Tumor and Stem Cell Biology

Cancer Research

Cyclin D1/Cyclin-Dependent Kinase 4 Interacts with Filamin A and Affects the Migration and Invasion Potential of Breast Cancer Cells

Zhijiu Zhong, Wen-Shuz Yeow, Chunhua Zou, Richard Wassell, Chenguang Wang, Richard G. Pestell, Judy N. Quong, and Andrew A. Quong

Abstract

Cyclin D1 belongs to a family of proteins that regulate progression through the G_1-S phase of the cell cycle by binding to cyclin-dependent kinase (cdk)-4 to phosphorylate the retinoblastoma protein and release E2F transcription factors for progression through cell cycle. Several cancers, including breast, colon, and prostate, overexpress the *cyclin D1* gene. However, the correlation of cyclin D1 overexpression with E2F target gene regulation or of cdk-dependent cyclin D1 activity with tumor development has not been identified. This suggests that the role of cyclin D1 in oncogenesis may be independent of its function as a cell cycle regulator. One such function is the role of cyclin D1 in cell adhesion and motility. Filamin A (FLNa), a member of the actin-binding filamin protein family, regulates signaling events involved in cell motility and invasion. FLNa has also been associated with a variety of cancers including lung cancer, prostate cancer, melanoma, human bladder cancer, and neuroblastoma. We hypothesized that elevated cyclin D1 facilitates motility in the invasive MDA-MB-231 breast cancer cell line. We show that MDA-MB-231 motility is affected by disturbing cyclin D1 levels or cyclin D1-cdk4/6 kinase activity. Using mass spectrometry, we find that cyclin D1 and FLNa coimmunoprecipitate and that lower levels of cyclin D1 are associated with decreased phosphorylation of FLNa at Ser2152 and Ser1459. We also identify many proteins related to cytoskeletal function, biomolecular synthesis, organelle biogenesis, and calcium regulation whose levels of expression change concomitant with decreased cell motility induced by decreased cyclin D1 and cyclin D1-cdk4/6 activities. *Cancer Res; 70(5); 2105–14. ©2010 AACR.*

Introduction

The canonical function of cyclin D1 is to promote progression through the G_1-S phase of the cell cycle by binding to cyclin-dependent kinase 4 (cdk4) to phosphorylate and inactivate the retinoblastoma protein and release E2F transcription factors. Several human cancers, including breast, colon, and prostate, as well as hematopoietic malignancies, overexpress the *cyclin D1* gene (1–3). However, there is no correlation between cyclin D1 overexpression and regulation of E2F target genes by microarray analysis nor between cdk-dependent cyclin D1 activity and tumor development, suggesting that the role of cyclin D1 in oncogenesis is at least partially independent of its function as a cell cycle regulator (4, 5). Cyclin D1 has recently been associated with cell adhesion and motility in primary bone macrophages (6). Studies in cyclin $D1^{-/-}$ mouse embryo fibroblasts revealed that cyclin D1 inhibits Rho-activated kinase II and thrombospondin 1 to promote cell migration (7). The cdk inhibitor $p16^{INK4a}$ has also been shown to inhibit the migration of erythroleukemia and endothelial cells (8, 9). Indeed, $p16^{INK4a}$ colocalized in the ruffles and lamellipodia of migrating endothelial cells together with cyclin D1, cdk4/6, and the $\alpha_v\beta_3$-integrin machinery.

Filamin A (FLNa), a member of the nonmuscle actin-binding protein family, is a widely expressed molecular scaffold protein that regulates signaling events involved in cell motility and invasion by interacting with integrins, transmembrane receptor complexes, adaptor molecules, and second messengers (10, 11). FLNa has recently been shown to bind cyclin B1/cdk1 in a yeast two-hybrid system using recombinant glutathione *S*-transferase (GST)-cyclin B1 protein as bait and a 10.5-day-old embryonic mouse library as prey (12). Using truncated recombinant FLNa and cyclin B1 protein fragments, the regions of interaction between FLNa and cyclin B1 were shown to be located within amino acids 1–40 of cyclin B1 and the FLNa NH_2-terminal region in repeat 9. In addition to cyclin B1, filamins have been reported to bind with more than 30 proteins, and because many filamin-interacting proteins are membrane receptors for cell signaling molecules, filamins may be involved in coordinating a variety of signal transduction pathways (13). For example, FLNa has been shown to be a substrate for calcium-calmodulin–dependent kinase II,

Authors' Affiliation: Kimmel Cancer Center, Department of Cancer Biology, Thomas Jefferson University, Philadelphia, Pennsylvania

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Andrew A. Quong, Kimmel Cancer Center, 233 South 10th Street, Room 804, Philadelphia, PA 19107. Phone: 215-503-9273; Fax: 215-503-9274; E-mail: andrew.quong@mail.jci.tju.edu.

doi: 10.1158/0008-5472.CAN-08-1108

interacting with filamentous actin to promote migration of human neck squamous cell carcinoma cells (14). FLNa has also been shown to interact with prostate-specific antigen and regulate androgen receptor (15, 16). In addition, FLNa has been shown to be a key element in transforming growth factor-β signaling through its association with SMADs (17) and in A549 lung carcinoma cells undergoing epithelial-mesenchymal transition (18). FLNa has also been associated with a variety of cancers including prostate cancer, melanoma, human bladder cancer, and neuroblastoma (10, 19, 20).

We hypothesized that elevated cyclin D1 facilitates motility in the highly invasive and metastatic MDA-MB-231 breast cancer cell line. Although there are many proteins that have been shown to affect migration and invasion in this cell line, our focus on these molecules is due to the fact that many of the known proteins affect mitogenic signals that affect the levels of cyclin D1, and there are several known kinases affecting FLNa and the increasing evidence that FLNa plays a key role in many processes such as epithelial-mesenchymal transition. In our studies, we found that the cell motility of MDA-MB-231 cells can be affected by altering cyclin D1 levels or cyclin D1-cdk4/6 kinase activity. Using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS), we found that cyclin D1 coimmunoprecipitates with the actin cytoskeleton protein FLNa and that the phosphorylation state of FLNa was concomitantly affected when either cyclin D1 levels or cyclin D1-cdk4/6 kinase activity was altered. We also found that lower levels of cyclin D1 are associated with decreased phosphorylation of FLNa at Ser2152 and Ser1459. We also analyzed the effects of decreasing cyclin D1 and cyclin D1-cdk4/6 activity on the global phosphoproteome. Our analyses revealed changes in protein expression in many proteins related to cytoskeletal function, biomolecular synthesis, organelle biogenesis, and calcium regulation concomitant with decreased cell motility induced by decreased cyclin D1 and cyclin D1-cdk4/6 activity.

