Subcellular Localization and Ser-137 Phosphorylation Regulate Tumor-Suppressive Activity of Profilin-1
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*Running Title: Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

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Background: The actin-binding protein profilin-1 is a eukaryotic protein essential for growth, with poorly understood antitumor function.

Results: Profilin-1’s antitumor activity requires nuclear localization and is inhibited by Ser-137 phosphorylation.

Conclusion: Profilin-1 has spatially-defined functions and is post-translationally regulated.

Significance: Our data support a model to reconcile profilin-1’s seemingly oppositional cellular functions and may have implications for novel anticancer therapies.

ABSTRACT

The actin-binding protein profilin-1 (Pfn1) inhibits tumor growth and yet is also required for cell proliferation and survival, an apparent paradox. We previously identified Ser-137 of Pfn1 as a phosphorylation site within the poly-L-proline (PLP)-binding pocket. Here we confirm that Ser-137 phosphorylation disrupts Pfn1 binding to its PLP-containing ligands with little effect on actin-binding. We find in mouse xenografts of breast cancer cells that mimicking Ser-137 phosphorylation abolishes cell cycle arrest and apoptotic sensitization by Pfn1 and confers a growth advantage to tumors. This indicates a previously unrecognized role of PLP-binding in Pfn1’s antitumor effects. Spatial restriction of Pfn1 to the nucleus or cytoplasm indicates that inhibition of tumor cell growth by Pfn1 requires its nuclear localization, and this activity is abolished by a phosphomimetic mutation on Ser-137. In contrast, cytoplasmic Pfn1 lacks inhibitory effects on tumor cell growth but rescues morphological and proliferative defects of PFN1 null mouse chondrocytes. These results help reconcile seemingly opposed cellular effects of Pfn1, provide new insights into the antitumor mechanism of Pfn1, and implicate Ser-137 phosphorylation as a potential therapeutic target for breast cancer.
Pfn1 (~14kDa) is an actin-binding protein involved in actin polymerization and remodeling. It was the first identified member of the profilin gene family and is ubiquitously expressed. Pfn1 facilitates filamentous actin (F-actin) polymerization by binding and exchanging nucleotides on globular actin (G-actin), and by ushering ATP-bound G-actin to the barbed ends of actin filaments (1-4). Pfn1 is required for cytokinesis (5,6), and is essential for the development and survival of many eukaryotes including mouse, fly and yeast (6-8). Besides G-actin, Pfn1 also has a separate binding site for poly-L-proline (PLP) motifs, which are present in many cellular proteins. Whereas some of the known PLP ligands of Pfn1 are actin-binding factors (e.g. Ena/VASP, N-WASP, Arp2, and mDia), others are involved in signaling, membrane trafficking, synaptic scaffolding and nuclear functions (2,4). Thus Pfn1 may participate in diverse cellular processes depending on its interaction with different PLP ligands. For instance, we have identified huntingtin (Htt), a PLP-containing protein that causes Huntington disease (HD), as a novel Pfn1 ligand. Direct interactions between Htt and Pfn1 inhibit mutant Htt aggregation, thereby implicating Pfn1 as a potential modifier of HD pathogenesis (9).

Remarkably, despite being essential for cell growth and survival, Pfn1 also has antitumor functions. Its expression is decreased in multiple types of carcinoma (breast, bladder, and pancreas) (10-14), and its ectopic re-expression inhibits the proliferation and survival of several cancer cell lines in vitro and in vivo (12,14-16). Recently, low Pfn1 expression was correlated with poor outcome of bladder and pancreatic cancer patients (13,14). However, unlike classic tumor suppressor genes, homozygous deletion and somatic mutations of the PFN1 gene are extremely rare and have not been causally linked to cancer. Though this is consistent with PFN1 being an essential gene, the mechanistic basis of the opposing functions of Pfn1 are completely unknown. On a cellular level, Pfn1’s antitumor effect has been attributed to cell cycle arrest in G1 phase and sensitization to apoptosis (17). However, at a molecular level, its antitumor function remains poorly understood.

Pfn1 is predominantly cytoplasmic. However, it is also present in the nucleus and, after binding G-actin, is exported back into the cytoplasm by Exportin-6 (18). Nuclear Pfn1 has been functionally implicated in gene expression regulation, based on its association with transcriptionally active genes (19), its presence in nuclear speckles/Cajal bodies (20,21), and its association with nuclear proteins such as the transcription factor p42POP (22) and the pre-mRNA splicing regulatory factor SMN (21). It is also required for actin-dependent RNA synthesis by respiratory syncytial virus (23). However, unlike the well-characterized role of cytoplasmic Pfn1 as an actin assembly factor, its nuclear functions are poorly understood.

