Corrections were made to account for differences in gain, exposure time and detector sensitivity for image acquisition as well as for differences in fluorophore brightness (differences in labeling efficiency between the two fluorophores were not corrected for; the Alexa Fluor 488 efficiency was 1.25 x > that of the 594.

Cell invasion assay.

Cell invasion assays were performed as described earlier (17). Briefly, cells (\approx 40 hours post siRNA transfection) were seeded into cell culture inserts for invasion assays. In bottom wells chemokines CXCL12, CXCL11, CXCL8 and CCL21 were added at 200 ng/ml concentration in 500 μ l volume. The migrated cells in the center of the membrane were counted in 5 consecutive fields (each one with an area of $300 \times 200 \mu\text{m}^2$) under a microscope at 200 \times power. The mean number of traversing cells was calculated and expressed as the mean plus or minus the standard error from triplicate samples. Treatment effects were considered significant by a Student's t-test comparing two means giving p values < 0.05 .

Cell proliferation assay.

CXCR4 overexpressing cells (\sim 50% confluent) transfected with or without 100 nM PI4KIII α siRNA for 6 hrs in duplicate 60-mm dishes were harvested 44 hrs later and seeded in 12 wells (1×10^4 cells/well) of a Cell-Bind 96-well, black culture plate in complete growth medium (RPMI-1640, 10% FBS, 1% penicillin/streptomycin). After 24 hr (to allow adhesion) cells were assessed for growth at the indicated times (post-seeding) by the CyQuant NF cell proliferation assay (Molecular Probes, Life Technologies) from quadruplicate wells.

Immunohistochemical analysis

Human prostate primary and metastatic biopsy tumor tissues were obtained from an institutional human investigative committee approved protocol. Analysis of human tumor tissues for cytokeratin (Cat #C2562, lot#033M4760V from Sigma-Aldrich) and PI4KIII α were performed as in previously described procedures (18).

Mass spectrometry analysis of lipid rafts:

All analyses were performed on a Thermo QExactive MS (ThermoFisher Scientific, Watham, MA). Heavy and light SILAC sample pairs were pooled, reduced with dithiothreitol and alkylated with iodoacetamide. Following digestion with trypsin, peptides were separated by reversed phase chromatography using an Easy 1000 nano UHPLC system (Thermo) and Acclaim PepMap 100, 75 μm x 2 cm trap with Acclaim PepMap RSLC, 75 μm x 15 cm column (Dionex). Peptides were eluted with a 2 h gradient from 5% to 30% acetonitrile with pH maintained by 0.1% formic acid. Column effluent was analyzed directly by MS/MS using HCD fragmentation. Data analysis was carried out in R version 3.0.0. Identified proteins were submitted to Ingenuity Pathway Database (IPA®, QIAGEN Redwood City, www.ingenuity.com) to identify relevant pathways.

Statistical analysis:

Statistical analysis was performed with GraphPad Prism 6 by two-tailed unpaired t test. Statistical significance ($p < 0.05$) was determined by comparing means of control and experimental groups. One-Way ANOVA (analysis of variance) was performed on gene expression profiles of clinical samples (Figure 6A and supplemental figure 8), Tukey post-test being performed on multiple comparisons to determine the significance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are:

SILAC	stable isotope labelling with amino acids in cell culture
CXCR4	CXC motif containing G protein coupled receptor 4
PI4KIIIα	phosphatidylinositol 4-kinase III alpha isoform
MS	Mass spectrometry