Materials and Methods

Cell culture. Human breast carcinoma MDA-MB-231 cells (American Type Culture Collection) were grown in DMEM containing penicillin and streptomycin (each 100 mg/L) and supplemented with 10% fetal bovine serum (FBS) at 37°C in 5.0% CO_2. The Stable Isotopic Labeling by Amino Acids in Cell Culture (SILAC) Flex DMEM (Invitrogen) was prepared as per manufacturer's recommendation using heavy amino acids $^{13}C_6{}^{15}N_4$ Arg and $^{13}C_6$ Lys, and the cells were used in SILAC-based mass spectrometry experiments (21).

Cell invasion/migration assay. The cell invasion assay was conducted using BD Biocoat Matrigel 24-well invasion chambers with filters coated with extracellular matrix on the upper surface (BD Biosciences). The experiments were done according to the manufacturer's protocol. Experiments were done in triplicate (mean ± SE). Cells (2.5×10^4) were added to the upper chamber and allowed to invade for 24 h. The experiments were done according to the manufacturer's protocol.

Wound healing assay. MDA-MB-231 cells were grown to 70% confluence in six-well plates. Linear scratches were made with a micropipette tip across the diameter of the well, and dislodged cells were rinsed with PBS. Cell culture medium was replaced with fresh DMEM containing 10% FBS. The cells were allowed to grow and the width of the wound was monitored at the specified times for the degree of wound healing.

Fluorescent immunocytochemistry. Cells were prepared as in the wound healing assay and probed with mouse anti–cyclin D1 (DCS-6, 1:50; Cell Signaling Technology) and anti-FLNa (1:100; Santa Cruz Biotechnology) primary antibodies in 1% bovine serum albumin (BSA) at 4°C overnight. Then cells were incubated with goat anti-mouse IgG conjugated to Alexa 488 and goat anti-rabbit IgG conjugated to Alexa 647 (1:2,000; Invitrogen Corporation) in 1% BSA for 1 h. Cells were stained with 100 ng/mL 4′,6′-diamidino-2-phenylindole hydrochloride in PBS for 2 min to visualize the nuclei. Images were captured digitally using a Zeiss LSM 510 META confocal microscope.

Lysis buffers, immunoprecipitation, and Western blot. Whole-cell lysates for Western blots were prepared in a modified radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 7.5), 150 mmol/L sodium chloride, 1% NP40, 0.5% sodium deoxycholate, and 0.1% SDS] supplemented with 1 mmol/L sodium orthovanadate and protease inhibitor cocktail (Complete EDTA-free protease inhibitor cocktail from Roche Applied Science). For the immunoprecipitation studies, whole-cell lysates were prepared in COPRE lysis buffer [20 mmol/L HEPES (pH 7.9), 50 mmol/L sodium chloride, 0.1% NP40, 10% glycerol, 1 mmol/L DTT]. COPRE lysates (500 μg) were incubated with 1 μg of antibodies for 1 h at 4°C before adding 40 μL of protein G magnetic beads (Invitrogen) for an overnight incubation at 4°C. The following antibodies were used for Western blotting: anti–cyclin D1 (Ab-3) polyclonal antibody (Lab Vision/Neomarker); anti–cyclin D1 (DCS-6) monoclonal antibody, anti-cdk4 (C-22) and anti-actin (C-11) polyclonal antibodies, anti-FLNa and anti–phospho-FLNa (Ser2152) polyclonal antibodies (Cell Signaling Technology); and ImmunoPure horseradish peroxidase–conjugated goat anti-rabbit antibodies (Pierce Biotechnology). Primary antibodies were used at 1:1,000 dilution and secondary horseradish peroxidase–conjugated antibodies were used at 0.05 μg/mL.

Perturbation of the cyclin D1-cdk complex by p16^{INK4} peptides. Peptides corresponding to amino acids 84–103 of human p16^{INK4a} protein with a COOH-terminal sequence of 16 amino acids encoding the *Antennapedia* homeodomain (Penetratin) were synthesized. Peptide 20 (DAAREGFLATLVVLHRAGARRQIKIWFQNRRMKWKK) with the substitution of Asp92 with alanine has a lower IC_{50} to inhibit cyclin D1-cdk4/6 phosphorylation of a GST-pRb protein *in vitro* and to arrest cell cycle progression in G_1 than the corresponding peptide containing the wild-type sequence, and peptide 21 (DAAREGFLDTLAALHRAGARRQIKIWFQNRRMKWKK) carrying the substitution of Val95 with alanine and Val96 with alanine has an increased IC_{50} *in vitro* and has lost ~60% of the cell cycle inhibitory capacity (22, 23).

These peptides were added to the cell culture medium at a concentration of 20 μmol/L.

Perturbation of the cyclin D1-cdk complex by cyclin D1 siRNA. Cyclin D1 expression was inhibited using validated Stealth siRNAs (Invitrogen). Two different inhibiting RNAs were used in our studies and were found to have different efficiencies of cyclin D1 knockdown. They were CCND1(51) (AUGGUUUCCACUUCGCAGCACAGGA) and CCND1(52) (UUAGAGGCCACGAACAUGCAAGUGG). The Invitrogen predesigned negative control siRNAs were also used. Transfections were done with Lipofectamine 2000 as per manufacturer's recommendation, 200 pmol of siRNA and 5 μL of Lipofectamine 2000 for each well of a six-well plate (Invitrogen).

Proteomics. The identification of phosphoproteins was accomplished using two complementary methods. For both methods, cells were first grown in SILAC medium as described. In the first method, after treatment with the inhibitory peptides, phosphoproteins were isolated using the Qiagen PhosphoProtein Purification kit as per manufacturer's instructions. In the second method, phosphopeptides were isolated using PhosSelect (Sigma-Aldrich) as per manufacturer's instructions. Proteins separated on gels were identified using LC-MALDI on a 4800 Proteomics Analyzer (Applied Biosystems, Inc.) and the phosphopeptides were analyzed using electrospray ionization-mass spectrometry (ESI-MS) on a Proteome X workstation (Thermo-Fisher). Peptide identification was done on an in-house Mascot Server for the LC-MALDI spectra and on Sequest for the ESI-MS spectra. Additional information can be found in Supplementary Data.

Bioinformatics analysis. Function information of identified phosphoproteins was obtained from the Swiss-Prot database. To determine if any types of proteins are overrepresented, enrichment analysis of their gene ontology (GO) terms was done. To find statistically overrepresented GO categories among phosphoproteins identified in this study, we used the BiNGO plugin for Cytoscape (24). The required data set files were created as described in the BiNGO User Guide. The enrichment analysis was done using the "HyperGeometric test" with correction for multiple hypothesis testing using the following parameters: GO_Biological_Process ontology, annotation for *H. sapiens*. GO terms that were significant with P value of <0.05 were determined to be overrepresented.

