Corrections

BIOCHEMISTRY

The authors note that all instances of “δ-catenin” should instead appear as “p120-catenin.”

www.pnas.org/cgi/doi/10.1073/pnas.1411381111

GENETICS

The authors note that the accession number for Bioproject is PRJNA247931 and the accession number for SRA Study is SRP042027.

www.pnas.org/cgi/doi/10.1073/pnas.1412162111

NEUROSCIENCE
Correction for “GABA<sub>A</sub> receptor target of tetramethylenedisulfotetramine,” by Chunqing Zhao, Sung Hee Hwang, Bruce A. Buchholz, Timothy S. Carpenter, Felice Lightstone, Jun Yang, Bruce D. Hammock, and John E. Casida, which appeared in issue 23, June 10, 2014, of Proc Natl Acad Sci USA (111:8607–8612; first published May 27, 2014; 10.1073/pnas.1407379111).

The authors note that the author name Felice Lightstone should instead appear as Felice C. Lightstone. The corrected author line appears below. The online version has been corrected.

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Quantitative phosphoproteomic analysis reveals system-wide signaling pathways downstream of SDF-1/CXCR4 in breast cancer stem cells

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Contributed by Gerhard Wagner, March 19, 2014 (sent for review November 10, 2013)

Breast cancer is the leading cause of cancer-related mortality in women worldwide, with an estimated 1.7 million new cases and 522,000 deaths around the world in 2012 alone. Cancer stem cells (CSCs) are essential for tumor reoccurrence and metastasis which is the major source of cancer lethality. G protein-coupled receptor chemokine (C-X-C motif) receptor 4 (CXCR4) is critical for tumor metastasis. However, stromal cell-derived factor 1 (SDF-1)/CXCR4-mediated signaling pathways in breast CSCs are largely unknown. Using isotope reductive dimethylation and large-scale MS-based quantitative phosphoproteome analysis, we examined protein phosphorylation induced by SDF-1/CXCR4 signaling in breast CSCs. We quantified more than 11,000 phosphorylation sites in 2,500 phosphoproteins. Of these phosphoproteins, 87% were statistically unchanged in abundance in response to SDF-1/CXCR4 stimulation. In contrast, 545 phosphosites in 266 phosphoproteins were significantly increased, whereas 113 phosphosites in 74 phosphoproteins were significantly decreased. SDF-1/CXCR4 increases phosphorylation in 60 cell migration- and invasion-related proteins, of them 43 (>70%) phosphoproteins are unrecognized. In addition, SDF-1/CXCR4 upregulates the phosphorylation of 44 previously uncharacterized kinases, 8 phosphatases, and 1 endogenous phosphatase inhibitor. Using computational approaches, we performed system-based analyses examining SDF-1/CXCR4-mediated phosphoproteome, including construction of kinase–substrate network and feedback regulation loops downstream of SDF-1/CXCR4 signaling in breast CSCs. We identified a previously unidentified SDF-1/CXCR4-PKA-MAP2K2-ERK signaling pathway and demonstrated the feedback regulation on MEK, ERK1/2, b-catenin, and PPP1Cs in SDF-1/CXCR4 signaling in breast CSCs. This study gives a system-wide view of phosphorylation events downstream of SDF-1/CXCR4 signaling in breast CSCs, providing a resource for the study of CSC-targeted cancer therapy.

Breast CSC Isolation and Identification. A subpopulation of human mammary epithelial (HMLER) (CD44high/CD24low) cancer cells was isolated from human mammary epithelial HMLER cancer cells by flow cytometry. A small percentage of HMLER (CD44high/CD24low)β3 cells with trypsin/Accutase-sensitive and fast-adsorption characters were consequently isolated (Fig. L4). These sible, site-specific protein phosphorylation is critical to the signaling networks that regulate CSC self-renewal, differentiation, and metastasis. Protein-reversible phosphorylation has been extensively analyzed in examining one or a few protein phosphorylation events that affect CSC signaling (1). However, the phosphoproteome composed by protein kinase-driven and phosphatase-regulated signaling networks largely controls CSC fate. Therefore, large-scale analysis of differentially regulated protein phosphorylation is central to understanding complex cellular events, such as CSC maintenance and dissemination.