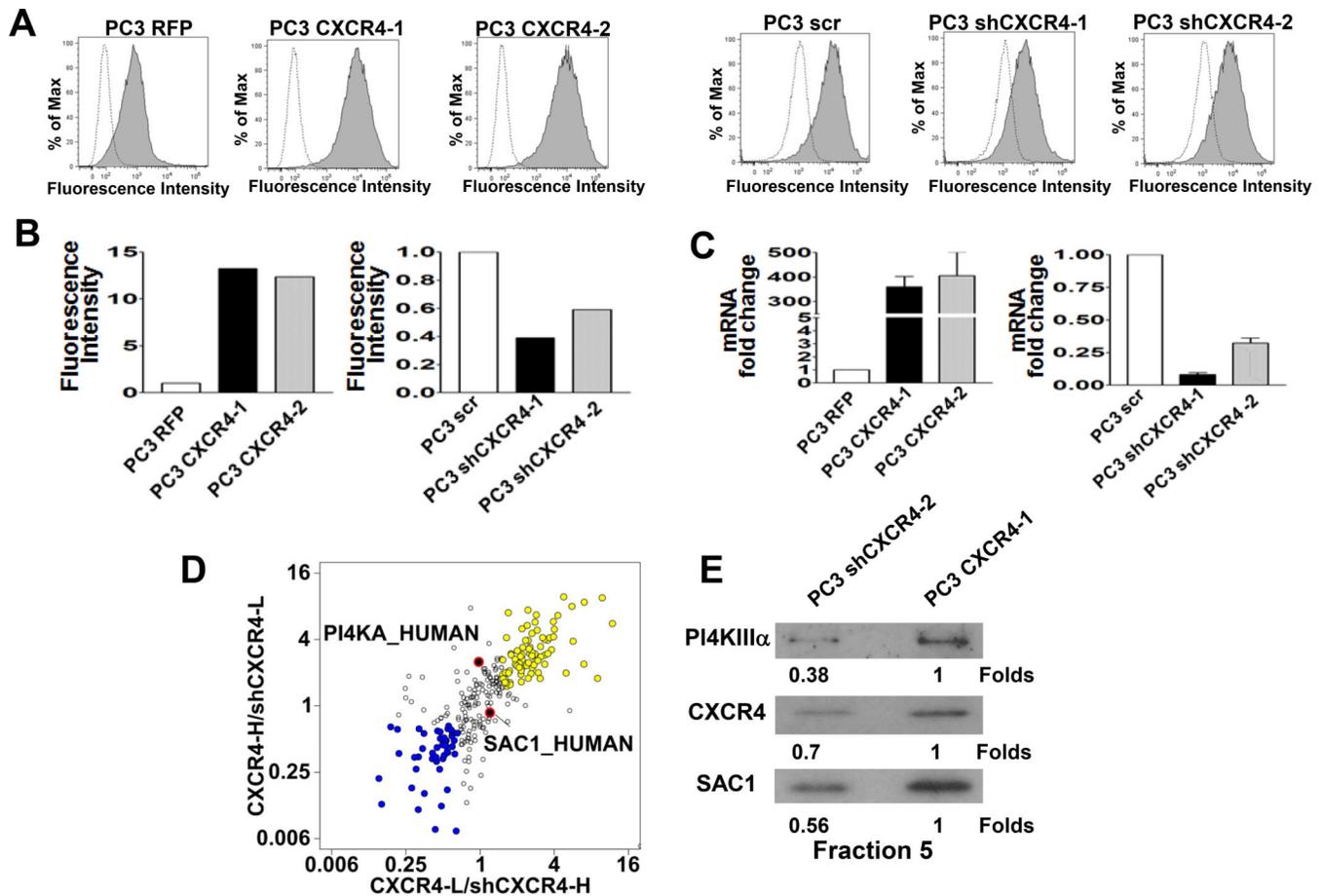
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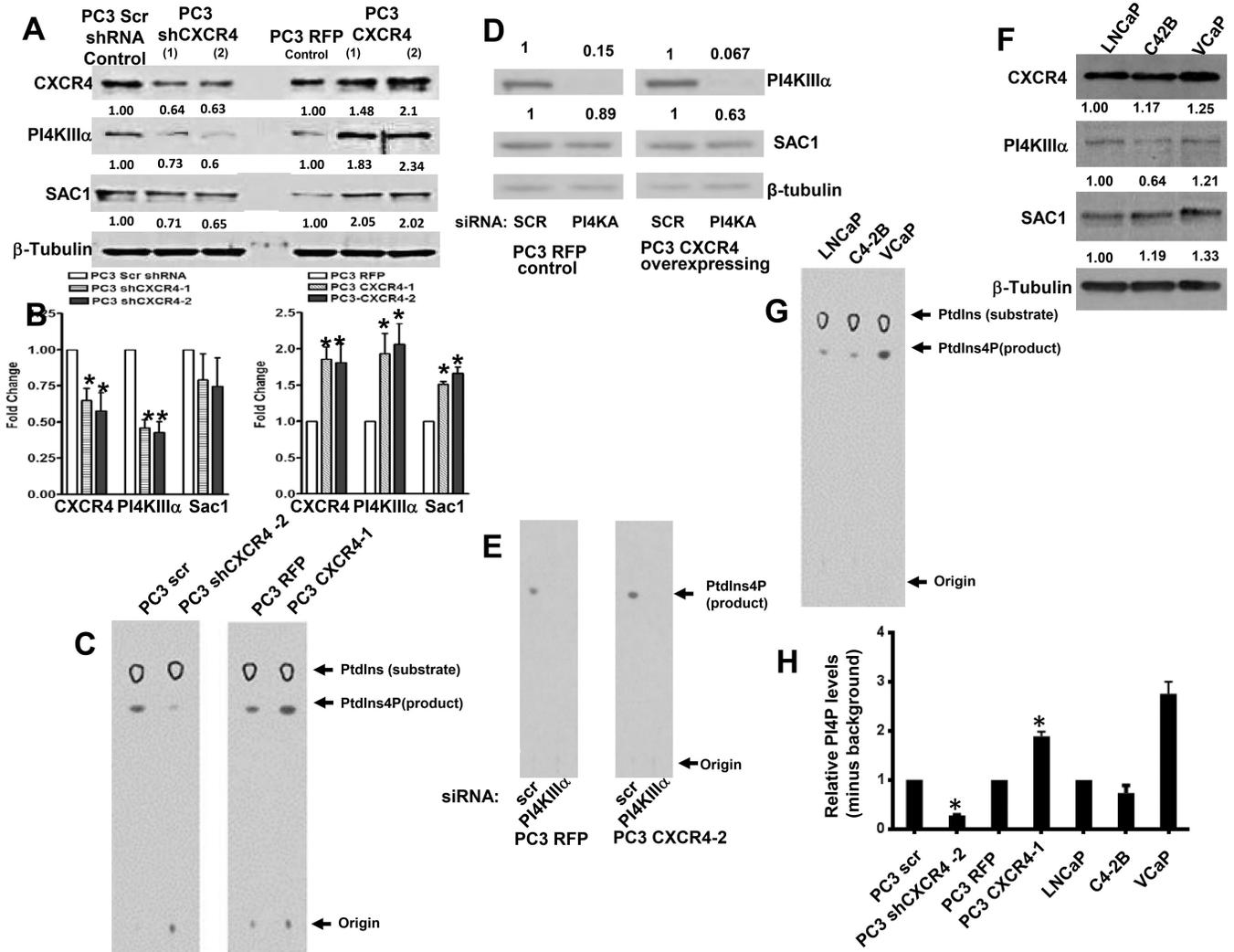
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**Figure 1.**