Results

Decreased Cyclin D1 and Cyclin D1-cdk4/6 Kinase Activity Reduces the Invasion and Migration Potential of MDA-MB-231 Breast Cancer Cells

Previous studies have shown that cyclin $D1^{-/-}$ mouse embryo fibroblasts display increased cellular adherence, defective motility, and impaired wound response compared with those with restored cyclin D1 levels (7). To determine if cyclin D1 also helps control motility in the highly migratory MDA-MB-231 breast cancer cells, cyclin D1 mRNA and protein expression was inhibited using two available siRNAs, CCND1(51) and CCND1(52). A control siRNA with random sequence was used as negative control. Western blot data show that both cyclin D1–specific siRNAs inhibit cyclin D1 protein expression, albeit at different levels. CCND1(52) inhibits cyclin D1 by ~50% and CCND1(51) inhibits cyclin D1 by >90% compared with cells transfected with control siRNA (Fig. 1A).

Using these siRNAs, the role of cyclin D1 in MDA-MB-231 cell migration was assessed with a wound healing assay. In Table 1, we report the average width of the wound for the cells treated with the two cyclin D1–specific siRNAs and that for the cells treated with control siRNA relative to the initial wound width 12 hours after scratching. Cells treated with the cyclin D1–specific siRNAs had significantly wider wounds compared with cells treated with control siRNA ($P < 0.001$).

We next examined the role of cyclin D1 in the ability of MDA-MB-231 cells to invade in Matrigel-coated modified Boyden invasion chambers. Transfection with either CCND1 (51) or CCND1(52) siRNA almost completely abolished the ability of MDA-MB-231 cells to cross the membrane. Very few cells transfected with either cyclin D1–specific siRNA crossed the membrane (<1 cell per field of view) when assayed 12 hours after transfection (Table 1). In contrast, in cells transfected with control scrambled siRNA, an average of 38.4 cells were seen per field of view.

To determine if cell migration is also dependent on cdk4/6 activity, we inhibited kinase activity by introducing two peptides derived from $p16^{INK4a}$ into the culture media of MDA-MB-231 cells (22, 23). The p16 ^{INK4} family of proteins inhibit cdk4 and cdk6 kinase activities through direct interaction with the kinase subunit only. The $p16^{INK4a}$ p21 peptide contains two alanine substitutions at Val95 and Val96 of the $p16^{INK4a}$ protein, and the $p16^{INK4a}$ p20 peptide contains a substitution of Asp92 to alanine. These peptides have been shown to be taken up from the tissue culture medium (22). In these studies, cyclin D1-cdk4/6 kinase activity was first blocked by incubating cells with 20 μmol/L of p20 or p21 $p16^{INK4a}$ peptide for 24 hours. The monolayer was then scratched to create a wound, and the monolayer washed and incubated with peptides for an additional 24 hours. The wound was completely healed in the untreated cells after 24 hours, whereas in the cells treated with either p20 or p21 peptide, there was incomplete wound healing and the difference for p20- and p21-treated cells and wild-type was statistically significant, with $P < 4.0 \times 10^{-5}$ (Table 1). The p20 peptide has been shown to be a stronger kinase inhibitor than p21 (22). Consistent with this, p20 was more effective than p21 at inhibiting the wound healing activity of these cells.

FLNa Binds to Cyclin D1 *In vitro*

Although the mechanism by which cyclin D1 influences cellular migration is not well understood, several studies using cells from cyclin D1–deficient mice have been reported (6, 7, 22). To identify proteins that interact directly with cyclin D1, immunoprecipitation experiments were conducted using MCF-7 cells transfected with FLAG-tagged cyclin D1. The immunoprecipitated proteins were first separated on an SDS-PAGE gel and stained with Coomassie R250. Bands were excised and digested with trypsin, and the proteins identified by MALDI-MS/MS. A novel binding partner, the actin cytoskeleton protein FLNa, was identified in a band

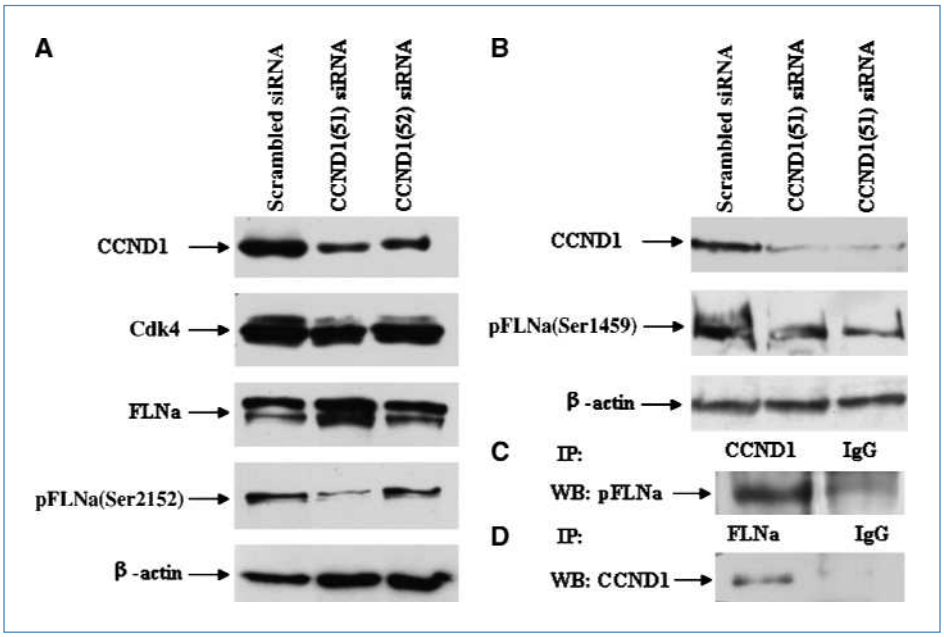

Figure 1. MDA-MB-231 cells were transfected with scrambled, CCND1(51), and/or CCND1(52) siRNA. The amount of cyclin D1 protein was lower after transfection with cyclin D1–specific siRNA compared with scrambled siRNA. The amount of cdk4 and FLNa was not affected, whereas the amounts of pFLNa(Ser2152) (A) and pFLNa (Ser1459) (B) decreased in cells transfected with cyclin D1–specific siRNA, with greater effect with CCND1(51). FLNa is a binding partner of cyclin D1. C, anti–cyclin D1 immunoprecipitations were done using MDA-MB-231 protein lysate. Phosphorylated FLNa was detected by Western blot of the immunoprecipitated fraction. D, anti-FLNa immunoprecipitations were done using MDA-MB-231 protein lysate. Cyclin D1 was detected by Western blot of the immunoprecipitated fraction.

that migrated above the 220-kDa molecular weight marker, consistent with the molecular weight of 280 kDa of FLNa. FLNa has been shown to be critical for cellular motility, as it promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins and various transmembrane proteins.