To unveil the signal transduction downstream of SDF-1/CXCR4 signaling in CSCs, in this study we have carried out isotope reductive dimethylation and large-scale liquid chromatography tandem mass spectrometry (LC-MS/MS)-based phosphoproteomic profiling and quantification in human breast CSCs upon SDF-1/CXCR4 stimulation. The phosphorylation events presented here include SDF-1/CXCR4-mediated phosphorylation sites in several key kinases and phosphatases, and several important signaling pathways in breast CSCs.

Results

Significance

Tumor metastasis is the major cause of cancer lethality, whereas the underlying mechanisms are obscure. Breast cancer stem cells (CSCs) are essential for breast cancer relapse and metastasis and stromal cell-derived factor 1 (SDF-1)/chemokine (C-X-C motif) receptor 4 (CXCR4) is a key regulator of tumor dissemination. We report a large-scale quantification of SDF-1/CXCR4-induced phosphoproteome events and identify several previously unidentified phosphoproteins and signaling pathways in breast CSCs. This study provides insights into the understanding of the mechanisms of breast cancer metastasis.


The authors declare no conflict of interest.

Data deposition: The five Excel files (Files S1–S5) and MS raw data have been deposited at http://gwagner.med.harvard.edu/intranet/PNAS_Manuscript_2014/.

See Commentary on page 7503.

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enriched HMLER (CD44\textsuperscript{high}/CD24\textsuperscript{low})\textsuperscript{FA} subpopulation cells were subsequently identified to characterize breast CSC properties with significantly increased tumoursphere growth capacity in vitro (Fig. S1), potent tumor growth capacity in mouse model in vivo (Fig. 1B), and increased drug resistance (>10-fold) compared with non-CSCs (Fig. 1C). The enriched breast CSCs, which express high levels of CXCR4, were used in this study (Fig. 1D).

Phosphoproteomic Profiling and Quantification. SDF-1 (100 ng/mL) induced significant phosphorylation increase of both Tyr and Ser/ Thr at 10 min in these breast CSCs (Fig. S2A and B). Previous studies reported that CXCR7 is another receptor of SDF-1 (5). To rule out the possible effects of SDF-1/CXCR7 signaling and other potential SDF-1-induced signaling, we compared phosphorylation events induced by 100 ng/mL SDF-1 for 10 min in breast CSCs with or without transient CXCR4 knocked-down (Fig. S2C) by isotope reductive dimethylation and MS-based phosphoproteomic profiling (Fig. 2A and Fig. S3). We quantified 11,131 phosphorylation sites of 2,567 phosphoproteins. Of these phosphosites, 87% were statistically unchanged in abundance in response to SDF-1/CXCR4 stimulation. In contrast, SDF-1/CXCR4 increases phosphorylation of 545 phosphosites in 266 phosphoproteins at least 2.5-fold and decreases phosphorylation of 113 phosphosites in 74 phosphoproteins (Fig. 2B). Distribution of tyrosine phosphorylation (p-Tyr) in the total phosphosites was 1.87% (Fig. 2B), which is consistent with theoretical prediction and previous observations (6). In contrast, SDF-1/ CXCR4 used high percentage of p-Tyr (6.08%, Fig. 2B) in mediated phosphosites, indicating that the relatively rare p-Tyr plays important roles in SDF-1/CXCR4 signaling in breast CSCs.

Of the classified proteins with increased phosphorylation mediated by SDF-1/CXCR4, the total percentage of proteins involved in cell adhesion, migration, cytoskeleton, actin, and microtubule association is up to 22.5% (60 of 266, Fig. 2C), perfectly matching the established critical roles of SDF-1/ CXCR4 in cell migration and invasion, and tumor metastasis. Of these 60 cell mobility-related proteins, 43 proteins (>70%) have previously not been characterized as dependent on SDF-1/ CXCR4 signaling. Interestingly but not unexpectedly, kinases represent the largest percentage of a single item among these classified proteins mediated by SDF-1/CXCR4 signaling (9.8% or 26 of 266, Fig. 2C). The roles of 20 unique phosphosites in PLECl are unknown (Fig. S4A). The remarkable phosphorylation change in GTPase activating proteins, cell junction proteins, histone modification enzymes, transporters and endogenous inhibitors suggests that SDF-1/CXCR4 signaling is implicated in many cellular activities that have previously not been recognized (Fig. 2C).