Characterization of PC3 low and high CXCR4 cells and proteomics analysis. A) Left panel: overlay of control (PC3-RFP) and CXCR4-overexpressing (PC3-CXCR4-1 and -2) cells receiving only APC-labeled goat anti-mouse secondary antibody (---) (Cat # 115-136-146, lot # 109425 from Jackson Immuno research laboratories) or both secondary plus mouse anti-CXCR4 primary antibody (Cat # MAB172 from R and D Systems) (—, shaded); right panel: overlay of control (scr shRNA ---) and knockdown (shCXCR4-1 and -2 —, shaded) cells treated as indicated in the left panel. B) Quantification of data presented in panel A. C) Q-PCR analysis confirms respective high and low CXCR4 mRNA levels in CXCR4 and shCXCR4 cells. For shCXCR4 the vertical axis is actually (fold change)⁻¹. D) Scatterplot from proteomics analysis of SILAC heavy/light CXCR4/shCXCR4 lipid raft protein ratios. Yellow color represents proteins overexpressed and blue color represents proteins underexpressed in PC3 CXCR4 cells compared to PC3 shCXCR4 cells. E) Western-blot analysis of lipid-raft-containing sucrose gradient fraction (#5) from stable CXCR4-overexpressing and -knockdown PC3 cell lines confirms their respective CXCR4/PI4KIII α overexpression and knockdown; Numbers refer to relative band amounts measured by densitometry.

**Figure 2.**

PI4KIII α expression and activity correlate with CXCR4 in prostate cancer cells. A) Representative WBs, from 3 independent experiments with similar results, of RIPA⁺⁺ whole cell lysates analyzed show a significant down regulation (~ 30–40%) in CXCR4, PI4KIII α and Sac1 levels in shCXCR4 cell lines (1 and 2), whereas they significantly increase (~ 2 fold) in the CXCR4 cell lines (1 and 2). B) Quantitation of WB band intensities from 3 experiments with similar results. *, indicates statistically significant difference, $p < 0.05$ by unpaired t test. C) WBs showing levels of CXCR4, PI4KIII α and Sac1 in three PC cell lines C4–2B and VCap vs the LNCaP cell line. A representative blot from three independent experiments with a densitometric quantitation was shown. D) Representative autoradiograms from TLC analysis of PI4KIII α lipid kinase activity in CXCR4 and shCXCR4 cell line lysates vs. their respective parental cell controls, and in three PC cell lines. E) Relative activity from panel D) from 3 independent experiments were quantitated by densitometry and normalized to the PC3 scrambled (scr) shRNA or PC3 RFP control samples or, for the PC cell lines, to LNCaP. F) Representative WB of control scrambled (SCR) and PI4KIII α siRNA-transfected cell lysates from CXCR4-overexpressing and control (RFP) PC3 cells