Because of the role of FLNa in cell motility and also because FLNa is a known phosphoprotein, we hypothesized that our observation of the cyclin D1/cdk4 effect on cell migration is mediated through phosphorylation of FLNa. Whereas there are many potential sites of phosphorylation on FLNa, only two (Ser2152 and Ser2523) of the 28 have been associated with cytoskeletal reorganization.[1] Phosphorylation at Ser2152 has been shown to be required for Pak1-mediated membrane ruffling and for regulation of cellular migration by ribosomal S6 kinase, a key kinase in the Ras-mitogen-activated protein kinase pathway (25, 26). We therefore checked to see if FLNa phosphorylated at Ser2152 immunoprecipitates with endogenous cyclin D1 in MDA-MB-231 cells. We found that FLNa phosphorylated at Ser2152 (pFLNa) coprecipitated with cyclin D1 using an antibody against phosphorylated FLNa in a Western blot of the cyclin D1 precipitate (Fig. 1C), establishing the interaction of phosphorylated FLNa and endogenous cyclin D1 in MDA-MB-231 cells. As further proof, we immunoprecipitated endogenous FLNa and then used an antibody against cyclin D1 in a Western blot of the FLNa precipitate and found that cyclin D1 coprecipitated with FLNa, verifying the interaction between FLNa and cyclin D1 (Fig. 1D). These results show that cyclin D1 and pFLNa (Ser2152) are binding partners in MDA-MB-231 protein lysates.

[1] http://www.phosphosite.org/proteinAction.do?id=2546&showAllSites=false

Cyclin D1 and FLNa colocalize in MDA-MB-231 cell ruffles. Although cyclin D1 is primarily located in the nucleus, there have been some reports that there are significant levels in the cytoplasm, particularly near the cell membrane. To further investigate if cyclin D1 and FLNa are interacting at the cell membrane and that this colocalization is likely a functional interaction related to migration, we performed immunofluorescence double labeling of cyclin D1 and FLNa. In Fig. 2, we show using confocal microscopy that the signal of cyclin D1 was mainly shown in the nucleus, with lower but significant signal in the cytoplasm and cell membrane. FLNa was observed to be localized primarily in the cytoplasm and cell membrane. We observed that cyclin D1 and FLNa proteins colocalize strongly in cell ruffles in those cells that migrated into the gaps created by wounding, indicating that the colocalization of cyclin D1 with FLNa is in migrating cells, but not in nonmigrating cells.

Mechanism of Cyclin D1–Dependent Migration and Invasion

We have shown that decreased cyclin D1 protein expression and cyclin D1-cdk4/6 activity decrease the invasion and migration potential of MDA-MB-231 cells. We have also shown that cyclin D1 and FLNa precipitate together in an immunoprecipitation assay. Because cyclin D1 is known as a regulatory subunit of a dimeric holoenzyme that includes cdk4, we next measured the effects of cyclin D1 knockdown on the levels of protein expression of cdk4, FLNa, and pFLNa. MDA-MB-231 cells were transfected with either control siRNA or cyclin D1–specific siRNA [CCND1(51) and CCND1(52)] for 48 hours and protein expression was then measured by Western blot (Fig. 1A). As expected, cyclin D1 protein expression was lower in cells transfected with cyclin D1–specific siRNA (when compared with the actin loading control, it is

Table 1. Summary of wound healing and invasion assays using MDA-MB-231 cells transfected with cyclin D1–specific siRNA or $p16^{INK4a}$ peptides

Treatment	Width of wounds after 12 h (% of initial ± SD)	No. of invading cells after 24 h (avg ± SD)
Scr siRNA (M)	0.12 ± 0.08	38.4 ± 11.6
CCND1(51)	0.72 ± 0.22*	0.15 ± 0.48*
CCDN1(52)	0.42 ± 0.16*	0.2 ± 0.52*

Treatment	Width of wounds after 24 h (% of initial ± SD)
wt	0.04 ± 0.02
p20	0.53 ± 0.07*
p21	0.38 ± 0.04*

*$P < 0.05$.

reduced by 80% and 40%, respectively), but neither FLNa nor cdk4 protein expression was affected. However, the level of phosphorylated FLNa was lower in cells transfected with both cyclin D1–specific siRNAs. These data support the hypothesis that FLNa phosphorylation is cyclin D1 dependent.

Identification of Other Cyclin D1/cdk4–Dependent Phosphoproteins

To identify other proteins whose phosphorylation status is dependent on cyclin D1 and cdk4/6, we used the SILAC *in vivo* labeling strategy combined with phosphoprotein enrichment to identify proteins that are differentially expressed 48 hours after introduction of the $p16^{INK4a}$ p20 peptide to inhibit cyclin D1-cdk4/6. We identified approximately 150 phosphoproteins. By comparing the peak intensities of the isotopically labeled peptides, we were able to determine that 44 were upregulated and 19 were downregulated, responding with at least a 1.5-fold change in expression level in response to cyclin D1-cdk4/6 inhibition (Table 2). The majority of these phosphoproteins have been previously associated with some type of cancer either *in vitro* or *in vivo*. We used Biological Network Gene Ontology tool (BiNGO), a Java-based tool, to determine which Gene Ontology (GO) categories are statistically overrepresented in the set of identified phosphoproteins (24). Although it is currently not possible to analyze the entire phosphoproteome in a single experiment and the enrichment process may result in an unbalanced enrichment of certain types of proteins, we assume that particular functional groups containing a larger-than-expected number of affected proteins are isolated based on the underlying physiologic process and not due to the enrichment process. Figure 3 shows the graphical representation of the results. The colored nodes are those determined to be overrepresented with statistical significance. We found that by looking at the closest branch points of the overrepresented GO_biological processes, four major categories are represented: (*a*) cellular organization and biogenesis/localization, which contains cytoskeleton-related processes; (*b*) metabolic processes; (*c*) cell motility; and (*d*) metabolic processes. Table 3 lists the Gene ID residing under each of these categories. Many of these proteins are classified under multiple processes. Specifically, many of the proteins classified under cellular organization

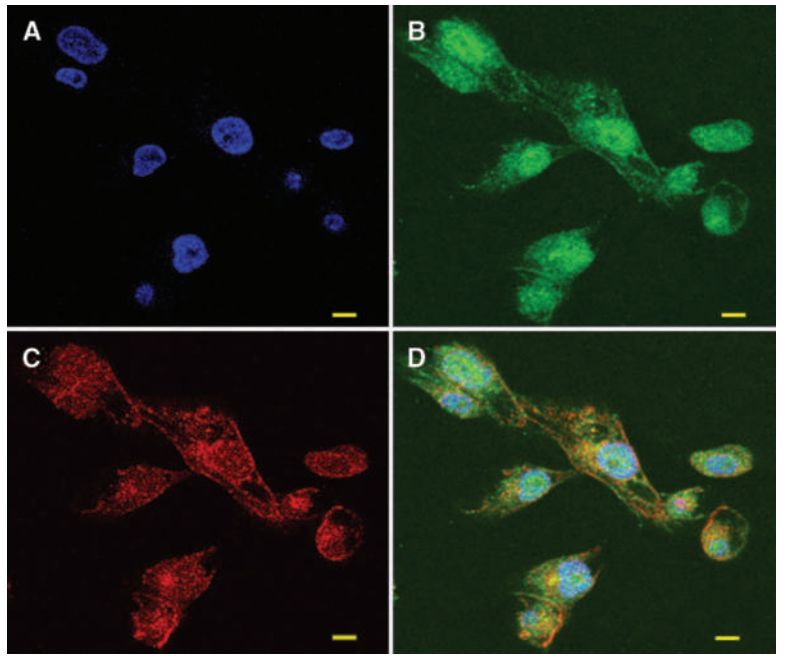

Figure 2. Cyclin D1 and FLNa colocalize in cell ruffles. MDA-MB-231 cells were stained for cyclin D1 (green), FLNa (red), and DNA (blue). Colocalization of cyclin D1 and FLNa is indicated by the yellow color in the merged picture. Bar, 10 μm.