Recent studies suggest that Pfn1 functions are regulated by phosphorylation. For example, phosphorylation of Pfn1 at Tyr-129 occurs in vascular endothelial cells stimulated with vascular endothelial growth factor, and this is required for efficient actin polymerization at the cell leading edges and for stimulus-induced angiogenesis (24). We originally described Pfn1 phosphorylation on Ser-137 (9,25), and found that this inhibits Pfn1 binding to the PLP-containing Htt protein, and its ability to suppress mutant Htt aggregation (9). Thus, Ser-137 phosphorylation may regulate Pfn1 by controlling its binding to PLP-containing ligands. We have now investigated how Ser-137 phosphorylation affects the tumor-inhibitory activities of Pfn1 in the context of breast cancer models. Ser-137 phosphorylation blocks Pfn1’s ability to inhibit cell cycle progression of breast cancer cells. It also inhibits the pro-apoptotic activity of Pfn1, and renders tumor cells more resistant to apoptosis in mouse xenografts. Importantly, tumor cell growth inhibition by Pfn1 requires its nuclear localization, whereas cellular proliferation depends on cytoplasmic Pfn1, and both functions are regulated by Ser-137 phosphorylation. Together, our study helps elucidate the antitumor mechanism of Pfn1 and highlights a critical regulatory effect of Ser-137 phosphorylation.

**EXPERIMENTAL PROCEDURES**
**Molecular Cloning** - Untagged and Myc-tagged Pfn1 in pcDNA3 were generated previously (9). N-terminally HA-tagged Pfn1 was PCR amplified and similarly cloned into pcDNA3. For lentiviral constructs, cDNAs encoding untagged Pfn1 were cloned into the pENTR1A vector and subsequently recombined into the pLenti-CMV/TO-Neo-DEST vector (Addgene, #17292) using LR Clonase II according to the manufacturer’s protocol (Invitrogen). Pfn1 fused with the nuclear localization (NLS) and export sequences (NES) were PCR amplified and cloned into the lentiviral pFLRu-NYFP-FH vector (26). Three tandem NLS repeats (DPKKKRRK, adapted from the Clontech pAcGFP1-Nuc) and a single NES sequence (MLVLDQKLELELDEQQ, adapted from the Clontech pCaspase3 sensor vector vector) were fused to the N-terminus of Pfn1 and cloned downstream of YFP in the pFLRu-NYFP-FH vector to generate YFP-NLS-Pfn1 and YFP-NES-Pfn1. To silence Pfn1, a 21-mer sequence (GTGTTTTGATCAACAAGAA) was cloned into the pFLRu-FH vector (26,27). A second shRNA (TACGTGAATGGGCTGACACTT) in the pLKO.1 vector was obtained from the RNAi consortium at the Genome Institute of Washington University. A control shRNA containing a non-targeting sequence (CAACAAGATGAAGAGCACAA) was cloned into the pFLRu-FH vector.

**Antibodies** - Primary antibodies used for Western blots are as follows: mouse anti-HA-tag (Covance, MMS-101P), mouse anti-Myc-tag (Santa Cruz, sc-40), mouse anti-β-actin (Santa Cruz, sc-47778; Cell Signaling, #3700), mouse anti-α-tubulin (Sigma, T9026; Cell Signaling, #3873), rabbit anti-GFP (Santa Cruz, sc-8334), mouse anti-GFP (Thermo Scientific, PIMA515256), rabbit anti-Pfn1 (Cell Signaling, #3237), rabbit anti-PARP (Cell Signaling, #9532), rabbit anti-cleaved PARP (Cell Signaling, #9541), rabbit anti-cleaved caspase-3 (Cell Signaling, #9661), rabbit anti-Rb (Cell Signaling, #9313). Primary antibodies used for immunofluorescence staining and immunohistochemistry are as follows: rabbit anti-Pfn1 (Cell Signaling, #3237), rabbit anti-cleaved caspase-3 (Cell Signaling, #9661), and mouse anti-Myc-tag (Santa Cruz, sc-40). Secondary antibodies for Western blots are alkaline phosphatase-conjugated (Sigma) and horseradish peroxidase-conjugated anti-rabbit and anti-mouse (Amersham Biosciences) antibodies. For immunofluorescence staining, Alexa Fluor® 488-conjugated goat anti-rabbit IgG was purchased from Invitrogen. For immunohistochemistry, VECTASTAIN Elite ABC kit (rabbit IgG) was purchased from Vector Laboratories.