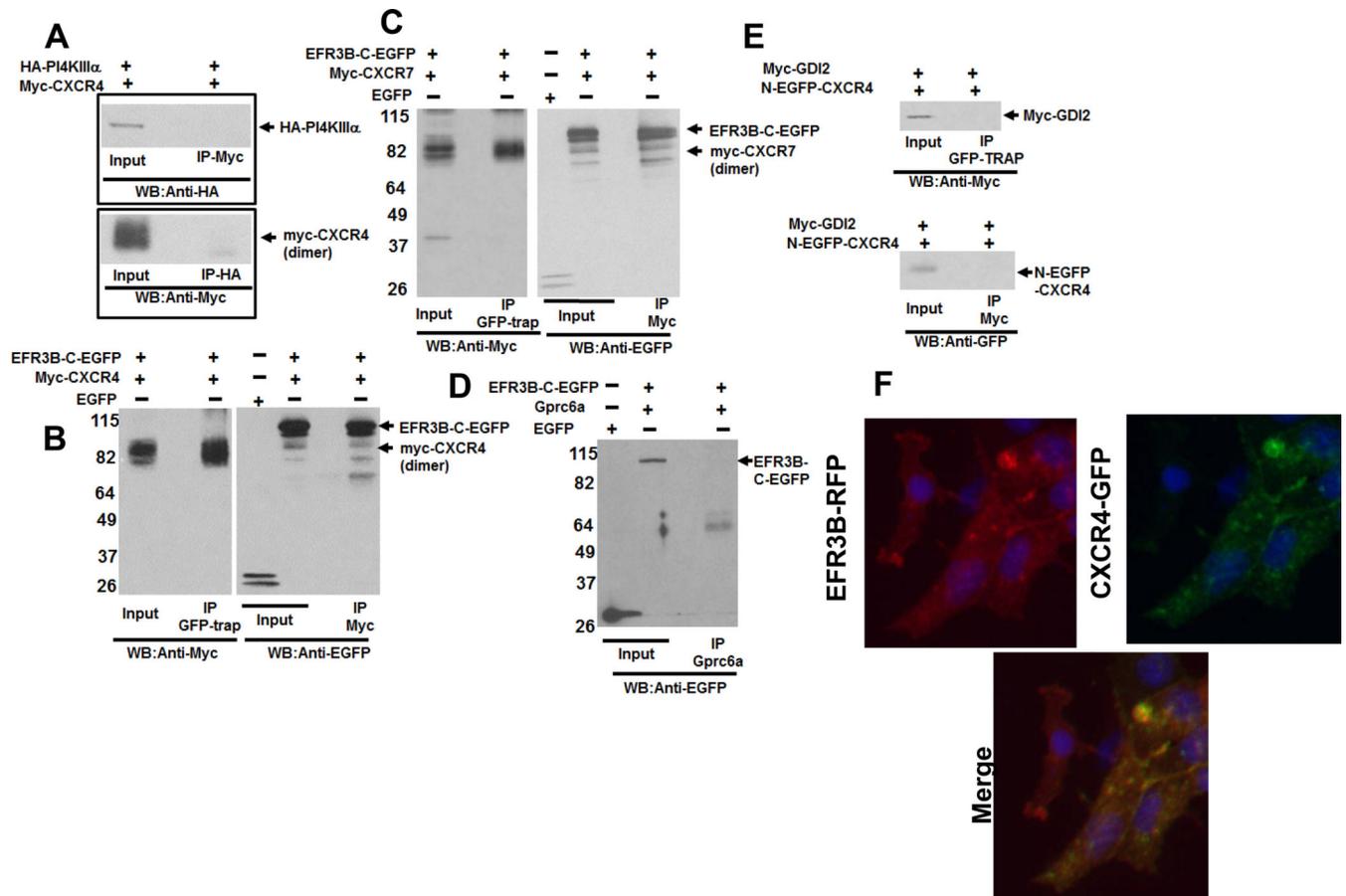
shows significant knockdown (~85–95%) of PI4KIII α , with smaller changes in Sac1 levels. Numbers indicate relative amounts determined by densitometry and normalized to β -tubulin loading control. G) Representative autoradiograms of in vitro kinase assays from TLC analysis of PI4KIII α lipid kinase activity in scrambled or PI4KIII α siRNA-transfected cell.

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**Figure 3.**

Chemokine receptors bind PI4KIII α partner EFR3B. Cos-7 cells were transfected with plasmids expressing fusion genes (as indicated in the figure), lysates were prepared with RIPA++ buffer and co-immunoprecipitation studies were performed followed by Western blot analysis. A) PI4KIII α and CXCR4 showing no direct binding, B) direct binding between EFR3B and CXCR4, C) direct binding between EFR3B and CXCR7, D) no binding between EFR3B and Gprc6a (osteocalcin receptor), E) a negative control showing a no interaction between GDI2 and CXCR4. F) Immunofluorescence data showing co-localization of EFR3B-GFP with CXCR4-GFP in cos-7 cells.