Table 2. Phosphoproteins whose level of expression was altered by at least 1.5-fold, listed by inhibition of cyclin D1-cdk4/6 activity using $p16^{INK4a}$ peptides in MDA-MB-231 cells

Gene symbol	Name	GenBank accession no.	Fold change ($p16^{INK4}$ peptide vs no peptide)	Association with cancer
c11orf30	EMSY (chromosome 11 ORF 30)	Q7Z589	2.3	x
CALU	Calumenin precursor (crocalbin)	O43852	2.9	x
EEF1B2	Eukaryotic translation elongation factor 1β2	P24534	2.4	x
EIF5	Eukaryotic translation initiation factor 5	P55010	2.8	x
ENO1	Enolase 1α	P06733	2.4	x
ERBB2IP	Erbb2 interacting protein	Q96RT1	2.6	x
FAM120A	Family with sequence similarity 120A	Q9NZB2	2.4	
GATA4	GATA binding protein 4	P43694	2.8	x
HSPB1	Heat shock 27-kDa protein 1	P04792	2.2	x
ITIH2	Inter-α (globulin) inhibitor H2	P19823	2.5	x
ITSN2	Intersectin2 (ITSN2, SH3P18)	Q9NZM3	2.3	
KRT1	Keratin 1 (epidermolytic hyperkeratosis, CK1)	P04264	2.1	x
KRT10	Keratin 10 (epidermolytic hyperkeratosis)	P13645	2.2	x
LAMB2	Laminin β2 (laminin S)	P55268	7.9	x
LASP1	LIM and SH3 protein 1	Q14847	2.9	x
LRIT1	Leucine-rich repeat–containing protein 21 precusor (LRC21)	Q9P2V4	2.2	
MARCKS	Myristoylated alanine-rich protein kinase C substrate	P29966	2.2	x
MLL	Myeloid/lymphoid or mixed lineage leukemia	Q03164	2.7	x
MYO7A	Myosin VIIA	Q13402	2.1	
NOL1	Nucleolar protein 1, 120 kDa	P46087	2.5	x
PCYT1A	Phosphate cytidylyltransferase 1, choline, α	P49585	2.3	
PCYT1B	Phosphate cytidylyltransferase 1, choline, β	Q9Y5K3	2.1	
PDLIM4	PDZ and LIM domain 4	P50479	2	x
PKP4	Plakophilin-4	Q99569	2.5	x
PPFIA2	Liprin-α2 (LIPA2, PTPRF-interacting protein α2)	Q75334	2.4	x
PRSS1	Protease, serine, 1 (trypsin 1)	Q07477	3.7	x
RCN1	Reticulocalbin-1 precusor	Q15293	2.5	x
RHPN2	Rhophilin-like protein	Q8IUC4	10.1	
RPL10	60S ribosomal protein L10	P27635	2.4	x
RPL13	Ribosomal protein L13	P26373	3.6	x
RPL27A	Ribosomal protein L27a	P46776	2.2	x
RPS6	40S ribosomal protein S6	P62753	2	x
SEC22A	Vesicle-trafficking protein SEC22a	Q96IW7	3	
SEC61B	Protein transport protein Sec61 β-subunit	P60468	2.1	
TALDO1	Transaldolase 1	P37837	6.3	x
TPI1	Triosephosphate isomerase 1	P60174	1.7	
TUBA1B	Tubulin, α-ubiquitous chain	P68363	2.1	
TUBA1C	Tubulin α6	Q9BQE3	3.1	
TUBB1	Tubulin β1	P07436	2.3	x
TUBB5	Tubulin β5	Q7JJU6	2.4	x
TUFM	Tu translation elongation factor, mitochondrial (EFTU)	P49411	2.4	
VPS13D	Vacuolar protein sorting-associated protein 13D	Q5THJ4	3.3	
YLPM1	YLP motif–containing protein 1 (YLPM1, ZAP113)	P49750	2.6	
ZNF148	Zinc finger protein 148	Q9UQR1	3.2	x
AFAP1	Actin filament–associated protein	Q8N556	–3.3	

(*Continued on the following page*)

Table 2. Phosphoproteins whose level of expression was altered by at least 1.5-fold, listed by inhibition of cyclin D1-cdk4/6 activity using $p16^{INK4a}$ peptides in MDA-MB-231 cells (Cont'd)

Gene symbol	Name	GenBank accession no.	Fold change ($p16^{INK4}$ peptide vs no peptide)	Association with cancer
AHI1	Abelson helper integration site 1 protein homolog (AHI-1, Jouberin)	Q8N157	–2.2	x
AHNAK	AHNAK nucleoprotein isoform 1	Q09666	–2.5	
ARHGEF2	Rho/rac guanine nucleotide exchange factor 2	Q9H023	–2.7	x
BCLAF1	BCL2-associated transcription factor 1	Q9NYF8	untreated only	
C11orf58	Small acidic protein	O00193	–7.1	
CLIC6	Chloride intracellular channel 6	Q96NY7	untreated only	
CST4	Cystatin-S precusor	P01036	–2.1	
DYNC1LI1	Dynein light chain-A	Q9Y6G9	–2.2	
EHA2	Ephrin receptor EphA2	P29317	untreated only	x
ERRFI1	Mitogen-inducible gene 6 protein	Q9UJM3	–2.6	x
FAM129B	Hypothetical protein LOC64855	Q5VVW7	–5.3	
FAM82C	Family with sequence similarity 82, member C	Q96TC7	untreated only	
FLNA	Filamin 1 (actin binding protein-280)	P21333	–1.5	x
G3BP1	Ras-GTPase–activating protein SH3-domain–binding protein	Q13283	–3.7	x
HN1	Hematologic and neurologic expressed 1 isoform 1	Q9UK76	–6.7	x
HRNR	Hornerin	Q86YZ3	–2.9	x
MACF1	Microtubule-actin cross-linking factor 1	Q96PK2	–2.1	x
NCOA3	Nuclear receptor coactivator 3	Q9Y6Q9	–2.1	x
NOP5/NOP58	Nucleolar protein NOP5/NOP58	Q9Y2X3	–2.8	x

and biogenesis/localization are involved with cell motility as well as cytoskeletal-related processes such as actin- and microtubule-based movement and actin binding, suggesting that increased protein synthesis and processes related to organelle and cytoskeletal components are important in the decreased cell migration phenotype resulting from cyclin D1-cdk4/6 repression. Many of the proteins categorized under metabolism are involved in protein synthesis.