**Cell culture** - All cell lines were purchased from ATCC with the exception of MDA-MB-231 cells stably expressing a tri-modal reporter fusion (firefly luciferase, red fluorescence protein and HSV1-sr39 truncated thymidine kinase (28). MDA-MB-231, HEK293, HEK293T, NIH3T3, and immortalized Pfn1(-/-) chondrocytes cells were all grown in DMEM supplemented with 10% fetal bovine serum and 100U/ml penicillin/streptomycin. For transient transfection of HEK293 and NIH3T3 cells, Lipofectamine 2000 was used according to manufacturer’s protocol (Invitrogen). To generate lentivirus, HEK293T (pFLRu-NYFP-FH-based) or HEK293FT (pLenti-CMV/TO-Neo-DEST-based) cells were co-transfected with vector DNA and packaging plasmids with Fugene 6 reagent based on manufacturer’s protocol (Roche). Media containing viruses were collected and clarified 2 days after transfection.

**Pull-down assays** - For PLP-binding of Pfn1, HEK293 cells grown on a 6-well dish were transfected with HA-tagged Pfn1 constructs (wt, S137A and S137D), and lysed in 200µl buffer containing 10mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Triton X-100, 1x protease inhibitor cocktail (EDTA-free Complete Mini, Roche), and 1x phosphatase inhibitor cocktail (PhosSTOP, Roche). MDA-MB-231 cells stably expressing NLS or NES-tagged Pfn1 (wt, S137A and S137D) were used. Cell lysates were centrifuged at 20,000g for 5 mins at 4°C, and supernatants were bound to 20µl control or PLP-coupled sepharose beads for 2hrs at 4°C. Beads were washed 4 times with the lysis buffer, and
boiled in SDS sample buffer for 5 mins. Samples were separated on a 12% SDS-PAGE gel, and bound HA-Pfn1, YFP-NLS-Pfn1 or YFP-NES-Pfn1 were analyzed by Western blot using an anti-HA antibody (1:2000) or anti-GFP antibody (1:2000). For actin-binding of NLS or NES-tagged Pfn1, MDA-MB-231 stable cells grown on 10cm dishes were lysed in 200µl RIPA buffer containing protease and phosphatase inhibitors and bound to ~20µl anti-GFP antibody-conjugated sepharose beads (Abcam, ab69314) for 2hrs at 4°C. Beads were washed, boiled in SDS sample buffer, and analyzed by SDS-PAGE as above. Co-immunoprecipitated actin was analyzed by Western blot.

**Subcellular fractionation** – MDA-MB-231 cells were grown on 10cm dishes and harvested by trypsinization. Cell pellets were divided into cytoplasmic and nuclear fractions using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Scientific, P178833) according to manufacturer’s protocol. Cytoplasmic and nuclear fractions were analyzed by Western blots at a 1:5 volume ratio.

**Analysis of F-actin/G-actin ratio** - HEK293 cells grown on 60mm dishes were transfected with pcDNA3 or different Myc-tagged Pfn1 constructs. Two days after transfection, cells were harvested and lysed in 200µl F-actin stabilization buffer (50mM PIPES, pH 6.9, 50mM NaCl, 5mM MgCl2, 5mM EGTA, 5% glycerol, 0.1% Triton X-100, 0.1% Nonidet P-40, 0.1% Tween-20, 0.1% β-mercaptoethanol, 1mM ATP and 1x EDTA-free Complete Mini protease inhibitor cocktail (Roche)). Lysates were centrifuged at 150,000g for 1 hour at 37°C. Supernatants containing soluble G-actin were collected, and boiled in SDS sample buffer. Pellets were resuspended in 200µl ice-cold distilled water containing 1µM cytochalasin D (Calbiochem), and incubated on ice for 1 hour to depolymerize F-actin. As a control, F-actin stabilizing Jaspalakinolide (0.1µM) (Calbiochem) was added to cultured cells for 30 mins at 37°C prior to harvesting and fractionation. After boiling in SDS sample buffer for 5 mins, same volumes of both fractions (G-actin in the supernatant and F-actin in the pellet) were separated on a 12% SDS-PAGE gel, and levels of endogenous actin and over-expressed Pfn1 were analyzed by Western blot using antibodies against β-actin (1:5000) and Myc-tag (1:5000).

