ZF21 PROTEIN REGULATES CELL ADHESION AND MOTILITY

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Cell migration on an extracellular matrix (ECM) requires continuous formation and turnover of focal adhesions (FAs) along the direction of cell movement. However, our knowledge of the components of FAs and the mechanism of their regulation remains limited. Here, we identify ZF21, a member of a protein family characterized by the presence of a phosphatidylinositol 3-phosphate-binding FYVE domain to be a new regulator of FAs and cell movement. Knockdown of ZF21 expression in cells increased the number of FAs and suppressed cell migration. Knockdown of ZF21 expression also led to a significant delay in FA disassembly following induction of synchronous disassembly of FAs by nocodazole treatment. ZF21 bound to focal adhesion kinase (FAK), localized to FAs, and was necessary for dephosphorylation of FAK at Tyr397, which is important for disassembly of FAs. Thus, ZF21 represents a new component of FAs, mediates disassembly of FAs, and thereby regulates cell motility.

The cells in most tissues are surrounded by an extracellular matrix (ECM) and interaction of the cells with the ECM plays a pivotal role in maintaining cell morphology and tissue architecture and in mediating signals regulating a variety of cellular functions, such as proliferation, motility, survival, and differentiation (1-3). Integrins are the major receptors for proteins of the ECM and they transmit ECM-mediated signals to a variety of intracellular machineries (4). Binding of cells to the ECM induces clustering of integrins and triggers recruitment of multiple cellular proteins to the inner surface of the plasma membrane (4,5). Formation and reorganization of such adhesion structures are particularly important for cell migration and these structures are regulated dynamically along the direction of cell movement (6,7). The formation of adhesion structures during the spreading and migration of cultured cells on ECM layers has been studied extensively (8,9). During cell migration,
primitive adhesion structures can be observed at
the migration front as relatively small focal
complexes along the periphery of ruffling
membranes (10). Most of these structures are
short lived, but some grow to become more
stable adhesion links to the actin cytoskeleton
so as to permit the generation of force for cell
movement via actin contraction (10,11).

Activation of Rho/ROCK signals at the
adhesion complex promotes actin
polymerization and leads to the development of
mature focal adhesions (FAs) (12). Although the
formation of FAs at the migration front is
important for the generation of a driving force
to pull the cell body forward, the FAs gradually
migrate to the rear of the cell owing to
movement of the cell body. To continue
migration forward, these FAs at the rear must be
disassembled to allow detachment from the
ECM. Thus, it is important to understand how
formation and disassembly of FAs are regulated
during cell migration.

Multiple cellular proteins are recruited to the
cytoplasmic domains of integrins, where they
mediate both physical connection to the
cytoskeleton and functional connections to
cellular signalling pathways (13). These
cytoplasmic effectors include paxillin, vinculin,
α-actinin, and talin, which together act as a
scaffold to connect integrins to the cytoskeleton
(13-17). Clustering of integrins leads to
successive phosphorylation of FAK, c-Src and
adaptor proteins, such as p130Cas and CrkII at
FAs (18-21). Activation of the Rho/ROCK
cascade stimulates formation of stress fibres that
stabilize the adhesion structures, which mature
into stable FAs (12,22).

While our knowledge of the processes
regulating the assembly of FAs has accumulated,
the regulation of their disassembly remains less
well understood. However, previous studies
have revealed that extension of microtubules to
FAs is essential to initiate their disassembly and
that disruption of microtubules by treatment of
cells with nocodazole stabilizes FA structures
(23-25). Treatment of cells with nocodazole and
subsequent retraction of the drug from the
culture media triggers synchronous disassembly
of FAs. Therefore, nocodazole is an excellent
tool to dissect the steps of FA assembly and
disassembly. Calpain-2, a calcium-dependent
endopeptidase, has been implicated in the
destruction of FAs via cleavage of integrin, FAK,
talin, and α-actinin etc (26). Dephosphorylation
of FAK, paxillin, and p130CAS by protein
tyrosine phosphatases, such as PTP-PEST,
SHP-2 or PTP-1B, also induces disassembly of
FA complexes (27-29). At the final stage of FA
disassembly, integrins are internalized in a
dynamin-dependent manner (23,30). However,
it is apparent that such information explains
only a portion of the full process of disassembly
of FAs.

In this study, we identify ZFYVE21 (ZF21)
to be a new constituent protein of FAs that acts
as a key regulator of their disassembly. We
previously identified ZF21 as a protein that
could bind to the cytoplasmic (CP) tail of
membrane type-1 matrix metalloproteinase
(MT1-MMP) (31), a potent invasion-promoting
protease (32). A cDNA fragment encoding ZF21
was isolated as a result of a yeast two-hybrid
screen using the CP tail as bait (31), although
the interaction may not represent a
physiological one because we were unable to detect direct binding of ZF21 to MT1-MMP in vitro or in vivo (unpublished results). During that study, however, we observed that ZF21 could modulate cell adhesion. We show here that knockdown of ZF21 expression enhances adhesion of cells to the ECM and that it suppresses their migration. We also show that ZF21 is required to initiate the disassembly of FAs. Finally, we demonstrate that ZF21 localizes to FAs, where it can bind and prevent the dephosphorylation of FAK.

**Experimental Procedures**

**Cell cultures-** HT1080, MDA-MB231 and Cos-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM (Invitrogen), supplemented with 10% fetal bovine serum, penicillin and streptomycin (Invitrogen). All cells were cultured at 37°C under a 5% CO₂, 95% air atmosphere.

**Antibodies and reagents-** A polyclonal anti-ZF21 antibody was prepared by affinity purification of immune sera collected following the immunization of a rabbit or a chicken with the recombinant protein expressed in Escherichia coli. We used commercially available antibodies to detect Myc (Roche), EEA1 (14, BD Biosciences), Transferrin receptor (H68.4, Zymed), β-Tubulin (E7, Development Studies Hybridoma Bank), Venus (anti-GFP, A11122, Invitrogen), Actin (C4, Millipore), Integrin-β1 (18, BD Biosciences), Integrin-β1 (for adhesion blocking, 6S6, Millipore), FAK (BD Biosciences), Tyr397-phosphorylated FAK (Biosource or Abcam), Vinculin (VIN-11-5, Sigma), Talin (8D4, Sigma), SHP-2 and PTP-1B (PTP Sampler Kit, BD Bioscience). Nocodazole (Sigma) was used at 5 μM. Type I-A collagen was purchased from Nitta Gelatin. All other chemical reagents were purchased from Sigma-Aldrich or Wako, unless otherwise indicated.