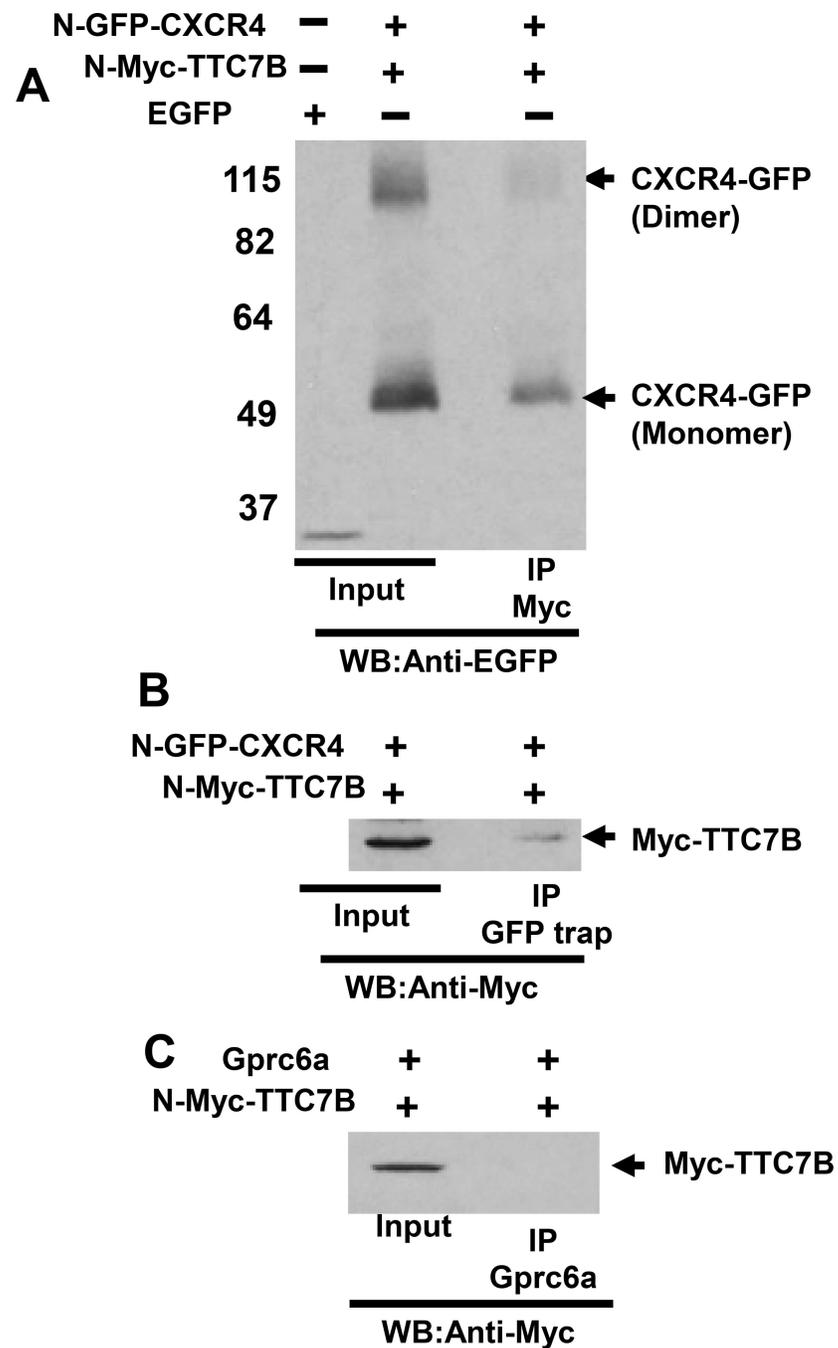
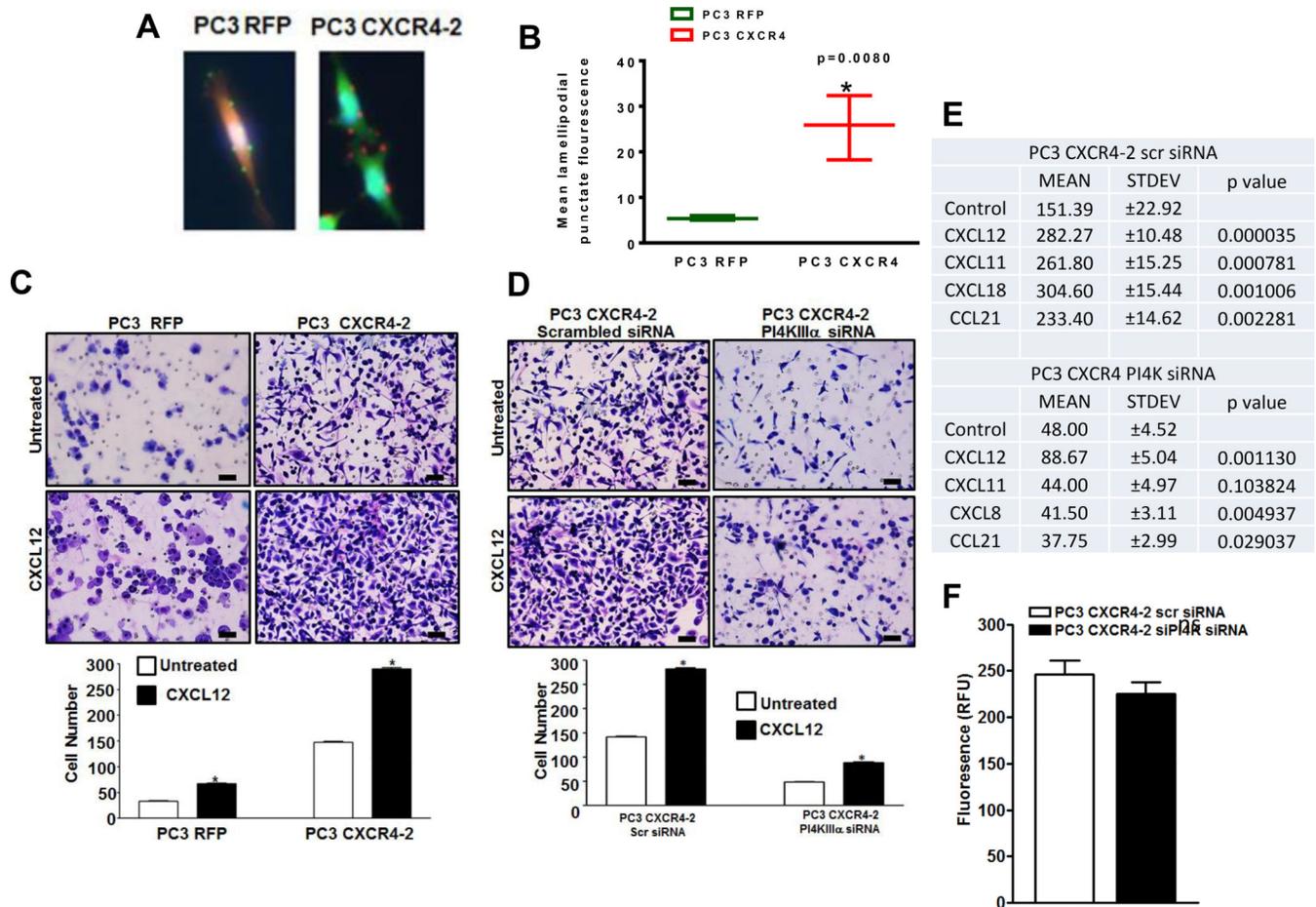