**Immunocytochemistry** - Cultured cells were grown on glass coverslips coated with poly-D-lysine and fibronectin (5µg/ml). For anti-Pfn1 staining, cells were fixed in 4% paraformaldehyde for 30 mins at room temperature, washed with PBS and permeabilized in PBS/0.1% Triton X-100. After blocking in 2% bovine serum albumin (BSA) (in PBS/0.1% Triton X-100) for 1 hour, cells were incubated with primary antibody (1:100) at 4°C for overnight, washed and incubated with Alexa Fluor-conjugated secondary antibodies (1:500) for 2 hrs at room temperature. For Rhodamine-phalloidin staining, cells were fixed with 4% paraformaldehyde, washed, blocked, and incubated for 20mins in 100nM Rhodamine-phalloidin (Cytoskeleton Inc.) along with the secondary antibody. Cells were washed with PBS/0.1% Triton X-100 following secondary antibody binding, counterstained with DAPI (1µg/ml), and mounted using the ProLong Gold antifade reagent (Invitrogen). Fluorescent images were acquired using the Zeiss LSM 5 PASCAL confocal system equipped with the following lasers: 405nm, 488nm, 543nm.

**Immunohistochemistry** - Resected tumors from the xenografted mice were fixed in formalin for 24 hrs and embedded in paraffin blocks. Slides of 4µm thickness were sectioned and baked at 60°C for 30 mins prior to staining. They were deparaffinized in xylene, and hydrated in graded ethanol and deionized H2O. Tissues were subjected to antigen unmasking by microwaving in 10mM Citrate buffer, pH 6.0 for 10 mins, followed by washing in deionized H2O and PBS. They were treated with 3% hydrogen peroxide for 15 mins, followed by washing in deionized H2O and PBS. They were treated with 3% hydrogen peroxide for 15 mins, followed by washing in deionized H2O and PBS. They were treated with 3% hydrogen peroxide for 15 mins, followed by washing in deionized H2O and PBS. They were treated with 3% hydrogen peroxide for 15 mins, followed by washing in deionized H2O and PBS. They were treated with 3% hydrogen peroxide for 15 mins, followed by washing in deionized H2O and PBS.
Slides were developed using the 3,3'-diaminobenzidine (DAB) peroxidase substrate kit (Vector Labs), counterstained with hematoxylin, and mounted. Bright-field whole-slide imaging was performed using the Olympus NanoZoomer 2.0-HT system (Hamamatsu). Image acquisition and analysis were performed using the NDP.scan 2.5 and NDP.view software, respectively.

Cell Synchronization and DNA Content Analysis – For serum starvation, MDA-MB-231 cells were grown in DMEM media containing 0.1% FBS for 48 hrs, followed by release in DMEM growth media containing 10% FBS for 24 hrs. For double thymidine block, MDA-MB-231 cells were cultured in growth media containing 2mM thymidine (Sigma) for 18 hrs, followed by 9 hrs of release in thymidine-free media. They were blocked again with 2mM thymidine for 16 hrs followed by various hours of release. Cells were harvested by trypsinization, rinsed with PBS and fixed in 70% ice-cold ethanol. After storing at -20°C for overnight, cells were pelleted down for 5 mins at 200g, rehydrated in PBS, centrifuged again, and resuspended in staining solution containing 20µg/ml propidium iodide and 200µg/ml RNase A in PBS/0.1% Triton X-100. DNA content was measured using the 488nm laser of a BD Biosciences flow cytometry analyzer with a Cytek BluFL2 or BluFL3 detector. BluFL2 detector was used for cells expressing untagged Pfn1, whereas BluFL3 detector was used for those expressing YFP and YFP-tagged Pfn1 constructs (to avoid emission overlap between propidium iodide and YFP). Cell cycle analysis was performed using the Watson model of FlowJo.

In Vitro Growth Assays – For 2D culture, MDA-MB-231 stable cells were seeded at 10,000 cells per well in 12-well dishes, with triplicate wells per stable line. Cells were quantified by the MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma) viability assay 1 day and 7 days post-seeding. Briefly, they were incubated with 0.5mg/ml MTT in growth media for 3 hrs at 37°C, followed by solubilization of MTT formazan for 30 mins with 1ml isopropanol containing 4mM HCl and 0.1% Triton X-100 at room temperature. 200µl solvent was transferred to a transparent 96-well plate, and the absorbance at 590nm was read on a fluorescence plate reader (Tecan Infinite M1000) with 620nm as the reference wavelength.