Kinases, Phosphatases, and Endogenous Phosphatase Inhibitors. To analyze the effects of SDF-1/CXCR4 signaling, we arranged the phosphorylation affected kinases and phosphatases in a circle and color-coded them according to the phosphorylation responses (Fig. 3A). We found only 23% (14 of 60) phosphoproteins contain known phosphosites. Thus, most of the kinases and phosphatasess identified here have not been previously described in SDF-1/CXCR4 signaling.

The p-Thr202 and p-Tyr204 sites of ERK1, two well-established SDF-1/CXCR4-regulated phosphosites, increased 1.6-fold in the phosphoproteome (Fig. S4). The p-Thr185 and p-Tyr187 of ERK2 increased 1.4-fold. In Western blot analyses, we confirmed that SDF-1/CXCR4 increases phosphorylation of these two sites in ERK1/2 (Fig. S4B) under the same conditions as in the phosphoproteome analyses described here. These experimental results are consistent with the fact that activated kinases are generally more rapidly dephosphorylated by phosphatases compared with nonkinase signal effectors due to the tighter control on active kinases in cells (7, 8). We further analyzed the kinases in the phosphoproteome and found that 24 kinases showed increased phosphorylation 1.6- to ∼2.5-fold. Of them ERK1 and PAK4 are known kinases in SDF-1/CXCR4 signaling (Fig. 3A and Fig. S4B). These 24 kinases were tracked and stringently recovered. Combined with the aforementioned 26 kinases (Fig. 3C), SDF-1/CXCR4 increases phosphorylation of 50 kinases in total (Fig. 3A).

To evaluate these results, we compared the phosphorylation change of GSK3β (Ser9), PKAC (Thr197), and δ-catenin (Tyr228) in Western blot assays with/without CXCR4 transient knockdown (Fig. 3B). Indeed, we observed that SDF-1 increases...
phosphorylation of these sites whereas CXCR4-specific knockdown effectively neutralizes the SDF-1 induction effects. The identified set of 50 kinases contains 6 kinases established to be associated with SDF-1/CXCR4 signaling: (FAK (Tyr576), ERK1 (Thr202/Tyr204), PKA (Thr197), PKC-δ, and Rps6K1) and six overlapped phosphosites (9).

Of the 50 kinases identified, 44 (88%) were previously unknown as dependent on SDF-1/CXCR4 signaling (Fig. 3A). Notably, 92% proteins (46 of 50) show at least twofold increase in phosphorylation at one or more sites. Of all 87 phosphosites of the kinases detected here, only 17 were already known. SDF-1/CXCR4 phosphorylation at one or more sites. Of all 87 phosphosites of the kinases detected here, only 17 were already known. SDF-1/CXCR4 significantly increases phosphorylation of these sites whereas CXCR4-specific knockdown effectively neutralizes the SDF-1 induction effects. The identified set of 50 kinases contains 6 kinases established to be associated with SDF-1/CXCR4 signaling: (FAK (Tyr576), ERK1 (Thr202/Tyr204), PKA (Thr197), PKC-δ, and Rps6K1) and six overlapped phosphosites (9).

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Overall, these data significantly extend the current knowledge of SDF-1/CXCR4 signaling-mediated phosphorylation change of kinases and phosphatases toward a system-wide view.

**Phosphorylation in Protein Complexes.** Phosphorylation regulation of components in protein complexes is essential for signal effector activities, signaling regulation, and cellular processes (10). We found up to 10 established protein complexes encompassing 21 proteins present where phosphorylation increased in SDF-1/CXCR4 signaling (Fig. 4). Five protein complexes play important roles in cell motility, and an over 15-fold phosphorylation increase was found in δ-catenin, which is involved in a complex with DLG1 and critical for cell polarization, migration, and invasion (11, 12). This is consistent with the important role of SDF-1/CXCR4 in the regulation of cell trafficking. In 5 proteins of AMPKβ1, MYPT1, PKAcα, PPP1Ca, and δ-catenin, 7 of 11