**Plasmids-** Complementary DNAs (cDNAs) encoding ZFYVE21 (ZF21), FAK, Paxillin, Zyxin, SHP-2 or PTP-1B were amplified from HT1080 cells by RT-PCR. ZF21 mutants were obtained by PCR using human ZF21 as a template. FAK mutants were obtained by PCR using human FAK as a template. To prepare ZF21 or its mutant fused to the N terminus of Venus protein, m1Venus cDNA (provided by Dr. A. Miyawaki, RIKEN, Japan) was amplified by PCR and cloned into the SacII site of the pLenti6/V5-DEST vector (Invitrogen). To prepare FAK, Paxillin or Zyxin fused to the C terminus of Venus protein, m1Venus cDNA was amplified by PCR and cloned into the SpeI site of the pLenti6/V5-DEST vector. To prepare Zyxin fused to the C terminus of monomeric cherry protein (mCherry), mCherry cDNA (provided by Dr. R. Tsien, Howard Hughes Medical Institute, University of California, San Diego, CA, USA) was amplified by PCR and cloned into the SpeI site of the pLenti6/V5-DEST vector. To express proteins for each experiment, the mammalian expression vector pLenti6/V5-DEST or the E.coli expression...
vector pDEST15 (Invitrogen) was used.

Knockdown experiments using shRNA-shRNA sequences used for knockdown of target proteins were as follows: ZF21, 5′-caccgcagttgttgcccgagtcggagcaatg-3′; FAK #1, 5′-caccgcatctgccatcagttgtgaagaagcttcaatg-3′ and FAK #2, 5′-caccgcaacccatctgatggtaatcagttgcggtgc-3′. shRNA expressing lentiviral vectors were generated and used according to the manufacturer’s instructions (Invitrogen). For rescue experiments of ZF21 knockdown, an expression construct encoding a mutant ZF21 mRNA refractory to shRNA was generated by site-directed mutagenesis and the mutant protein was expressed with a Myc tag at the COOH terminus using the pENTR vector (Invitrogen).

Immunofluorescence microscopy- Cells were fixed in −20°C methanol for 10 min and permeabilized using 0.1% Triton-X100 in PBS for 10 min. After the samples were blocked in PBS containing 5% goat serum and 3% BSA, they were incubated with primary antibodies. All primary antibodies were visualized with either an Alexa 488- or 568-conjugated goat anti-mouse or anti-rabbit antibody (Invitrogen). Images of cells were captured with Leica ASMDW with CCD camera (Leica).

Cell fractionation- Cells were homogenized in a buffer containing 250 mM sucrose and 25 mM Tris-HCl (pH 7.5). After centrifugation at 8,000 x g for 15 min, the supernatants were centrifuged further at 105,000 x g for 1 hr. The pellet was used as the membrane fraction and the supernatant as the cytoplasmic fraction.

Adhesion assay- For the cell adhesion assay, the surface of 12-well plates was coated with poly-L-Lysine, fibronectin, collagen I or vitronectin at a concentration of 1 µg/ml and nonspecific binding sites were blocked with 0.5 mg/ml BSA. Cells suspended in serum-free DMEM were seeded onto the plates at 3 x 10⁴ cells per well and incubated for 30 min. After removal of nonadherent cells, adherent cells were trypsinized and counted.

The effect of blocking of integrin-β1 on cell adhesion was analyzed as follows. 96-well plates were coated with poly-L-Lysine, collagen I, fibronectin or vitronectin (1 µg/ml) and nonspecific binding sites were blocked with 0.5 mg/ml BSA. Cells suspended in serum-free DMEM were seeded onto the wells (2 x 10⁴ cells per well) and incubated for 30 min in the presence or absence of 1 µg/ml integrin-β1 blocking antibody. After removal of nonadherent cells by washing, the remaining cells were stained with Hoechst 33342. Finally, fluorescence was quantified in a FLUOstar OPTIMA (BMG LABTECH).

Cell migration assay- Both sides of transwells with 8-µm pore size filters (Corning) were precoated with fibronectin (5 µg/ml) for 1 hr at 37°C. DMEM (400 µL) containing 10% FBS was added to the lower chamber, and 200 µL of a cell suspension (5 x 10⁴ cells) was placed in the upper chamber.
The plates were incubated at 37°C in a 5% CO₂ atmosphere for 6 hr. Cells in the lower chamber were then stained with 0.5% crystal violet solution and counted using a light microscope at 200 x magnification. Values are averages of 3 fields.

Cell surface protein pull-down assay- A pull-down assay of cell surface proteins was performed as described previously (33). Briefly, cells were washed three times with chilled phosphate-buffered saline (PBS) containing 1 mM MgCl₂ and 0.1 mM CaCl₂ and then incubated with sulfo-NHS-biotin (Pierce) (0.1 mg/ml) in the same buffer at 4°C for 30 min. The reaction was terminated by incubating the cells with 100 mM glycine in PBS. The cells were lysed in TNETS buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, 1% SDS), and the biotinylated proteins were precipitated with streptavidin–conjugated Sepharose beads (Amersham-Pharmacia). The samples were analyzed by Western blot using each antibody.

TIRF microscopy- For total internal reflection fluorescence (TIRF) microscopy, living cells were observed using an APON 60×OTIRFM objective on an IX81 microscope equipped with a TIRF illuminator and fibre optic coupled laser illumination (Olympus).