Soft Agar Assay - Base layer of 0.6% agar was prepared by diluting 5% agar in DMEM/10% FBS growth media in 12-well plates (1ml/well). MDA-MB-231 cells were trypsinized, counted, and mixed with 0.4% agar (diluted in growth media) at 3000 cells/well density and plated as the top layer (1ml/well). 0.5ml growth media was added to each well after solidification. Triplicate wells were set up for each MDA-MB-231 stable line, and the same experiment was independently conducted three times. 0.005% crystal violet was used to stain colonies for 2 hours in the 37°C incubator before imaging.

Mouse Xenografts - The animal experiment was carried out in strict accordance with the guidelines recommended for care and use of laboratory animals by the National Institutes of Health. The Animal Studies Committee at Washington University (St. Louis, MO, USA) approved all animal protocols used in this study. NOD/SCID female mice were purchased from Charles River and kept under standard institutional care. They were at 5-week old at the time of surgery. MDA-MB-231 stable cells were trypsinized, washed 2 times in PBS and kept on ice prior to injection. Mice were anesthetized, incised, and injected bilaterally in their 4th mammary glands with 1 x 10^6 MDA-MB-231 cells per side. The final volume of injection was 50 µl per mammary gland. The incision was sutured, and the mice were monitored for tumor growth weekly via bioluminescence and caliper. For each stable cell line, 5 mice were injected and kept in the same cage. At 8 weeks post-injection, each mammary tumor was resected, weighed, and split. Half was subjected to rapid snap-freezing for Western blotting and the remainder was fixed overnight in 10% neutral buffered formalin (Fisher Scientific). Tumor samples were embedded in paraffin, and 4-µm sections were prepared for IHC.

In vivo bioluminescence Imaging - Bioluminescence imaging (BLI) of xenografted mice was performed using an IVIS Lumina device (Caliper). Imaging and signal quantification were
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

controlled by the acquisition and analysis software LivingImage 3.2 (Caliper) and Igor (Wavemetrics). For primary tumors, mice were imaged one day after tumor cell implantation and weekly thereafter for 8 weeks in total. For metastasis, mice were imaged once during the 9th week after primary tumor resection. Prior to imaging, mice were anesthetized with 2.5% isoflurane in a holding tank and injected intraperitoneally with D-luciferin (Biosynth) at 150mg/kg in PBS. They were awake for 5 mins, anesthetized again with 2.5% isoflurane in the holding tank, and then transferred inside the imaging chamber of the Lumina device with 1.5% isoflurane and O₂ flow through a nose cone. 15 mins after D-luciferin injection, images were acquired with a fixed series of exposure times and the same binning (1 min, 30 sec, 10 sec, 5 sec, 1 sec and 5 min; binning, low). For image analysis, an oval-shaped region of interest of the same size was used to calculate photon flux of all tumors at all time points. Data were expressed as total photon flux (photons/sec).

Statistical analysis - One way ANOVA analysis was conducted to assess the overall differences of the cell growth rates in 2D cultures and the end-point tumor weights from the mice. Multiple comparisons were conducted using the Tukey’s method following one-way ANOVA to assess the differences between individual groups. Unpaired student t-test was used to determine the difference between Pfn1-expressing tumors and those in the control group with regard to bioluminescence signals and tumor volumes. All statistical analyses were performed using GraphPad Prism 5.0 and R (version 2.15.2). P values <0.05 were considered significant.

RESULTS

Mimicking Ser-137 phosphorylation specifically inhibits Pfn1 binding to PLPs - In prior work we found that Ser-137 phosphorylation of Pfn1 blocks its binding to Htt protein, which has two PLP motifs (9). Ser-137 resides within the PLP-binding site of Pfn1 (Fig. 1A). Thus Ser-137 phosphorylation may inhibit general PLP-binding via direct steric hindrance or electrostatic repulsion conferred by the negative charge. To test this, we transiently expressed wild type, phospho-resistant (S137A), and phosphomimetic (S137D) forms of Pfn1 in HEK293 cells and measured their binding to PLP-conjugated sepharose beads. The S137D mutation abolished Pfn1’s binding to PLPs whereas the S137A mutation did not (Fig. 1B). Our prior co-immunoprecipitation experiment suggested that Ser-137 phosphorylation does not affect G-actin binding of Pfn1 at physiological salt concentration (9). When transiently overexpressed, wild type Pfn1 and its S137A or S137D mutants similarly decreased the F-actin/G-actin ratio of HEK293 cells (Fig. 1C) and redistributed F-actin (stained by phalloidin) from the cell body to the periphery of NIH3T3 cells (Fig. 1D). An actin-binding Pfn1 mutant (Y59A) (15), however, failed to redistribute F-actin in NIH3T3 cells, suggesting that such an effect of Pfn1 requires actin-binding (Fig. 1D). Though the net effects of Pfn1 on F-actin levels and localization depend on cellular context and relative abundance of other actin-regulatory factors (4), our results strongly suggest that Ser-137 phosphorylation does not directly affect Pfn1’s G-actin-binding. Rather, it appears to be a selective inhibitory event for Pfn1’s PLP-binding.