A majority of these proteins have a known role either directly in affecting cell motility or in a related role such as cell adhesion and cytoskeletal relationship. Many of these proteins also play roles in transcription, translation, and Ca^{2+} binding, suggesting that these mechanisms are important in the increased cell motility phenotype induced by $p16^{INK4a}$.

In addition to looking at the global changes in phosphorylated proteins, we looked for specific changes in FLNa phosphorylation.

Table 3. Categorization of overrepresented phosphoproteins under the major GO_processes identified by BiNGO analysis

Cell motility	Cellular component organization and biogenesis			Localization			Metabolic processes	
FLNa	ARHGEF2	LASP1	SH3KBP1	ARHGEF2	MYO7A	TUBA1C	ARHGEF2	RPL27A
HSPB1	C11ORF30	MAACF1	STMN1	ERBB2IP	NOP5/NOP58	TUBB1	EEF1B2	RPS6
LAMB2	EIF5	MLL	SYTL4	FLNa	NUP153	TUBB5	EIF5	STMN1
MACF1	EIF5B	MYO7A	TLN1	G3BP1	SEC22A	TXNDC1	EIF5B	TALDO1
MARCKS	ENO1	NOP5/NOP58	TUBA1B	HSPB1	SEC61B	VPS13C	ENO1	TPI1
TLN1	ERBB2IP	NUUP153	TUBA1C	ITSN2	SH3KBP1	VPS13D	HSPB1	TUBA1B
TUBB1	FLNa	PLEC1	TUBB1	LAMB2	STMN1		PCYT1A	TUBA1C
TUBB5	HSPB1	SEC22A	TUBB5	LASP1	SYTL4		PCYT1B	TUBB1
	ITSN2	SEC61B	TXNDC1	MACF1	TLN1		PGM2L1	TUBB5
	LAMB2	9-Sep		MARCKS	TUBA1B		RPL10	TUFM
							RPL13	

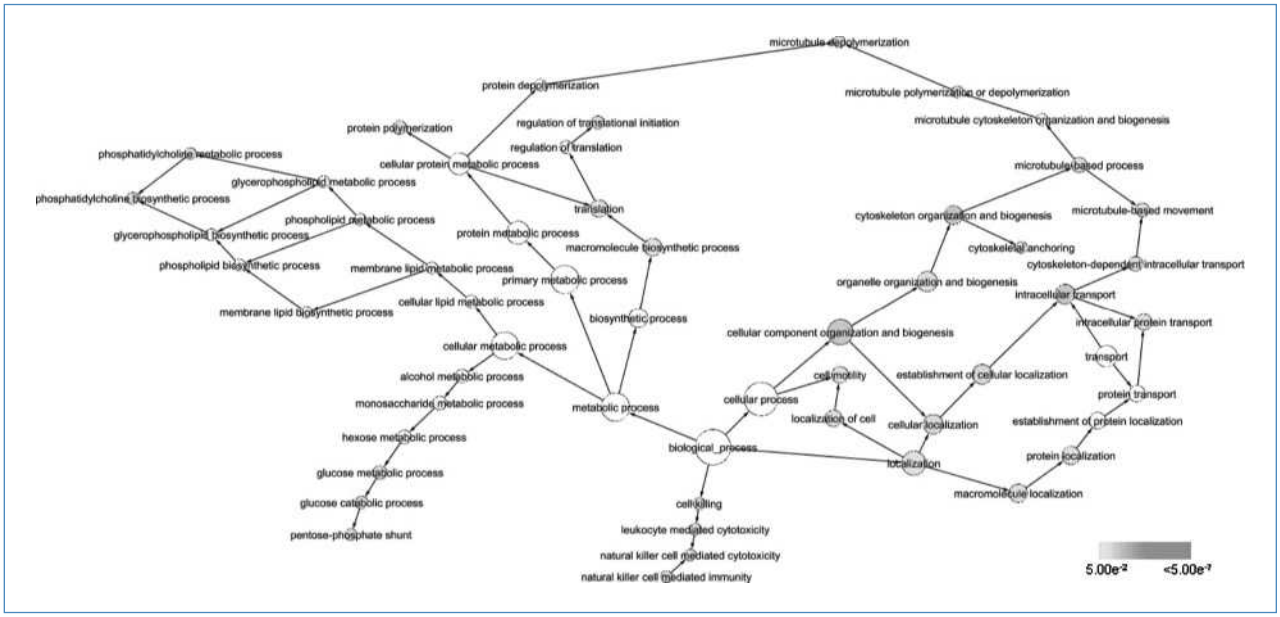

Figure 3. Pathway output from the BiNGO analysis. Gray circles indicate GO_processes determined to be overrepresented, with statistical significance as depicted in the legend.

We identified decreased levels of phosphorylated FLNa at Ser1459 on cyclin D1-cdk4/6 repression [Supplementary Fig. S1 shows the spectrum of the singly and doubly charged daughter ions of phosphorylated FLNa peptide (*m/z* 650.2, in the doubly charged state)]. Ser1459 has been previously identified as a site of FLNa phosphorylation but has never been linked to a specific kinase (27). Incubation with $p16^{INK4a}$ or cyclin D1 knockdown using siRNA caused a decrease of ~50% of pFLNa(Ser2152) as determined via Western blot and of pFLNa(Ser1459) in the mass spectrometry experiments. To validate this result, we obtained an antibody specific to pFLNa(Ser1459), and as shown in Fig. 1B, treatment of the cells with siRNA against cyclin D1 caused an ~50% decrease in the amount of pFLNa(Ser1459).

Discussion

We showed that cyclin D1 siRNA and inhibition of the cyclin D1-cdk4/6 kinase complex through peptide treatment resulted in decreased motility and an impaired wound healing response in the invasive MDA-MB-231 breast cancer cell line. We also showed that cyclin D1 bound to the actin binding protein FLNa and that the amount of phosphorylated FLNa(Ser2152) and FLNa(Ser1459) decreased concomitant with the decreased migration resulting from cyclin D1 siRNA transfection. We found that cyclin D1 and FLNa coimmunoprecipitated as well as colocalized in migrating cells, which suggested that the interaction was likely functional as the interaction occurred at the cell membrane where phosphorylated FLNa could recruit other molecules that were required for cytoskeleton reorganization (11). These findings identify a migration-related function of cyclin D1 in breast cancer cells and also provide new information about the mechanism for affecting motility in these cells.