Immunoprecipitation- Cells were lysed with immunoprecipitation (IP) buffer (1% NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM Na₃VO₄ and protease inhibitor cocktail Set III). Endogenous ZF21 in the lysate was captured with chicken anti-ZF21 IgY and immunoprecipitated using chicken IgY precipitating agarose beads (Millipore). Venus tagged proteins were immunoprecipitated using agarose beads conjugated to anti-GFP antibody (MBL). The beads were washed three times with IP buffer and bound proteins were eluted with Laemmli sample buffer.

The pull-down assay- Cells cultured on a plastic plate were washed with ice-cold PBS, scraped off in RIPA buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM MgCl₂, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 % glycerol, 1 mM Na₃VO₄ and protease inhibitor cocktail Set III) on ice. Lysates were centrifuged for 5 min at 20,000 x g at 4°C. A fraction of the cleared lysates was incubated with 10 μg of GST-ZF21 or GST-ZF21 mutant bound to glutathione-conjugated Sepharose beads at 4°C for 6 hr. Pellets containing the beads were collected, washed three times with ice-cold RIPA buffer, and subjected to SDS-PAGE followed by Western blot analysis using the indicated antibodies.

Hypo-osmotic shock- Hypo-osmotic shock for cells was performed as previously described (34). Briefly, cells were grown 48 hr on 10 cm dishes. Cells were washed with PIPES buffer (20 mM PIPES, 100 mM KCl, 5 mM MgCl₂, 3 mM EGTA, pH 6.0) and exposed to a hypo-osmotic condition in 20% PIPES buffer for 10 min. After the cells were disrupted, most cellular contents were removed...
and washed with PIPES buffer, pH 7.0. The adhesion plaques remaining on the dish were resolved with Laemmli sample buffer and analyzed by Western blot using each antibody.

**Focal adhesion disassembly assay** - The focal adhesion disassembly assay was performed as described previously (23). Briefly, cells were grown on fibronectin-coated glass coverslips and treated with 5 μM nocodazole for 30 min to depolymerize microtubules. After the drug was removed, cells were incubated to resume polymerization of microtubules. Cells were fixed in -20°C methanol for 10 min, rehydrated in PBS followed by permeabilizing with 0.1% Triton-X100 in PBS for 5 min, and subjected to immunohistocemistry.

**FAK dephosphorylation assay** - The FAK dephosphorylation assay was performed as described previously (23). Briefly, cells were grown on fibronectin-coated plastic dishes and treated with 5 μM nocodazole for 30 min to depolymerize microtubules. After the drug was removed, cells were incubated to resume polymerization of microtubules. Cells were resolved with Laemmli sample buffer and analyzed by Western blot analysis.

**Statistical analysis** - Data represent the mean ± SD. The unpaired Student’s t-test was used for analyzing differences between experimental groups.

**RESULTS**

*The FYVE domain of ZF21 mediates membrane localization* - ZF21 is a member of a protein family characterized by the presence of a Fab1p-YOPB-Vps27p-EEA1 (FYVE) domain (35), which binds phosphatidylinositol 3-phosphate (PtdIns(3)P) within the plasma membrane, but contains no other previously described functional motifs (Fig. 1A). The FYVE domain-containing family of proteins comprises 38 members that only exhibit conserved amino acid sequences within this FYVE domain. This finding suggests that family members exhibit divergent functions, and certain of these proteins have been shown to modulate signaling processes, membrane trafficking, or cytoskeletal reorganization (36). Two isoforms of ZF21 are predicted (encoding 234 or 252 amino acids) based on the presence of two mRNA splice variants. ZF21 mRNAs are expressed nearly ubiquitously in mouse tissues (Fig. S1A). We detected the shorter variant of the ZF21 protein in all human cell lines that we analyzed by Western blot, whereas the expression of the longer variant was more variable and not always present (Fig. S1B). In the following experiments, we studied the shorter variant ZF21 (WT) and a mutant thereof (HN) containing two amino acid substitutions in the consensus sequence for binding PtdIns(3)P (Fig. 1A) (37,38).

We detected expression of the endogenous ZF21 in human fibrosarcoma-derived HT1080 cells as a 27 kDa protein by Western blot analysis using a polyclonal antibody generated against the recombinant protein (Fig. 1B). Biochemical fractionation of the cells revealed that ZF21 was enriched in the plasma membrane fraction, as was the transferrin...
receptor (TfR) used as a positive control membrane protein, although we did detect some expression of ZF21 in the cytoplasmic fraction (Fig. 1B). To examine whether the FYVE domain is crucial for membrane association, we first created fusion proteins comprising the m1Venus protein fused to the C terminus of ZF21(WT) or ZF21(HN) and stably expressed these fusions in HT1080 cells. We used a lentiviral vector to express the exogenous proteins and to knock down expression of the endogenous proteins using shRNAs. Although the m1Venus-tagged ZF21(WT) was enriched in the membrane fraction, the m1Venus-tagged ZF21(HN) protein was not (Fig. 1C), suggesting that the FYVE domain is important for membrane localization. Immunohistochemical analysis of the subcellular localization of m1Venus-tagged ZF21(WT) (WT-m1Venus) revealed a vesicle-like distribution of the signal within the cells (arrowheads), most of which co-localized with EEA-1, a marker protein for endosomes (Fig. 1D). In contrast, a similar analysis of the m1Venus-tagged FYVE domain mutant (HN-m1Venus) revealed a more diffuse cytoplasmic localization (Fig. 1E).

**ZF21 affects cell motility by regulating cell adhesion**—We next observed that constitutive knockdown of the expression of ZF21 reduced the rate of migration of MDA-MB231 (Fig. 2A) and HT1080 (Fig. 2B) cells. However, forced expression of a Myc-tagged wild type ZF21 (WT-myc) in cells in which expression of ZF21 had been knocked down restored cell migration, whereas forced expression of a Myc-tagged HN mutant (HN-myc) in such cells did not.