Mimicking Pfn1 phosphorylation on Ser-137 promotes tumor growth in vivo - Pfn1 has been found to suppress breast cancer cell growth in mouse xenografts. This requires actin binding (15), but the role of PLP-binding has not been clearly defined. Our ability to mimic Ser-137 phosphorylation and selectively block PLP-binding by Pfn1 allowed us to test its role in this process. We stably expressed Pfn1(wt), Pfn1(S137A), or Pfn1(S137D) in MDA-MB-231 breast carcinoma cells with the bacterial β-glucuronidase (GUS) as a negative control. Western blot showed that exogenous Pfn1 proteins were expressed at levels 2-3 fold above endogenous Pfn1 (Fig. 2A). We injected stable MDA-MB-231 cells into the mammary fat pads of immunodeficient female NOD/SCID mice, and monitored tumor burden weekly with bioluminescence imaging (utilizing stably expressed firefly luciferase (28)) and caliper measurement (feasible 4 weeks post injection). Compared to the GUS control group, mice injected
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

with cells expressing Pfn1(wt) and Pfn1(S137A) exhibited significantly slower tumor growth (Fig. 2B-D). In contrast, tumors formed by cells expressing Pfn1(S137D) grew significantly faster than all other lines (Fig. 2B-D). We confirmed these findings by end-point tumor weight measurements (Fig. 2E). Taken together, our results suggest Ser-137 phosphorylation, via disrupting PLP-binding, inhibits Pfn1 antitumor function and promotes tumor formation in vivo.

Mimicking Pfn1 phosphorylation on Ser-137 inhibits tumor cell apoptosis in vivo - Pfn1 potentiates apoptosis in multiple cancer cell lines (29-31). To test if Ser-137 phosphorylation inhibits Pfn1’s pro-apoptotic activity, we examined the xenograft tumors for cleaved caspase-3 (c-Casp-3) and cleaved PAPR (c-PARP) using western blot. Tumors expressing Pfn1(wt) and Pfn1(S137A) contained significantly higher levels of c-Casp-3 (>4-fold) and c-PARP (2-fold) compared to the Gus control. In contrast, tumors expressing Pfn1(S137D) contained lower levels of both markers compared to the Gus control (80% decrease for c-PARP and 60% decrease for c-Casp-3) (Fig. 3A-B). The changes in c-Casp-3 levels were further confirmed using immunohistochemistry (Fig. 3C). Tumors expressing Pfn1(wt) and Pfn1(S137A) both contained more c-Casp-3-positive cells than the Gus control tumor. Cells expressing Pfn1(S137A) exhibited more intense staining than those expressing Pfn1(wt), indicating that Pfn1(S137A) might be especially effective in promoting apoptosis. In contrast, we detected much fewer positive cells in Pfn1(S137D)-expressing tumors (Fig. 3C). These data suggest that in vivo Ser-137 phosphorylation not only abolishes the pro-apoptotic activity of Pfn1, but also makes tumors more resistant to apoptosis, causing the fast-growing phenotype of Pfn1(S137D)-expressing tumors. Interestingly, neither c-PARP nor c-Casp-3 was detectable by Western blot when the same stable MDA-MB-231 cells were grown in 2D cultures (Fig. 3D). When triggered with etoposide (a DNA topoisomerase II inhibitor and an apoptotic inducer), both markers appeared. However, Pfn1 expression had no effect on their levels regardless of the phosphorylation status of Ser-137 (Fig. 3D). This indicates that Pfn1 sensitizes tumor cells to apoptosis preferentially in an in vivo microenvironment in response to appropriate stimuli.