Figure 4. CXCR4 binding with PI4KIII α adaptor TTC7B. Cos-7 cells were transfected with plasmids expressing fusion genes (as indicated in the figure), lysates were prepared with RIPA++ buffer and co-immunoprecipitation studies were performed followed by Western blot analysis. A) Western blot analysis showing reciprocal interaction between CXCR4 and TTC7, B) Absence of interaction between Gprc6a and TTC7.

**Figure 5.**

PI4KIII α regulates chemokine stimulated invasion of prostate cancer cells A) Representative immune fluorescence images showing punctate staining near invasive protrusions in PC3 cell lines displaying invasive phenotype. Merged images (merges) were obtained from overlays of the same image field from red, green and blue channels. PC3 RFP cells were stained with anti-PI4P antibody and Alexa Flour 488 (Green color) conjugated secondary antibody. PC3 CXCR4-2 cells have green fluorescence and were stained with anti-PI4P antibody and Alexa Flour 594 (Red color) conjugated secondary antibody. B) Quantification of the mean PI4P punctate fluorescence intensity (FI) performed on several point-like sources from 3 different fields shows a large increase in punctiform intensity in CXCR4-2 cells ($p=0.008$) vs control (RFP) PC3 cells. Shown are the means \pm S.E. ($N=3$) from 3 different fields; *, denotes statistical significance ($p < 0.05$) by two-tailed unpaired t test using GraphPad Prism 6.05. C) CXCL12-stimulated invasion assay of control PC3 (RFP) and CXCR4 over-expressing (CXCR4-2) cells under naïve conditions, showing 4-fold increased invasion of CXCR4-2 vs. RFP control cells under both basal and stimulated conditions. Bar graph presents the mean \pm SE; *, statistically significant, $p < 0.01$ $n=3$). D) Cell invasion assay of CXCR4-2 cells under PI4KIII α siRNA-transfection conditions giving significant knockdown (~85–95%) of PI4KIII α shows 3-fold reduced CXCL12-stimulated invasion for the PI4KIII α siRNA-treated vs. control scrambled (Scr) siRNA-treated cells.