The cells in which expression of ZF21 had been knocked down following expression of the shRNA showed reduced motility and exhibited more adherent morphology on the culture dish compared to the control cells. This suggested to us that ZF21 might regulate cell adhesion and thereby affect cell motility. To test this, MDA-MB231 cells that had been transduced with the shZF21- or control shLacZ-expressing vectors were suspended and seeded on plastic plates that had been coated with different ECM of proteins. Adhesion of the cells to poly-L-lysine was unaffected by knockdown of ZF21 expression (Fig. 2C). However, adhesion of the cells to fibronectin, collagen I and vitronectin was enhanced significantly following shRNA-mediated knockdown of the expression of ZF21 (shZF21), compared to the cells expressing the irrelevant control shRNA (shLacZ) (Fig. 2D, 2E, and 2F, respectively). Forced expression of WT-myc mitigated the enhanced cell adhesion observed in the ZF21 knockdown cells to a level observed in the cells expressing the control shRNA. These results indicated that ZF21 protein regulates adhesion of cells to ECM proteins.

**ZF21 increases cell surface expression of integrin-β1**—Integrins are the major receptors for ECM components (4). We next made use of an antibody that blocks binding of integrin-β1 to the ECM so as to examine whether ZF21 affects integrin-mediated cell adhesion. The enhanced adhesion of the cells to collagen I,
fibronectin or vitronectin observed in the ZF21 knockdown cells was nearly fully suppressed by exposure of the cells to this blocking antibody (Fig. 3A).

We next analyzed the level of the integrin-β1 protein in whole cell lysates and in preparations of membrane proteins prepared from MDA-MB231 cells expressing either the control or ZF21 knockdown shRNA. The level of expression of integrin-β1 in whole cell lysates was unaffected by following expression of either the control or ZF21-targeted shRNA vector or in cells also expressing the WT-myc fusion protein (Fig. 3B, integrin-β1 lower panel). To detect integrin-β1 expressed on the surface of these cells, proteins on the surface were labeled with biotin, affinity-purified using streptavidin beads, and subjected to Western blot analysis (Fig. 3B, integrin-β1, upper panel). The relative level of the expression of integrin-β1 on the cell surface versus the level in the whole cell lysate was quantified following repetition of the experiments and is presented in Fig. 3C. Cell surface expression of integrin-β1 was enhanced significantly following knockdown of the expression of ZF21 and expression of myc-tagged ZF21 in the knockdown cells reduced integrin-β1 expression to a level similar to that observed in the control (shLacZ) cells (Fig. 3C). We concluded that ZF21 did not affect the cell-surface expression of other membrane proteins, because its knockdown did not affect the levels of biotin-labeled transferrin receptor (TfR) or MT1-MMP (Fig. 3B and 3C). Thus, ZF21 specifically regulates expression of integrin-β1 on the cell surface.

Knockdown of ZF21 increases the number of focal adhesions- Since integrin-β1 is an integral component of FAs, changes in the level of its membrane localization following knockdown of ZF21 might be due to effects of this knockdown on the formation and/or turnover of FAs. FAs were visualized by immunohistochemistry using antibodies against FA-resident proteins such as vinculin, talin, and FAK. MDA-MB231 cells expressing either the shLacZ or shZF21 vectors were cultured on a glass slide and subjected to immunohistochemistry using each antibody. Vinculin (Fig. 4A-D) and talin (Fig. 4E-H) showed a punctate distribution at the periphery of the cells presumably representing FAs and the areas enclosed by the boxes are enlarged in the right panels. The number of FA-like punctates appeared to be increased in the ZF21 knockdown cells compared to the control cells. FAs were further visualized using an antibody that specifically recognizes FAK that is phosphorylated at Y397. The immunohistochemical signal is presented both in a form overlaid upon the optical microscope image (Fig. 4I and 4L) and alone (Fig. 4J and 4M). This signals exhibited a punctate distribution similar to that of vinculin and talin. The areas enclosed by the boxes in Fig. 4J and Fig. 4M are enlarged in Fig. 4K and Fig. 4N, respectively. The number of FAs was counted and compared between control (shLacZ, Fig. 4O) and ZF21 knockdown (shZF21, Fig. 4P) cells. The average number of FAs per cell was significantly increased in the knockdown cells compared to the number observed in the
control cells. Thus, the effect of ZF21 on the amount of integrin-β1 expressed on the cell surface likely reflects an effect of ZF21 protein upon the formation and/or disassembly of FAs.

ZF21 regulates disassembly of FAs—ZF21 presumably affects the level of FAs by modulating either their formation or disassembly. Treatment of cells with nocodazole disrupts microtubules and stabilizes FAs by preventing their disassembly (23). Subsequent depletion of the drug restores microtubules and triggers the synchronous disassembly of FAs. To identify ZF21-dependent regulatory step in relation to the nocodazole-sensitive step, we treated MDA-MB231 cells expressing shLacZ, shZF21, or shZF21+myc-tagged ZF21 with nocodazole. Nocodazole treatment of the cells disrupted fibrous microtubule structures as confirmed by immunostaining of the cells with an antibody against microtubules (Fig. 5; B, F, and J). FAs were visualized by immunostaining with an antibody recognizing pY397-FAK and the level of expression of ZF21 did not affect the number of FAs observed in cells treated with nocodazole (Fig. 5; A, E, I for images, and M and N for quantification). These results indicate that ZF21 did not affect formation of FAs and instead suggested that it might regulate disassembly of FAs at some step after the nocodazole-dependent step. Subsequent depletion of nocodazole from the culture media and 30 min incubation induced re-formation of microtubule structures as confirmed by immunostaining (Fig. 5; D, H, and L). The number of FAs in the control cells decreased markedly as observed in Fig. 5C and Fig. 5M. In parallel with the decrease in the number of FAs, the area covered by the FAs also decreased (Fig. 5N, shLacZ). In contrast, the decrease in the number of FAs (Fig. 5, G and M) and in the area covered by the FAs (Fig. 5N) was significantly delayed in cells in which expression of ZF21 was knocked down. Thus, ZF21 appears to play a role during microtubule-induced disassembly of FAs.