Preventing or Mimicking Ser-137 phosphorylation both inhibit cell cycle arrest by Pfn1 - Pfn1 has been reported to cause G1 arrest of the cell cycle in the MDA-MB-231 cells (17). To test if this partially contributes to the antitumor effect of Pfn1 in mouse xenografts, we examined levels of geminin, a cell cycle marker absent in G1 but present throughout S and G2/M phases. Western blot showed that tumors expressing Pfn1(wt) contained ~60% less geminin than the Gus control tumors (Fig. 4A), consistent with the Pfn1(wt)-expressing cells being arrested in G1 phase. However, tumors expressing Pfn1(S137A) or Pfn1(S137D) contained similar levels of geminin to the Gus control tumors. DNA content analysis of synchronized cells in 2D cultures confirmed this since only cells expressing Pfn1(wt) were significantly arrested in G1 phase (33% increase in cell number compared to Gus control cells) (Fig. 4B-C). This is also consistent with the cell growth rates in 2D cultures (under which condition apoptosis of these cells was minimal) as measured by the MTT assay (Fig. 4D). Thus, inhibition of cell proliferation by Pfn1, at least in the MDA-MB-231 cells, may require Ser-137 in a freely phosphorylatable and dephosphorylatable state. We conclude that Pfn1(wt) might inhibit tumor growth through effects on proliferation and apoptosis together, whereas Pfn1(S137A) functions mainly by sensitization to apoptosis.

Cell cycle arrest and growth inhibition by Pfn1 requires nuclear localization - Though predominantly a cytoplasmic protein, a fraction of Pfn1 is present in the nucleus, and has been implicated in gene transcription and pre-mRNA processing (19,20,23). Thus Pfn1 may have distinct functions depending on its subcellular localization. To test how subcellular localization influences Pfn1’s antitumor activity, we fused Pfn1 to YFP along with nuclear localization (NLS) or nuclear export sequences (NES), and stably expressed the fusion proteins (termed NLS-Pfn1 and NES-Pfn1) in MDA-MB-231 cells (Fig. 5A).
Western blot showed that NLS-Pfn1 fusions were expressed at levels approximately 25% of endogenous Pfn1, whereas NES-Pfn1 fusions were expressed at levels similar to endogenous Pfn1 (Fig. 5B).

Next, we tested if the NLS or NES-tagged Pfn1 maintains the structural integrity of the untagged Pfn1 by testing ligand-binding. Both NLS and NES-tagged Pfn1 bind actin and PLPs in the MDA-MB-231 stable cells (Fig. 5C-D). As for untagged Pfn1, neither S137A nor S137D affected the actin-binding of NLS or NES-tagged Pfn1 (Fig. 5C). However, S137D specifically abolished PLP-binding of both NLS and NES-tagged Pfn1. Of note, S137A subtly increased PLP-binding of NLS-Pfn1 and significantly increased PLP-binding of NES-Pfn1 (Fig. 5D), consistent with the idea that Ser-137 undergoes certain degree of phosphorylation in both compartments, resulting in reduced PLP binding.

We next compared the in vitro growth rates of the MDA-MB-231 stable cells in 2D cultures (Fig. 6A). NLS-Pfn1 inhibited cell growth compared to the YFP control. NES-Pfn1, however, failed to do so. DNA content analysis of synchronized cells indicated that wild type NLS-Pfn1 arrested cells in G1 phase (Fig. 6B). 3hrs after double thymidine block, NLS-Pfn1(wt) caused a 14% decrease of cells in S phase compared to YFP control cells, indicating a delay in G1/S transition. At 6hr, when most YFP control cells completed DNA synthesis, 35% more NLS-Pfn1(wt) cells were in S phase and 18% fewer were in G2/M phase, reflecting slower S phase entry and completion. By 12hr, most YFP control cells had completed mitosis and reentered G1 phase. However, 46% more NLS-Pfn1(wt) cells were still in G2/M phase and 20% fewer in G1 phase. The slower cycling rate of NLS-Pfn1(wt) cells was also reflected at 26hr post-release when 50% fewer were found in G2/M phase compared to YFP cells, presumably due to their delay in G1/S transition in two consecutive cycles. In contrast, no cell cycle arrest and proliferative inhibition were observed in MDA-MB-231 cells expressing NLS-Pfn1(S137A) or NLS-Pfn1(S137D) (Fig. 6A-B), similar to the behaviors of their untagged counterparts under the same experimental conditions (Fig. 4).