Recent studies suggest a role for cyclin D1 in cellular migration (6, 28). The bone marrow macrophages isolated from cyclin D1–deficient mice displayed decreased motility (6). It was also shown that cyclin $D1^{-/-}$ mouse embryo fibroblasts exhibited increased adherence and decreased cellular motility compared with wild-type cells (7). Further evidence showed that these cyclin D1–related phenotypic changes were regulated through a member of the Rho family of small GTPases that is known to play an important role in the regulation of cell motility. In these cells, the activity of Rho-activated kinase (ROCKII) was increased. Thrombospondin 1, a matrix glycoprotein that inhibits cellular metastasis, was also shown to be regulated by cyclin D1 in these cells. The cdk inhibitor p27KIP has also been determined to be required for cyclin D1 regulation of cellular migration mouse embryo fibroblasts (28). In T47D breast cancer cells, cyclin D1 was shown to act similarly. A fewer number of T47D cells transfected with cyclin D1 siRNA were able to across the membrane in the Boyden chamber migration assay compared with control cells (29).

In our studies, we find that decreased cyclin D1 levels in MDA-MB-231 cells also lead to decreased wound healing capacity as well as decreased invasion potential. We also provide evidence that cyclin D1 and pFLNa coimmunoprecipitate and cyclin D1 and FLNa colocalize in MDA-MB-231. The amounts of two phosphorylated forms of FLNa, pFLNa (Ser1459) and pFLNa(Ser2152), are dependent on the level of cyclin D1 protein. To our knowledge, this is the first report of a mediator of phosphorylation at Ser1459. FLNa has been shown to interact with the COOH-terminal pleckstrin homology domain of ROCK and that this complex localizes at protrusive cell membranes (30). The known association of FLNa with other small GTPases regulates actin remodeling, formation of filopodia, and membrane ruffles, which, taken

together with binding of FLNa to cyclin D1, suggests the mechanism for increased activity of ROCKII.

At least 28 sites of phosphorylation, including Ser2152 and Ser1459, have been identified in human FLNa.[2] With regard to Ser2152, FLNa has been reported to be a substrate for the serine/threonine kinase p21-activated kinase (Pak1) identified by yeast two-hybrid screening using the NH_2-terminal 1–270 amino acids of Pak1 as bait (31). In fibroblasts, FLNa has been identified as a substrate for one member of the Ras/mitogen-activated kinase pathway, p90 ribosomal S6 protein kinase 2 (25, 26). Ser1459 as a site of phosphorylation of FLNa has been identified in HeLa cell lysate that was enriched for phosphoproteins; however, the associated kinase was not identified (27, 32). Herein we have found that inhibition of cyclin D1-cdk4/6 activity resulted in a reduction of pFLNa (Ser1459) via mass spectrometry experiments and validated by Western blotting. A recent report by Lee et al. showed that cyclin B1/cdk1 can interact with and partially regulate the function of FLNa by phosphorylating Ser1436 *in vitro* and decrease its ability to cross-link actin *in vitro*. However, more experiments must be conducted to determine whether cyclin D1/cdk4 kinase directly phosphorylates Ser1459 or if it is part of a larger signaling pathway. An alternative hypothesis is that cyclin D1/cdk4 and FLNa are part of a complex and that phosphorylation of FLNa is accomplished by another molecule. FLNa could also be a potential scaffold for some unknown transmembrane receptors or signaling molecules.

We have identified approximately 65 phosphorylated proteins whose level of expression was significantly different in cells transfected with the $p16^{INK4a}$ peptide inhibitor of cyclin D1-cdk4/6 activity (Table 2). The majority of these phosphoproteins have been reported to play a role in some type of cancer either *in vitro* or *in vivo*. BiNGO was used to determine that localization, metabolism, cell motility, and cellular component organization/biogenesis Gene Ontology (GO) categories are statistically overrepresented in our set of identified phosphoproteins. Many of the proteins categorized under metabolism are involved in protein synthesis. Regulation of translation is critical for proper protein expression. Deregulation of protein translation is associated with abnormal gene expression leading to altered physiology, including cell growth and possibly cancer (33). Eukaryotic initiation factor 4E (eIF-4E) is one of the several initiation factors responsible for the regulation of translation in eukaryotes, and elevated level of eIF-4E is associated with many solid tumors including breast, prostate, and cervix cancers (33). In this study, we identify several other proteins related to protein translation that may play a role in increased cell motility. Signaling pathways identified as deregulated in some cancers are also represented in our data set.

In addition to FLNa, the other proteins associated specifically with cell motility are tubulin β1 and tubulin β5 (TUBB1 and TUBB5), microtubule-acting cross-linking factor 1 (MACF1), heat shock 27-kDa protein 1 (HSPB1), myristoylated alanine-rich protein kinase C substrate (MARCKS), talin 1 (TLN1), and laminin β2 (LAMB2). Except for TUBB1 and TUBB5, which, with actin and intermediate filaments, make up the cytoskeleton, and laminin β2, a component of the extracellular matrix, all of the proteins in this category are actin binding proteins. These data suggest that FLNa is only one of several other actin binding proteins involved in the cyclin D1–driven cell motility in MDA-MB-231 cells.

Taken together, our results show for the first time a direct interaction (and involvement) of cyclin D1 with the cytoskeletal protein FLNa, which is necessary for cell remodeling—a feature critical to cancer cell motility and hence migration and extravasation to other sites. Consistent with our results, there are reports that FLNa, in particular its phosphorylated form, plays an important role in breast cancer cell migration. FLNa phosphorylated at Ser2152 has been identified as a target of caveloin-1 in insulin-like growth factor-I–stimulated migration of MCF-7 breast cancer cells (34), and FLNa has been identified in the plasma of patients with metastatic breast cancer (35).

Disclosure of Potential Conflicts of Interest

A.A. Quong: employment, Food and Drug Administration. The other authors disclosed no potential conflicts of interest.

Grant Support

Pennsylvania Department of Health Breast and Cervical Cancer Section grant #05-07-09 (J.N. Quong and A.A. Quong), the Breast Cancer Research Foundation (A.A. Quong and J.N. Quong), and NIH grants R01CA70896, R01CA75503, R01CA86072, and R01CA107382 (R.G. Pestell). The Kimmel Cancer Center was supported by NIH Cancer Center Core grant P30CA56036 (R.G. Pestell). This project is funded in part by the Dr. Ralph and Marian C. Falk Medical Research Trust and a grant from the Pennsylvania Department of Health (R.G. Pestell). The Department specifically disclaims responsibility for any analysis, interpretations, or conclusions.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 03/25/2008; revised 12/06/2009; accepted 12/23/2009; published OnlineFirst 02/23/2010.