ZF21 is a component of focal adhesions—Since ZF21 regulates turnover of FAs, we examined whether ZF21 is a component protein of FAs in MDA-MB231 cells expressing m1Venus-tagged ZF21. Although most ZF21 protein is associated with intracellular vesicles (Fig. 1D), at least some fraction of ZF21 showed co-localization with talin at the periphery of the cells (Fig. 6A). To capture live images of ZF21 localized to sites of adhesion of the cell to the ECM, we employed Total Internal Reflection Fluorescence (TIRF) microscopy, with which we could visualize only fluorescence-labeled proteins located in the vicinity of sites of cell attachment to the slide glass in response to localized activation with a laser beam. We made use of mCherry-tagged Zyxin as a marker for FAs (Fig. 6B, red), while ZF21 was monitored by the fluorescence of the m1Venus tag (Fig. 6B, green). We observed co-localization of the fluorescent signals associated with the tagged ZF21 and Zyxin indicating that ZF21 localizes to FAs.

FAs comprise multiple components that interact to both form the physical structure of
FAs and to transduce signals. To test whether ZF21 interacts with other FA components, we performed a pull-down assay using a glutathione S-transferase (GST)-fused ZF21 protein. FAK, paxillin, or zyxin were expressed in Cos-1 cells as m1Venus-tagged forms. We observed binding of FAK, but not paxillin or zyxin, to GST-ZF21 based on Western blot analysis of proteins pulled down with the GST-ZF21 (Fig. 6C). We were also unable to pull down other proteins, such as talin, vinculin or α-actinin by this same GST-ZF21 pull down assay (unpublished data).

To delineate FAK domains that mediate binding to ZF21, we expressed various deletion constructs of FAK in Cos-1 cells as fusions with a V5-tag. Cell lysates were prepared and subjected to a pull-down assay using GST-ZF21 as bait (Fig. 6D). An N-terminal fragment that lacks the kinase domain (KD) and FAT domain failed to bind ZF21 (Fig. 6D, FERM). In contrast, the KD of FAK alone was sufficient to bind ZF21 (Fig. 6D, KD). Similarly, we analyzed the binding of ZF21 domains to FAK using the GST-fused deletion mutants (Fig. 6E). N-terminal (Nterm) and C-terminal (Cterm) fragments of ZF21 showed a greatly reduced ability to bind FAK, whereas the FYVE domain alone was sufficient to bind FAK (Fig. 6E).

We next tested whether the interaction between FAK and ZF21 might play a role in sequestering the latter in FAs. To test this idea, MDA-MB231 cells adhering to a culture dish were disrupted under hypo-osmotic conditions (HS), which allows most intracellular components to separate from the attached membrane fraction (34). Residual membrane fragments containing FAs were solubilized and subjected to Western blot analysis (Fig. 6F). EEA1, an endosomal protein, was largely absent from the residual membrane fragments after hypo-osmotic treatment of the cells compared to the untreated cells (Fig. 6F, lanes 4). In contrast, FAK and integrin-β1 were detected in the residual membrane fragments even after hypo-osmotic treatment of the cells (Fig. 6F, lane 4). Similarly to FAK and integrin-β1, endogenous ZF21 protein was detected in the residual fraction adhering to the dishes (Fig. 6F, lane 4). However, ZF21 was not detected in the residual adherent fraction of the cells if FAK expression was knocked down using shRNA (Fig. 6F, lanes 2 and 3 versus 5 and 6). This is not because knockdown of FAK expression destroyed whole cell adhesion structures, because a comparable amount of integrin-β1 was retained in the residual fraction of the knockdown cells (Fig. 6F, lane 5 and 6) as was observed in the control cells (Fig. 6F, lane 4).

**ZF21 regulates microtubule-dependent dephosphorylation of pY397-FAK.** De-phosphorylation of FAK at pY397 is crucial for the induction of FAs disassembly (39). Therefore, we tested whether ZF21 regulates dephosphorylation of FAK. MDA-MB231 cells expressing shLacZ, shZF21, or shZF21+myc-tagged ZF21 were treated with nocodazole and the level of pY397-FAK was monitored by Western blot analysis of cell lysates prepared after removal of the drug (Fig. 7A). Quantitative measurement of the levels of
phosphorylated FAK was carried out and is presented in Fig. 7B. The levels of pY<sup>397</sup>-FAK in the control cells (shLacZ) was decreased following removal of nocodazole, and reached 20% of the original level within 15 min (Fig. 7A and 7B). In contrast, the decrease in phosphorylation of FAK was significantly delayed in the ZF21 knockdown cells (shZF21), retaining a level of phosphorylation equivalent to approximately 60% of the original level even after 30 min. The effect of knockdown of by shZF21 on FAK phosphorylation was reversed completely by expression of the myc-tagged ZF21.

However, the decrease in the level of Y<sup>397</sup>-FAK after the re-growth of microtubules may reflect preferential degradation of the phosphorylated FAK rather than dephosphorylation. To exclude this possibility, we repeated the experiments in Fig. 7A in the presence of the phosphatase inhibitor, vanadate (Fig. 7C), and we found that vanadate inhibited the decrease in the level of Y<sup>397</sup>-FAK. Thus, ZF21 regulates the microtubule-induced dephosphorylation of FAK, and protein tyrosine phosphatases such as SHP-2 and PTP-1B which are reported to induce dephosphorylation of FAK (28,29) may be involved in this process. We examined whether these phosphatases associate with ZF21 and FAK by immunoprecipitating ZF21 and we observed co-precipitation of endogenous FAK and SHP-2, but not PTP-1B, by Western blot analysis (Fig. 7D). Thus, SHP-2 likely acts on FAK during microtubule-induced disassembly of FAs.

**DISCUSSION**

In this study, we identified ZF21 to be a regulator of adhesion to and migration on the ECM. Reduced expression of ZF21 led to enhanced adhesion to the ECM by preventing disassembly of FAs, thereby suppressing cell migration. ZF21 and FAK interact either directly or indirectly through specific domains, and this interaction is presumably important for recruitment of ZF21 to sites of cell adhesion. ZF21 is required for the microtubule-induced dephosphorylation of pY<sup>397</sup>-FAK at FAs and therefore reduced expression of ZF21 prevents the disassembly of FAs.