Results are from triplicate samples and show the mean \pm SE; *, statistically significant, $p < 0.01$ (n=3). (scale bar = 50 μm). E) Scrambled and PI4KIII α siRNA transfected cells were subjected to cellular invasion studies in the presence of CXCL12, CXCL11, CXCL8 and CCL21 chemokines. All invasion experiments were performed three times. F) Cell proliferation assay of CXCR4 overexpressing cells shows no significant change in growth inhibition between scrambled and PI4KIII α siRNA transfected cells (n=3).

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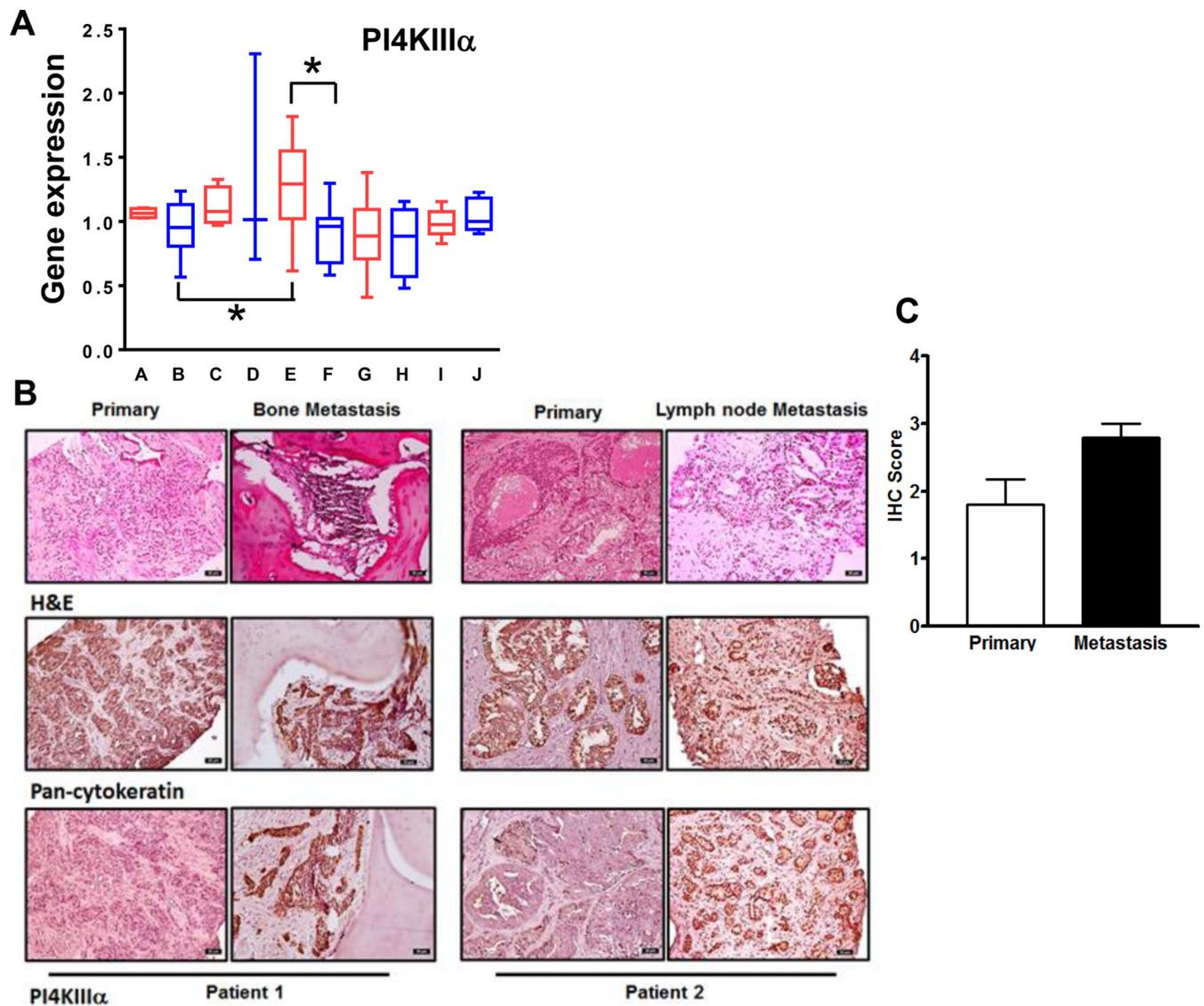