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**Fig. 3.** Phosphorylation in kinases, phosphatases, and phosphatase inhibitors. (A) For the proteins, the known kinases in SDF-1/CXCR4 signaling in both are in purple, known kinases in SDF-1/CXCR4 signaling not detected in this phosphoproteome are in gray, and phosphoproteins in phosphoproteome are in black. Recovered kinases (phosphorylation increase 1.6- to 2.5-fold) are in black with red underlining. Phosphosites with a red asterisk represent known active phosphosites in phosphoproteome. Phosphosites with a red asterisk represent known inhibitory phosphosites in this phosphoproteome. Known dual phosphosites in phosphoproteome are indicated by red dashed underlining. Conserved phosphosites in isozymes are underlined in black. The color bar in the key indicates phosphorylation increase fold. (B) Western blot analyses of SDF-1/CXCR4 regulated phosphorylation of kinases and effectors in A.
phosphosites match those previously described. Up to 36 phosphosites in these protein complexes have previously not been known to depend on SDF-1/CXCR-4 signaling (Fig. 4). Most of the protein complexes show a significant phosphorylation increase in multiple sites, such as δ-catenin (five sites), RBM15 (four sites), AKAP11 (three sites), and PKACA (two sites). In short, the biological functions of these 10 SDF-1/CXCR4–mediated protein complexes cover broad aspects of cellular processes, including cell migration, invasion, cell cycle, DNA repair, transcription, and signaling pathway regulation.

An MAPK Network. We examined the site-specific kinase–substrate and phosphatase–substrate of the 266 phosphoproteins with increased phosphorylation (Fig. 2B) and found 42 phosphosites of 28 phosphoproteins whose site-specific upstream kinases/phosphatases were established and detected in phosphoproteome (Table S2). SDF-1/CXCR4 mediates multiple upstream kinases of five phosphoproteins: PDK-1 and PKA for p-Thr197 of PKA (13, 14), ERK1 and GSK3β for p-Ser221 of Rps6ka1 (15, 16), ERK1/2 and GSK3β for p-Ser903 of NFκB (17), CDK1 and ERK for p-Ser56 of VIM (18), and CamKKII for p-Ser282 and Ser285 of Ets-1 (19). Both upstream kinase and phosphatase were recorded for 11 substrates (AMPKα1, CDK7, GSK3β, ERK1, PAK4, PKA, PKCδ, Rps6ka1, PP1Co, Rb1, and HNRNPK) in SDF-1/CXCR4 signaling.

Pathways are fundamental components of signaling transduction. To reconstitute signaling pathways downstream of SDF-1/CXCR4 signaling, we reconstructed kinase–phosphatase–substrate-based signaling pathways with the following three stringent approaches: (i) signal effectors showing phosphorylation increase upon SDF-1/CXCR4 stimulation in the phosphoproteome, (ii) establishing kinase/phosphatase–substrate interaction, and (iii)
establishing pathways in SDF-1/CXCR4 signaling (Tables S2 and S3). Using these approaches, we were able to infer the positive or negative regulation of phosphosites detected on proteins in our study. Furthermore, the results were compared with 45 phosphoproteomic experiments of mammalian cells reported in the literature to validate the prediction for the biological function (Table S3). These pathway reconstitution approaches lead to a construction of a SDF-1/CXCR4–mediated MAPK signaling network (Fig. 5).

Feedback Regulation on MEK, ERK, δ-Catenin, and PPP1Ca. To evaluate the feedback regulation, we examined the phosphosites of p(Ser217/Ser221) MEK1/2, p(Thr202/Tyr204)-ERK, and p(Tyr228)-δ-catenin and confirmed the feedback regulation of site-specific phosphorylation (Fig. 6A). SDF-1 significantly increases phosphorylation of p(Ser228)-MEK2, p(Thr202/Tyr204)-ERK, and p(Tyr228)-δ-catenin, and the phosphorylation at these sites decreased over longer time. To evaluate the reconstructed nested phosphate regulation loops (Fig. S5), we examined the p-Thr320 of PPP1Ca in SDF-1/CXCR4 signaling. We detected that phosphorylation of Thr320 increased upon SDF-1 stimulation and subsequently reversed over time (Fig. 6B). In addition, CXCR4 antagonist AMD3100 inhibits the SDF-1–induced phosphorylation increase of p-Thr320 in PPP1Ca (Fig. 6C), indicating that p-Thr320 of PPP1Ca is dephosphorylated by nested phosphatase regulation loops downstream of CXCR4 signaling. Taken together, these data experimentally confirmed the dephosphorylation feedback regulation of kinases of MEK and ERK, signal effector of δ-catenin, and phosphatase of PPP1Ca in SDF-1/CXCR4 signaling.