**ZF21 localizes to the plasma membrane via two different mechanisms.** The FYVE domain of ZF21 contains a consensus sequence motif that binds PtdIns(3)P within the lipid layer of the membrane. Amino acid substitutions in the FYVE domain of ZF21 abolish its ability to associate with the plasma membrane and cytoplasmic vesicles. In addition to this association with numerous intracellular vesicles, ZF21 protein also localizes to FAs, as we observed using TIRF microscopy to visualize proteins in close proximity to cell attachment sites on the slide glasses (Fig. 6B). We used a pull-down assay to show that ZF21 can bind FAK, but not paxillin or zyxin (Fig. 6C). The FYVE domain of ZF21 is important not only for the localization to endosomes but also for the binding to FAK (Fig. 6E). Enrichment of ZF21 at sites of cell attachment was further confirmed by analyzing membrane fragments attached to the culture dish after removal of most cellular material following
exposure of the cells to hypo-osmotic conditions (Fig. 6F). Enrichment of ZF21 to these sites of attachment was dependent on the presence of FAK.

Extension of microtubules to FAs induces disassembly of their structure (23,24). Presumably, microtubules convey critical protein components that trigger disassembly of FAs. Kinesin-1, which is a motor protein that transports vesicles toward the plus end of microtubules, is reported to be necessary for the disassembly of FAs (25). Since ZF21 associates with endosomes, ZF21 and its associated proteins may act as triggers for the microtubule-induced disassembly of FAs.

Knockdown of ZF21 in cultured cells did not affect the number of FAs in cells treated with nocodazole. These results indicate that ZF21 does not affect the formation of FAs. Successive depletion of the drug from the culture media induced disassembly of FAs and consequently decreased the number of FAs in control cells. However, knockdown of ZF21 expression in the cells prevented this decrease in FAs in response to nocodazole depletion, indicating that ZF21 plays a crucial role specifically in the disassembly of FAs after the microtubule-dependent step. This activity of ZF21 explains why we observed increased cell adhesion and suppression of motility on ECM following the knock down of expression of ZF21.

Disassembly of FAs requires the function of multiple proteins. Calpain-2 is required for the proteolytic degradation of components of FAs (26,40). Calpain-4 is a regulatory subunit for Calpain-1 or 2 and fibroblasts obtained from calpain-4-null mice exhibit accumulation of unusually large FAs and the cells exhibit reduced motility (41). Dephosphorylation of FAK and association of FAK with dynamin are required to reduce the level of cell surface integrins via endocytosis (23). Fibroblasts derived from FAK-null mice formed numerous immature FAs and the turnover of FAs was abrogated (42). Reflecting the abnormal turnover of FAs, the FAK-null cells showed a decreased rate of cell migration (42).

The level of pY^{397}-FAK decreased rapidly during synchronized disassembly of FAs after release of cells from nocodazole treatment, and treatment of cells with vanadate, a protein tyrosine phosphatase inhibitor, prevented this decrease. Protein tyrosine phosphatases, such as SHP-2 or PTP-PEST, have been reported to be responsible for the dephosphorylation of pY^{397}-FAK and to regulate the turnover of FAs (27,28). ZF21 interacts with FAK and SHP-2 in MDA-MB231 cells and knockdown of ZF21 expression abrogated the dephosphorylation of pY^{397}-FAK following nocodazole treatment of cells. So ZF21 appears to regulate either the recruitment or activity of SHP-2 during microtubule-induced disassembly of FAs.

In conclusion, we report that ZF21 is a regulator of cell adhesion. Since ZF21 regulates turnover of FAs, it also regulates cell motility on ECM. Thus, ZF21 is a potential new target for the treatment of cells that exhibit elevated motility and invasion, such as malignant tumor cells.
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**FOOTNOTES**

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The abbreviations used are: EEA1, Early endosome antigen 1; FYVE, Fab1p YOPB Vps27p, EEA1; FA, Focal adhesion; MT1-MMP, Membrane type-1 matrix metalloproteinase; TfR, Transferrin receptor; ZF21, ZFYVE21

FIGURE LEGENDS

Figure 1. ZF21 protein associates with the plasma membrane via the FYVE domain. (A) Schematic representation of ZF21(WT) and ZF21(HN) proteins. (B) Localization of ZF21 in the plasma membrane fraction. HT1080 cells were homogenized and separated into membrane and cytoplasmic fractions. Endogenous ZF21 was detected by Western blot analysis using anti-ZF21 antibody. Transferrin receptor (TfR) and β-tubulin represent markers for membrane and cytoplasmic proteins, respectively. (C) Association of ZF21 with the plasma membrane via the FYVE domain. WT ZF21 and its mutant were expressed in HT1080 cells as m1-Venus tagged forms (WT-m1Venus and HN-m1Venus, respectively) and analyzed similarly. (D, E) HT1080 cells stably expressing either WT-m1Venus (D) or HN-m1Venus (E) were cultured in 4-well chamber plates. EEA1, an endosome marker, was visualized with anti-EEA1 followed by a secondary antibody conjugated to Alexa Fluor 568 (red). Arrowheads (D) indicate ZF21 co-localized with EEA1. HN-m1Venus was distributed within the cytoplasm and did not co-localize with EEA1 (E). CCD images using a 60 x objective lens (scale bar, 10 μm).

Figure 2. ZF21 regulates adhesion to the ECM and cell migration. (A, B) Expression of ZF21 was knocked down using shRNA (shZF21) in MDA-MB231 (A) or HT1080 (B) cells. To rule out off-target effects of shZF21, ZF21-myc (WT-myc) or ZF21(HN)-myc mutant (HN-myc) were expressed in the knockdown cells. The cells were subjected to a migration assay using a transwell chamber equipped with filters coated with fibronectin and FBS was used as an attractant. Error bars indicate the mean ± standard deviation (n = 3). *p<0.005 (Student’s t-test). The level of ZF21 expression was detected by Western blot analysis as presented in the right hand panels. (C-F) MDA-MB231 cells used for migration assays in Fig. 2A were plated onto 12-well plates (3 x 10^4 cells per well) coated with poly-L-Lysine (C), fibronectin (D), collagen I (E) or vitronectin (F) and incubated for 30 min. Adherent cells were then counted. Error bars indicate the mean ± standard deviation (n = 3). * p<0.01, ** p<0.005 (Student’s t-test).