[2] http://www.phosphosite.org

References

1. Massague J. G_1 cell-cycle control and cancer. Nature 2004;432:298–306.
2. Drobnjak M, Osman I, Scher HI, Fazzari M, Cordon-cardo C. Overexpression of cyclin D1 is associated with metastatic prostate cancer to bone. Clin Cancer Res 2000;6:1891–5.
3. Jares P, Rey MJ, Fernandez PL, et al. Cyclin D1 and retinoblastoma gene expression in human breast carcinoma: correlation with tumour proliferation and oestrogen receptor status. J Pathol 1997;182:160–6.
4. Arnold A, Papanikolaou A. Cyclin D1 in breast cancer pathogenesis. J Clin Oncol 2005;23:4215–24.
5. Coqueret O. Linking cyclins to transcriptional control. Gene 2002;299:35–55.
6. Neumeister P, Pixley FJ, Xiong Y, et al. Cyclin D1 governs adhesion and motility of macrophages. Mol Biol Cell 2003;14:2005–15.
7. Li Z, Wang C, Jiao X, et al. Cyclin D1 regulates cellular migration

through the inhibition of thrombospondin 1 and ROCK signaling. Mol Cell Biol 2006;26:4240–56.
8. Yao WJ, Liang Y, Chen K, et al. Changes of biophysical behavior of k562 cells for p16 gene transfer. Clin Hemorheol Microcirc 2002;27: 177–83.
9. Alhaja E, Adan J, Pagan R, et al. Anti-migratory and anti-angiogenic effect of p16: a novel localization at membrane ruffles and lamellipodia in endothelial cells. Angiogenesis 2004;7:323–3.
10. Zhu TN, He HJ, Kole S, et al. Filamin A-mediated down-regulation of the exchange factor Ras-GRF1 correlates with decreased matrix metalloproteinase-9 expression in human melanoma cells. J Biol Chem 2007;282:14816–26.
11. Stossel TP, Condeelis J, Cooley L, et al. Filamins as integrators of cell mechanics and signalling. Nat Rev Mol Cell Biol 2001;2:138–45.
12. Cukier IH, Li Y, Lee JM. Cyclin B1/Cdk1 binds and phosphorylates Filamin A and regulates its ability to cross-link actin. FEBS Lett 2007; 581:1661–72.
13. Feng Y, Walsh CA. The many faces of filamin: a versatile molecular scaffold for cell motility and signalling. Nat Cell Biol 2004;6: 1034–8.
14. Bourguignon LY, Gilad E, Brightman A, Diedrich F, Singleton P. Hyaluron-CD44 interaction with leukemia-associated RhoGEF and epidermal growth factor receptor promotes Rho/Ras coactivation, phospholipase Cϵ-Ca^{2+} signaling, and cytoskeleton modification in head and neck squamous cell carcinoma cells. J Biol Chem 2006; 281:14026–40.
15. Lin JF, Xu J, Tian HY, et al. Identification of candidate prostate cancer biomarkers in prostate needle biopsy specimens using proteomic analysis. Int J Cancer 2007;121:2596–605.
16. Loy CJ, Sims KS, Yong EL. Filamin-A fragment localizes to the nucleus to regulate androgen receptor and coactivator functions. Proc Natl Acad Sci U S A 2003;100:4562–7.
17. Sasaki A, Masuda Y, Ohta Y, Ikeda K, Watanabe K. Filamin associates with Smads and regulates transforming growth factor-β signaling. J Biol Chem 2001;276:17871–7.
18. Keshamouni VG, Michailidis G, Grasso CS, et al. Differential protein expression profiling by iTRAQ-2DLC-MS/MS of lung cancer cells undergoing epithelial-mesenchymal transition reveals a migratory/invasive phenotype. J Proteome Res 2006;5:1143–54.
19. Bachmann AS, Howard JP, Vogel CW. Actin-binding protein filamin A is displayed on the surface of human neuroblastoma cells. Cancer Science 2006;97:1359–65.
20. Coughlin MF, Puig-de-Morales M, Bursac P, Mellema M, Millet E, Fredberg JJ. Filamin-A and rheological properties of cultured melanoma cells. Biophys J 2006;90:2199–205.
21. Ong SE, Blagoev B, Kratchmarova I, et al. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics 2002;1: 376–86.
22. Fahraeus R, Lane DP. The p16(INK4a) tumour suppressor protein inhibits $\alpha_v\beta_3$ integrin-mediated cell spreading on vitronectin by blocking PKC-dependent localization of $\alpha_v\beta_3$ to focal contacts. EMBO J 1999;18:2106–18.
23. Fahraeus R, Paramio JM, Ball KL, Lain S, Lane DP. Inhibition of pRb phosphorylation and cell-cycle progression by a 20-residue peptide derived from p16CDKN2/INK4A. Curr Biol 1996;6:84–91.
24. Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in biological networks. Bioinformatics 2005;21:3448–9.
25. Woo MS, Ohta Y, Rabinovitz I, Stossel TP, Blenis J. Ribosomal S6 kinase (RSK) regulates phosphorylation of filamin A on an important regulatory site. Mol Cell Biol 2004;24:3025–35.
26. Ohta Y, Hartwig JH. Phosphorylation of actin-binding protein 280 by growth factors is mediated by p90 ribosomal protein S6 kinase. J Biol Chem 1996;271:11858–64.
27. Beausoleil SA, Jedrychowski M, Schwartz D, et al. Large-scale characterization of HeLa cell nuclear phosphoproteins. Proc Natl Acad Sci U S A 2004;101:12130–5.
28. Li Z, Jiao X, Wang C, et al. Cyclin D1 induction of cellular migration requires p27(KIP1). Cancer Res 2006;66:9986–94.
29. Ostrander JH, Daniel AR, Lofgren K, Kleer CG, Lange CA. Breast tumor kinase (protein tyrosine kinase 6) regulates heregulin-induced activation of ERK5 and p38 MAP kinases in breast cancer cells. Cancer Res 2007;67:4199–209.
30. Ueda K, Ohta Y, Hosoya H. The carboxy-terminal pleckstrin homology domain of ROCK interacts with filamin-A. Biochem Biophys Res Commun 2003;301:886–90.
31. Vadlamudi RK, Li F, Adam L, et al. Filamin is essential in actin cytoskeletal assembly mediated by p21-activated kinase 1. Nat Cell Biol 2002;4:681–90.
32. Beausoleil SA, Villen J, Gerber SA, Rush J, Gygi SP. A probability-based approach for high-throughput protein phosphorylation analysis and site localization. Nat Biotechnol 2006;24:1285–92.
33. Thumma SC, Kratzke RA. Translational control: a target for cancer therapy. Cancer Lett 2007;258:1–8.
34. Ravid D, Chuderland D, Landsman L, Lavie Y, Reich R, Liscovitch M. Filamin A is a novel caveolin-1-dependent target in IGF-I-stimulated cancer cell migration. Exp Cell Res 2008;314:2762–73.
35. Alper O, Stetler-Stevenson WG, Harris LN, et al. Novel anti-filamin-A antibody detects a secreted variant of filamin-A in plasma from patients with breast carcinoma and high-grade astrocytoma. Cancer Sci 2009;100:1748–56.