SDF-1/CXCR4-PKA-MAP2K2-ERK Pathway. To further evaluate the reconstructed SDF-1/CXCR4–mediated MAPK-signaling network, we examined the SDF-1/CXCR4-PKA-MAP2K2-ERK pathway. In Western blot assays, we observed that SDF-1 treatment increased p(Thr197)-PKA, p(Ser222)-MAP2K2, and p(Thr202/Tyr204)-ERK1 whereas CXCR4 knockdown or CXCR4 antagonist AMD3100 neutralized these effects (Figs. 3B and 7A and Fig. S4B), indicating that SDF-1/CXCR4 mediates PKA, MAP2K2, and ERK1 signaling. Furthermore, we examined the PKA-MAPK cascade with PKA inhibitor 14-22-Amide and identified that SDF-1/CXCR4 regulates the PKA-MAP2K2-ERK pathway (Fig. 7B).

Discussion

Here we present the quantification and profiling of a large-scale phosphoproteome event in breast CSCs. We quantified 11,131 phosphosites in 2,567 phosphoproteins and found that SDF-1/CXCR4 signaling increases phosphorylation of 545 phosphosites in 266 phosphoproteins and decreases phosphorylation of 113 phosphosites in 74 phosphoproteins in breast CSCs. SDF-1/CXCR4 mediates 50 kinases, 8 phosphatases, and 1 phosphatase

Fig. 5. An MAPK network downstream of SDF-1/CXCR4 signaling in breast CSCs. The SDF-1/CXCR4–induced MAPK subnetwork shows reconstructed nested pathways with a five-tiered MAPK cascade and known dual phosphosites in MAP2K2 and ERK1. Black arrows show the known phosphorylation relationship in SDF-1/CXCR4 signaling in both. Green arrows represent the indirect phosphorylation relationship in the phosphoproteome. Pink arrows indicate biological function regulation. Red lines indicate dephosphorylation or inhibition. The green circle is the predicted functional complex (specific sites of the components are shown in Fig. 3). The light green MAPK cascade shows the five-tiered MAPK cascade in phosphoproteome.
Dephosphorylation feedback regulation on MEK1/2, ERK1/2, and β-catenin in breast CSCs. Breast CSCs were pretreated with 30 μg/mL AMD3100 for 1 h followed by with/without 100 ng/mL SDF-1 treatment for 20 min.

**Phosphorylation in both Core Phosphosites and Noncore Phosphosites in Kinases and Phosphatases.** Recent studies report that core phosphosites, which are implicated in fundamental cellular processes, are positionally conserved in eukaryotes, whereas noncore phosphosites evolved rapidly (20). To evaluate the SDF-1/CXCR4-regulated phosphosites in evolution, we analyzed them across six eukaryotic species of human, mouse, Arabidopsis, Drosophila, Caenorhabditis elegans, and yeast. We found that the regulated phosphosites of AMPK, CDC25, CDK1, CDK7, MAP2K2, ERK1, ERK3, PAK4, PDK1, PKA, Rps6ka1, and MP in phosphoproteome are highly conserved in all species of human, mouse, Arabidopsis, Drosophila, C. elegans, and yeast. Our observations are consistent with previous reports and find 29 additional cases (Tables S4 and S5 and Fig. 3A). Interestingly, we found that phosphorylation significantly increased at Thr511 of PKCδ and Thr309 of PKCe, which are homologous residues in the Thr-Pro (T-P) motif in a conserved TFCCGTG region in all 13 human PKC isoforms (Table S6). The conservation of regulated phosphosites (57%; 16 of 28) across species over 600 My in evolution suggests that these functional phosphosites have been ancient, strong selection constraints. These results and many other noncore phosphosites regulated by SDF-1/CXCR4 demonstrate that SDF-1/CXCR4 signaling employs both core phosphosites and noncore phosphosites in breast CSCs.

In addition, we found 190 of 545 phosphosites are in Ser/Thr-Pro (S/T-P) motifs in phosphoproteome, indicating that S/T-P turn motifs are broadly and frequently used and are essential for dynamic signaling transduction.