Figure 3. ZF21 regulates the level of integrin-β1 expressed on the surface of the cell. (A)
ZF21 regulates cell adhesion mediated by integrin-β1. MDA-MB231 cells prepared as in Fig. 2 were seeded onto 96-well plates (2 x 10⁴ cells per well) coated with 1 μg/ml collagen I, fibronectin or vitronectin and incubated for 30 min in the presence or absence of an anti-integrin-β1 blocking antibody. The adherent cells were stained with Hoechst 33342. The fluorescence was quantified in a FLUOstar OPTIMA (BMG LABTECH). Error bars indicate the mean ± standard deviation (n = 4). * p<0.05 (Student’s t-test). (B) Detection of proteins expressed on the surface of the cells. Expression of the indicated proteins in the same set of MDA-MB231 cells was analyzed by Western blot analysis using whole cell lysate (left). Proteins on the surface of cells were labeled with biotin, affinity purified and analyzed similarly (right). (C) Quantification of the levels of protein detected in Fig. 3 B (n=3). The experiment in Fig. 3C was repeated three times and the intensity of the antibody signals was quantified. The relative amount of each protein to that observed in the control cells is presented. * p<0.05 (Student’s t-test).

Figure 4. ZF21 modulates focal adhesions. (A-N) FAs formed by MDA-MB-231 cells. Cells expressing shLacZ (A, B, E, F, I-K) or shZF21 (C, D, G, H, L-M) were seeded onto glass coverslips in 10 % FBS containing DMEM. After 48 hr, vinculin (A-D), talin (E-H) or FAK phosphorylated at Tyr397 (I-N) was visualized with specific antibodies. The boxed areas in (A), (C), (E), (G), (J) and (M) are shown at higher magnification in the panels on (B), (D), (F), (H), (K) and (N), respectively. Scale bar, 10 μm. (I) and (L) are merged images of immunostaining and images derived by nomarski interference phase microscopy. (O, P) Quantitative analysis of the number of pY397-FAK dots. Number of FAs detected as pY397-FAK-positive dots per cell was counted for 100 cells and the experiment was independently repeated three times.

Figure 5. ZF21 is required for disassembly of FAs. (A-L) Immunostaining of MDA-MB-231 cells for pY397-FAK and microtubules. The same set of MDA-MB231 cells used in Fig. 3 was seeded onto fibronectin-coated glass coverslips. After 48 hr, cells were treated with nocodazole (5 μM) for 30 min. Then the cells were washed and cultured with nocodazole-free media for 0 (A, B, E, F, I and J) or 30 min (C, D, G, H, K and L). The cells were subjected to immunostaining for pY397-FAK (green, A, C, E, G, I and K) and microtubules (red, B, D, F, H, J and L), respectively. Cells also expressed shLacZ (A-D), shZF21 (E-H) or shZF21 + WT-myc (I-L). Scale bar, 10 μm. (M and N) Quantitative analysis of FAs. Number of FAs per cell was counted for 30 cells in three separate experiments (M). Total area of FAs per cell was also measured (N). Error bars indicate the mean ± standard deviation. * p<0.01 ** p<0.005 (Student’s t-test).

Figure 6. ZF21 is a component of focal adhesions and binds FAK. (A, B) Localization of ZF21 at FAs. (A) MDA-MB231 cells stably expressing ZF21-m1Venus were cultured in 4-well chamber plates. Talin, an endogenous FA marker, was visualized with anti-talin antibody followed
by a secondary antibody conjugated to Alexa Fluor 568 (red). Boxed areas are shown at a higher magnification below. Co-localization of ZF21 and endogenous talin at FAs is indicated by the arrows. (B) MDA-MB231 cells stably expressing both ZF21-m1Venus (green) and mCherry-zyxin (focal adhesion resident protein, red) were cultured onto a 35 mm glass-based dish and cell adhesion sites were specifically visualized by Total Internal Reflection Fluorescence Microscope (TIRFM). Boxed areas are shown at a higher magnification below. Co-localization of ZF21 and zyxin at FAs is indicated by the arrows. (C) Pull-down assay of FA proteins using GST-ZF21. Whole-cell lysates of Cos-1 cells expressing either m1Venus-FAK, -paxillin or -zyxin were incubated with GST-ZF21 or GST alone. Proteins bound to GST-ZF21 or GST were analyzed by Western blotting using anti-Venus or anti-GST antibody. (D) Pull-down assay of FAK truncation mutants using GST-ZF21. The domain structures of v5-tagged FAK derivatives are shown in the upper panel: full-length (Full), FERM and kinase domain (d-FAT), FERM domain (FERM) and kinase domain (KD). The amino acid positions are indicated. Whole-cell lysates of Cos-1 cells expressing each of the v5-tagged FAK derivatives were incubated with GST-ZF21. Proteins bound to GST-ZF21 were analyzed by Western blot using anti-v5 or anti-GST antibody. (E) Pull-down assay of FAK using GST-ZF21 truncation mutant. The domain structures of GST-tagged ZF21 derivatives are shown in the upper panel: full-length (Full), N-terminal domain (Nterm), FYVE domain (FYVE) and C-terminal domain (Cterm). The amino acid positions are indicated. Whole-cell lysates of HeLa cells were incubated with GST-ZF21 derivatives or GST alone. Proteins bound to GST-ZF21 were analyzed by Western blot using anti-FAK or anti-GST antibody. (F) Detection of proteins at cell adhesion sites. To detect ZF21 at cell adhesion sites, MDA-MB231 cells were subjected to hypo-osmotic shock (+HS) and residual cell fragments adhering to the dishes were collected for Western blot analysis. The control was the lysate of cells without hypo-osmotic treatment (-HS). Expression of endogenous FAK was knocked down using two different shRNAs (shFAK#1 and shFAK#2).