Figure 6. PI4KIII α and Sac1 expression in human prostate tumor samples. A) Box and whisker plot of PI4KIII α (gene name PI4KA) expression from expression profiling analysis of laser capture microdissection (LCM)-captured epithelial cell populations from 104 patient samples representing various stages of PC progression, from benign epithelium to metastatic disease, on cDNA microarrays (from NCBI GEO profiles dataset GDS3289). Shown are median, lower and upper quartiles, and lowest and highest values. Sample classes shown are: A, Benign prostatic hyperplasia (BPH) (n=4); B, Benign normal adjacent to prostate cancer foci (ADJ) + normal organ donor (NOR) (n=18); C, Atrophic lesion (ATR)-Proliferative inflammatory atrophy (PIA) + (ATR) (n=5); D, Metastatic prostate cancer (MET) naïve (n=3); E, (MET) only refractory (n=16); F, Localized prostate cancer (PCA) LCM Gleason pattern < 4 (n=13); G, (PCA) LCM Gleason pattern 4 & above (n=19); H, Prostatic intraepithelial neoplasia (PIN) (n=13); I, (Stromal) Benign nodules of BPH (n=7); and J, (Stromal) Normal (ADJ) (n=5). Y axis values derived from normalized, log₂-transformed

median fluorescence intensity ratios (635nm/532nm) of red Cy5-labeled patient prostate WTA-amplified cDNA)/green Cy3-labeled human Clontech Prostate Pool WTA-amplified cDNA) *, $P < 0.05$, indicates statistical significance, determined by the nonparametric ANOVA test ($p < 0.0001$) followed by Tukey post-test to compare all pairs of a column using GraphPad Prism software version 6.05 (GraphPad). B) Metastatic biopsy tissue along with matched primary tumor analysis of prostate cancer patient tissues for H and E staining, immunostaining with anti-pan-cytokeratin for tumor cell detection and anti-PI4KIII α . (C) Immunohistochemical analysis of pairs of primary and metastasis tissues from prostate cancer patients stained with anti-PI4KIII α antibodies. Nine pairs of patient tumor samples were analyzed for PI4KIII α expression.

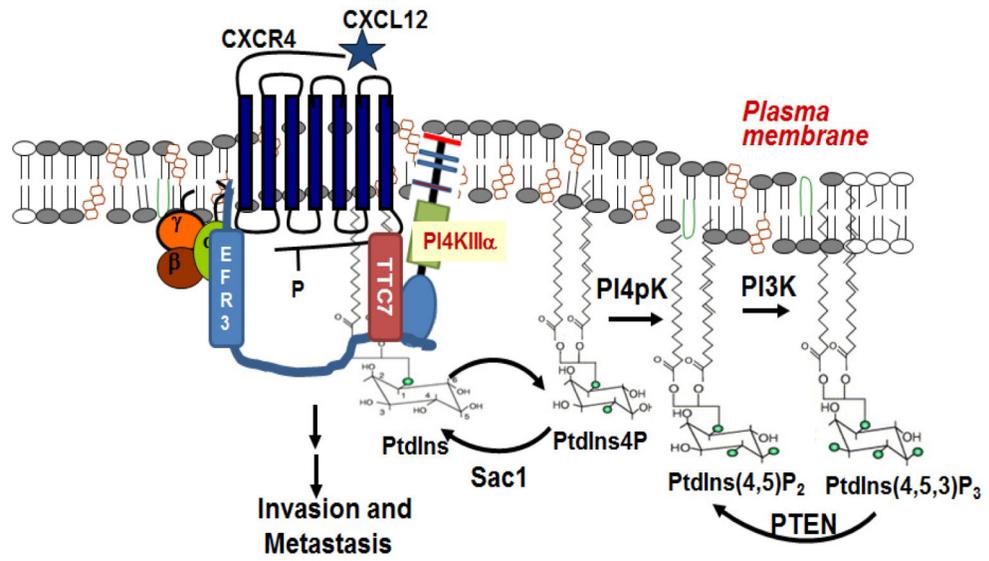


Figure 7.
A model for CXCR4 and PI4KIII α interaction in prostate cancer cells.

Table 1.

Cellular profiles of different PtdIns-4P species in Scr and PI4KIII α siRNA transfected PC3 CXCR4 cells. PI4P is a major PtdIns species in PC3 CXCR4 cells and PI4KIII α knockdown alters the cellular PtdIns-4P profiles.

PC3 CXCR4 cells	Cellular metabolite profiles of different PtdIns-4P species (% of total species)			
Transfection	PI4P	PI3,4P ₂	PI4,5P ₂	PI3,4,5P ₃
Scr siRNA	72.6	0.45	22	0.31
PI4KIII α siRNA	52.4	0.19	38.7	0.63

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