**Both Inhibitory Phosphosites and Dephosphorylation Feedback Regulation.** Previous studies reported that MP dephosphorylates p-Ser/Thr in MAP2K2, ERK1, ERK2, and ERK3 (21). Tyrosine-specific phosphatase PTPN12/13/14 dephosphorylate p-Tyr of ERK1/2, FAK, and δ-catenin (22, 23). The endogenous phosphatase inhibitor PPP1R2 (namely 1-2) negatively regulates MP by attenuating the catalytic activities PPP1Ca (24). We found that SDF-1/CXCR4 elevates phosphorylation in multiple phosphosites in MP, PTPN12, PTPN13, and PTPN14. In addition, we verified phosphorylation reverses over time in MEK2, ERK1/2, δ-catenin, and PPP1Ca in Western blot assays, which is in agreement with the reversed phosphorylation of total p-Tyr and p-Ser/Thr over time in Fig. S2, suggesting that the feedback regulation mechanism dynamically mediates signaling transduction in both a site-specific and phosphoproteome-wide format. We detected that SDF-1/CXCR4 increases phosphorylation in multiple inhibitory phosphosites: Tyr15/Tyr14 in CDK1 and Ser9 in GSK3β. These results suggest that SDF-1/CXCR4 not only activates multiple signaling pathways, but also induces inhibitor in breast CSCs. Using a computational approach, we constructed a SDF-1/CXCR4-induced signaling network, including MAPK cascade and its feedback regulation. We identified a previously uncharacterized signaling pathway of SDF-1/CXCR4-PKA-MAP2K2-ERK, and demonstrated the dephosphorylation feedback regulation on kinases of MEK and ERK1/2, phosphatase of PPP1Ca, and the signal effector of δ-catenin. This study extends our understanding of SDF-1/CXCR4 signaling in breast CSCs to a system-wide view.

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**Phosphorylation in both Core Phosphosites and Noncore Phosphosites in Kinases and Phosphatases.** Recent studies report that core phosphosites, which are implicated in fundamental cellular processes, are positionally conserved in eukaryotes, whereas noncore phosphosites evolved rapidly (20). To evaluate the SDF-1/CXCR4-regulated phosphosites in evolution, we analyzed them across six eukaryotic species of human, mouse, Arabidopsis, Drosophila, Caenorhabditis elegans, and yeast. We found that the regulated phosphosites of AMPK, CDC25, CDK1, CDK7, MAP2K2, ERK1, ERK3, PAK4, PDK1, PKA, Rps6ka1, and MP in phosphoproteome are highly conserved in all species of human, mouse, Arabidopsis, Drosophila, C. elegans, and yeast. Our observations are consistent with previous reports and find 29 additional cases (Tables S4 and S5 and Fig. 3A). Interestingly, we found that phosphorylation significantly increased at Thr511 of PKCδ and Thr309 of PKCe, which are homologous residues in the Thr-Pro (T-P) motif in a conserved TFCCGTG region in all 13 human PKC isoforms (Table S6). The conservation of regulated phosphosites (57%; 16 of 28) across species over 600 My in evolution suggests that these functional phosphosites have been ancient, strong selection constraints. These results and many other noncore phosphosites regulated by SDF-1/CXCR4 demonstrate that SDF-1/CXCR4 signaling employs both core phosphosites and noncore phosphosites in breast CSCs.

In addition, we found 190 of 545 phosphosites are in Ser/Thr-Pro (S/T-P) motifs in phosphoproteome, indicating that S/T-P turn motifs are broadly and frequently used and are essential for dynamic signaling transduction.

**Both Inhibitory Phosphosites and Dephosphorylation Feedback Regulation.** Previous studies reported that MP dephosphorylates p-Ser/Thr in MAP2K2, ERK1, ERK2, and ERK3 (21). Tyrosine-specific phosphatase PTPN12/13/14 dephosphorylate p-Tyr of ERK1/2, FAK, and δ-catenin (22, 23). The endogenous phosphatase inhibitor PPP1R2 (namely 1-2) negatively regulates MP by attenuating the catalytic activities PPP1Ca (24). We found that SDF-1/CXCR4 elevates phosphorylation in multiple phosphosites in MP, PTPN12, PTPN13, and PTPN14. In addition, we verified phosphorylation reverses over time in MEK2, ERK1/2, δ-catenin, and PPP1Ca in Western blot assays, which is in agreement with the reversed phosphorylation of total p-Tyr and p-Ser/Thr over time in Fig. S2, suggesting that the feedback regulation mechanism dynamically mediates signaling transduction in both a site-specific and phosphoproteome-wide format. We detected that SDF-1/CXCR4 increases phosphorylation in multiple inhibitory phosphosites: Tyr15/Tyr14 in CDK1 and Ser9 in GSK3β. These results suggest that SDF-1/CXCR4 not only activates multiple signaling pathways, but also induces
feedback regulation via both inhibitory phosphosites and dephosphorylation to mediate the signaling homeostasis.