Figure 7. Knockdown of ZF21 suppresses the microtubule-induced reduction in pY397-FAK. (A) Dephosphorylation of pY397-FAK in nocodazole-treated MDA-MB-231 cells. The same set of cells used in Fig. 3 was subjected to the analysis. Cell lysates were prepared at the indicated time points after deprivation of nocodazole. Total FAK and pY397-FAK were detected by Western blot analysis. (B) Experiments in Fig. 7 A were repeated three times (n=3) and the intensities of the bands were quantified. Relative levels of pY397 / total FAK to those at 0 min are presented. * p< 0.05 (Student’s t-test). (C) Phosphatase dependency of the dephosphorylation of pY397-FAK in nocodazole-treated MDA-MB-231 cells. The same set of cells used in Fig. 7A was subjected to the analysis. Cell lysates were prepared just after deprivation of nocodazole or after the further incubation for 30 min in the presence or absence of 0.5 mM Na3VO4 after deprivation of nocodazole. Total FAK and pY397-FAK were detected by Western blot analysis. (D)
Immunoprecipitation assay for endogenous ZF21 in MDA-MB231 cells. The lysates of MDA-MB231 cells were incubated with chicken anti-ZF21 IgY. The chicken anti-ZF21 IgY was precipitated with chicken IgY precipitating agarose beads (Millipore). Proteins bound to ZF21 were analyzed by Western blotting using each antibody.
Fig. 2

A

Number of migrated cells (Cells / 200x field)

ZF21
Actin

shLacZ  shZF21  shZF21 + WT-myc  shZF21 + HN-myc

MDA-MB231

B

Number of migrated cells (Cells / 200x field)

ZF21
Actin

shLacZ  shZF21  shZF21 + WT-myc  shZF21 + HN-myc

HT1080

C

Poly-L-lysine

MDA-MB231

D

Fibronectin

MDA-MB231

E

Collagen I

MDA-MB231

F

Vitronectin

MDA-MB231
**Fig. 3**

**Panel A**

Graph showing relative levels for shLacZ, shZF21, and shZF21 + WT-myc for Collagen I, Fibronectin, and Vitronectin. The x-axis represents different treatments, and the y-axis shows the relative level. Asterisks indicate statistical significance.

**Panel B**

Western blots for Integrin-β1, TfR, MT1-MMP, and ZF21 in cell surface and whole cell lysate samples for shLacZ, shZF21, and shZF21 + WT-myc.

**Panel C**

Bar graph showing surface/whole (relative level for shLacZ) for Integrin-β1, TfR, and MT1-MMP. Asterisk indicates statistical significance.
Fig. 4

Vinculin

shLacZ

shZF21

Talin

Nomarski / pY397-FAK

pY397-FAK

shLacZ

shZF21

Number of FAs / cell

% of cells

51-60
41-50
31-40
21-30
11-20
0-10

51-60
41-50
31-40
21-30
11-20
0-10
Fig. 5

| After washout | 0 min | 30 min |
|---------------|-------|--------|
|               | pY397-FAK | Microtubules | pY397-FAK | Microtubules |
| shLacZ        | ![Image A](image1) | ![Image B](image2) | ![Image C](image3) | ![Image D](image4) |
| shZF21        | ![Image E](image5) | ![Image F](image6) | ![Image G](image7) | ![Image H](image8) |
| shZF21 + WT-myc | ![Image I](image9) | ![Image J](image10) | ![Image K](image11) | ![Image L](image12) |

**M**

|      | shLacZ | shZF21 | shZF21 + WT-myc | shLacZ | shZF21 | shZF21 + WT-myc |
|------|--------|--------|-----------------|--------|--------|-----------------|
| 0 min | ![Chart](chart1) | ![Chart](chart2) | ![Chart](chart3) | ![Chart](chart4) | ![Chart](chart5) | ![Chart](chart6) |
| 30 min| ![Chart](chart7) | ![Chart](chart8) | ![Chart](chart9) | ![Chart](chart10) | ![Chart](chart11) | ![Chart](chart12) |

**N**

|      | shLacZ | shZF21 | shZF21 + WT-myc | shLacZ | shZF21 | shZF21 + WT-myc |
|------|--------|--------|-----------------|--------|--------|-----------------|
| 0 min | ![Chart](chart13) | ![Chart](chart14) | ![Chart](chart15) | ![Chart](chart16) | ![Chart](chart17) | ![Chart](chart18) |
| 30 min| ![Chart](chart19) | ![Chart](chart20) | ![Chart](chart21) | ![Chart](chart22) | ![Chart](chart23) | ![Chart](chart24) |
Fig. 6

A  MDA-MB231 / Epi

|          | ZF21-m1Venus | Talin | Merge |
|----------|--------------|-------|-------|
| Input    | -            | -     | -     |
| Pull down| -            | -     | -     |

B  MDA-MB231 / TIRFM

|          | ZF21-m1Venus | mCherry-Zyxin | Merge |
|----------|--------------|---------------|-------|
| Input    | -            | -             | -     |
| Pull down| -            | -             | -     |

C

|          | FAK         | Paxillin    | Zyxin    |
|----------|-------------|-------------|----------|
| Venus    | -           | -           | -        |
| GST      | -           | -           | -        |
| Input    | -           | -           | -        |
| Pull down| -           | -           | -        |

D

|          | v5          |
|----------|-------------|
| GST      | 1  248  415 684 914 1052 |
| FERM     | v5          |
| d-FAT    | v5          |
| Integrin-β1 | v5          |

E

|          | Full (1-234 aa) | Nterm (1-45 aa) | FYVE (41-105 aa) | Cterm (106-234 aa) |
|----------|-----------------|-----------------|-----------------|-------------------|
| GST      | -               | -               | -               | -                 |

F

|          | ZF21         | FAK           | Integrin-β1    | EEA1               |
|----------|--------------|---------------|----------------|--------------------|
| Input    | -            | -             | -              | -                  |
| Pull down| -            | -             | -              | -                  |

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