**SDF-1/CXCR4 Mediates Multiple MAPK Cascades in Breast CSCs.** The three-tiered kinase cascade of MAP3K-MAP2K-MAPK is ubiquitous in all eukaryotes and has an extremely wide range of functions in signal transduction (25). Increased phosphorylation at two or more sites in MAP3Ks (MAP3K11, Raf-1, and MLK7), two serine residues in MAP2Ks (Ser222 and Ser226 in MAP2K2), and conserved dual residues in MAPK (Thr202 and Tyr204 in ERK1) suggests that this three-tiered MAPK cascade may present multiple roles of switch, amplification, and feedback controller in SDF-1/CXCR4 signaling. The multiple MAP4K-MAP3K-MAP2K-MAPK-MAPKAPK five-tiered MAPK cascades presented here significantly extends the knowledge of SDF-1/CXCR4-mediated MAPK signaling.

In the graded pathway of SDF-1/CXCR4-PKC-α-Raf-1-MAP2K2-ERK3-MAPKAPK5 (26), phosphorylation of Thr368 of MAPKAPK5 increases 134-fold, which is consistent with the amplification effects of the MAPK cascade. The overexpressed H-Ras in HMLER (CD44high/CD24low) cells (27) may enhance the effects of Ras-mediated Raf-1-MAPK signaling, which may help to track SDF-1/CXCR4-regulated Raf-1-MAPK signaling pathways. MAP2K2 fills the gap of previously fragmentary or unknown SDF-1/CXCR4–induced ordered MAPK pathways and shows more complete pathways with highly overlapped phosphosites.

**Both G Protein-Dependent and -Independent Signaling.** Classical G protein-dependent ERK activation is rapid and transient because it is quickly quenched by the β-arrestin-mediated desensitization of the receptor. The β-arrestins scaffold the MAP kinase signaling molecules of MAP3K (Raf1), MAP2K (MEK1), and MAPK (ERK), leading to ERK1/2 phosphorylation and activation (28). The β-arrestin-mediated ERK responses are slower and more persistent. We found SDF-1/CXCR4 increases phosphorylation of ERK1/2 at 1 h (Fig. 6f), suggesting that β-arrestin-mediated ERK phosphorylation plays a role in SDF-1/CXCR4–induced persistent MAPK signaling activation. These results and many other G protein–dependent signaling events (Fig. 5 and Fig. S6) indicate that SDF-1/CXCR4 induces both G protein–dependent and -independent signaling in breast CSCs.

**Phosphorylation in Multiple Sites in Protein Complex Components.** A number of activated sites are located in multiple anchoring and scaffolding proteins AKAP2, AKAP11, and AKAP12, indicating that AKAP compartmentalization of PKA signaling pathways is widely used in various cellular processes, which is in agreement with established knowledge that AKAPs mediate PKA to appropriate substrate selection and pathway integration (29). Multisite covalent modification is omnipresent in components of these complexes, indicating that phosphorylation regulation in diverse distinct sites may be important (i) for compartmentalization/decomposition of functional complexes, (ii) for various functions in distinct signaling pathways, and (iii) acting as a node of pathway cross-talk or a switch of activation or inactivation of regulation loops.

**Phosphorylation of Cell Communication Regulators.** Recent studies reported that CSC communication with other cells in CSC niches is important for CSC relocation, self-renewal, proliferation, and differentiation (10). SDF-1/CXCR4 signaling significantly increases phosphorylation in gap junction alpha-1 protein (GJα1; Ser279 and Ser282), sodium-coupled neutral amino acid transporter-2 (SLC38A2; Ser10, Ser12, Ser18, Ser21, and Ser22), and sodium-dependent phosphate transporter-1 (SLC20A1; Ser417 and Tyr-418), which are important for cell communication. These data may provide novel hints to explore the potential roles of SDF-1/CXCR4 in cell communication.

**An SDF-1/CXCR4 Signaling Network in Breast CSCs.** Signals that are transmitted inside cells do not unidirectionally amplify through signaling pipelines, but rather are tightly orchestrated through highly interconnected networks by multilayered feedback regulation mechanisms. The characterization of the SDF-1/CXCR4–mediated phosphoproteome and experimental evaluation assays offer a systems-wide view of the phosphorylation signature of a key phase of the feedback signaling network downstream of CXCR4 signaling. Furthermore, it provides an opportunity to connect SDF-1/CXCR4–mediated kinases, phosphatase, phosphatase inhibitors, and other phosphoproteins, and effectors that have been reported. By integrating these phosphoproteins with phosphorylation increase in the phosphoproteome and the known factors in SDF-1/CXCR4 signaling (Tables S2, S3, and S7), we constructed an SDF-1/CXCR4–mediated signaling network in Fig. S6. Thus, SDF-1/CXCR4 signaling may be implicated in a broad range of biological procedures including cell adhesion, migration, invasion, chemotaxis, cell cycle, proliferation, apoptosis, angiogenesis, and cell communication.

**Phosphoproteomic Profiling in CSCs.** Understanding how breast CSCs tightly orchestrate signal transduction in response to extra- and intracellular stimuli defines a fundamental biological issue. Essential insights into this come from the definition of system-wide signaling network architectures, such as the large-scale SDF-1/CXCR4 signaling network in breast CSC presented here. A very recent study also used an LC–MS/MS–based approach to examine phosphorylation events downstream of SDF-1/CXCR4 in chronic lymphocytic leukemia cells (30). O’Hayre et al. used a Ni–nitrilotriacetic acid resin phosphoprotein enrichment approach and performed analyses with/without SDF-1 stimulation (30). The PHOS-select iron affinity beads and the strong cation exchange/imobilized metal affinity chromatography (SCX/IMAC) methods used in this study significantly increased phosphopeptide enrichment (~10-fold). Reductive dimethylation of proteins of wild-type (with CH3) and CXCR4 knockdown (with CD3) breast CSCs and SDF-1 treatment of both cells significantly increased the specificity of SDF-1/CXCR4 signaling. In their study, O’Hayre et al. (30) identified Raf1 and PDK1 as important signals downstream of SDF-1/CXCR4, which was confirmed in our study. However, we have constructed a much broader and more specific signaling network downstream of SDF-1/CXCR4 in breast CSCs. Our study evidenced that phosphoproteomic profiling is a powerful tool for the understanding of CSC signaling networks system-wide in complex tumor evolution procedures, such as tumorangiogenesis and tumor metastasis.

**Materials and Methods**

**Cell Cytometry.** The HMLER cell line was kindly provided by Robert Weinberg (Whitehead Institute for Biomedical Research, Boston). HMLER (CD44high/CD24low) cells were isolated from HMLER cells by flow cytometry using FITC-conjugated anti-CD44 (G44-26; Biosciences) antibody and PE-conjugated anti-CD24 (ML15; Biosciences) antibody. Cell cultures of mammary epithelial cell growth medium (MEGM) were ordered from Lonza.

**Animals.** HMLER (CD44high/CD24low) and MELC (CD44high/CD24low) cells were injected into mammary glands of 1.5-mo-old, nonobese diabetic SCID female mice (five mice for each group; Jackson Laboratories) for the dilution tumor formation assays, which was approved by the Harvard Medical Area Standing Committee on Animals.

**Cell Lysis, Protein Extraction, Reductive Dimethylation, Combination, and Digestion.** Five CXCR4 specific short hairpin RNA (shRNA) constructs (The RNAi Consortium, Broad Institute, Boston) were tested for their ability to knockdown CXCR4 expression by transfection with Lipofectamine Transfection 2000 reagents (Invitrogen). Thirty-six hours after transfection (with MEGM; Lonza), cells were cultured overnight in mammary epithelial cell basal medium (MEBM)
medium (Lanza) without growth factor additives. Before analysis, the cells were treated with 100 ng/mL SDF-1 for 10 min [37 °C, 5%(vol/vol) CO2]. HMLER (CD44low/CD24high) were cultured in MEBM without serum overnight before SDF-1 treatment. Cells were lysed via standard methods (21) and proteins were extracted. Reductive dimethylation of intact proteins were performed with NaCNBH3 (light, L) or NaCNBD3 (heavy, H) as previously described (31, 32). Light- and heavy-labeled proteins were combined and purified, followed by trypsin digestion via a standard protocol (31).

11. Wildenberg GA, et al. (2006) p120catenin and phosphorylation of intact proteins were performed with NaCNBH3 (light, L) or NaCNBD3 (heavy, H) as previously described (31, 32). Light- and heavy-labeled proteins were combined and purified, followed by trypsin digestion via a standard protocol (31).