Next, we tested if nuclear Pfn1 could inhibit the growth of MDA-MB-231 cells in soft agar (Fig. 6C). Three weeks post-seeding, cells expressing NLS-Pfn1(wt) and NLS-Pfn1(S137A) both formed fewer colonies than the YFP control cells, reminiscent of the effects of untagged Pfn1(wt) and Pfn1(S137A) in mouse xenografts, which both inhibited tumor growth (Fig. 2). Cells expressing NLS-Pfn1(S137D), however, formed a similar number of colonies to the YFP control, consistent with its lack of antitumor activity. NESPfn1 did not inhibit colony formation regardless of the phosphorylation state of Ser-137, consistent with its lack of anti-growth effects in 2D culture. Together, these results suggest that profilin-1 functions in the nucleus as an antitumor protein and is subjected to regulation by Ser-137 phosphorylation.

Given the distinct activities of nuclear vs. cytoplasmic Pfn1 with regard to tumor cell growth, we tested if their relative distribution within the cell changes as a result of G1/S arrest or apoptotic activation. We treated MDA-MB-231 cells with double thymidine and aphidicolin (DNA polymerase inhibitor) to induce G1/S arrest and DNA damaging agents SN-38 (a metabolite of irinotecan) and etoposide to induce apoptosis. We then separated the cells into cytoplasmic and nuclear fractions and quantified Pfn1 level by Western blot (Fig. 6D). We observed no clear effects of G1/S arrest or DNA damage-induced apoptosis on the relative distribution of Pfn1 between nucleus and cytoplasm. We conclude that while Pfn1 can influence these specific cell events, its subcellular localization is not governed by them per se.

Cytoplasmic Pfn1 rescues cellular defects of PFN1 null chondrocytes – Pfn1 is an essential protein for cytokinesis, proliferation and survival (6-8). In line with this, we observed severe growth inhibition of MDA-MB-231 cells upon acute Pfn1 knockdown by two different shRNAs (Fig. 7A-B). Systemic knockout of PFN1 is embryonically lethal in mice (7). Cartilage-specific PFN1 deletion is tolerable, but the mice develop chondrodysplasia, and cultured PFN1 null
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

chondrocytes show abnormally rounded shape, cytokinesis defects due to weakened actin-dependent traction force generation, and slower proliferation (5). We observed that re-expression of wild type Pfn1 in PFN1 null chondrocytes increased their proliferation, and normalized their morphology (Fig. 7C-D). To determine whether the cytoplasmic or nuclear Pfn1 functions were responsible for the rescuing effects, we expressed NLS-Pfn1 or NES-Pfn1. NES-Pfn1 rescued both the morphological and growth defects of PFN1 null cells whereas NLS-Pfn1 did not (Fig. 7C-D). This indicates that cytoplasm is the site of action for Pfn1’s essential cellular functions, in contrast to its effects on tumor growth, which occur in the nucleus. NES-Pfn1(S137A) more effectively rescued the proliferation defect of PFN1 null cells than NES-Pfn1(wt) and NES-Pfn1(S137D), suggesting that cytoplasmic Pfn1 function may be negatively regulated by Ser-137 phosphorylation.

DISCUSSION

Pfn1’s opposing cellular functions are spatially defined- Pfn1 is an essential cellular protein with paradoxical antitumor activities. Its systematic deletion is lethal in several eukaryotic organisms (mice, firefly and yeast) (6-8), and its genetic knockdown inhibits the growth of cultured mammalian cells (Fig. 7A-B) (32). Yet, Pfn1 also has anti-proliferative and pro-apoptotic activities, which have been mostly recognized in the context of cancer ((12,15-17) and Fig. 2-4). Recently, Pfn1 has been found to destabilize hypoxia-inducible factor (HIF)-1α and consequently inhibit angiogenesis, indicating the multifaceted nature of its antitumor functions. (14,33). However, until now it has remained puzzling how Pfn1 can simultaneously support and inhibit proliferation within the same cellular context. For instance, we have observed that both overexpression and knockdown of Pfn1 in the MDA-MB-231 cells cause growth inhibition (Fig. 4 and 7). In this paper, we have defined the nucleus as the site of action for Pfn1’s antitumor function, and the cytoplasm as where Pfn1 supports cell proliferation. This forms the basis for a simple “spatial confinement” model which helps explain the apparent Pfn1 paradox: its opposing cellular functions are compartmentalized between the nucleus and cytoplasm (Fig. 7E).

Though mainly in the cytoplasm, Pfn1 has been detected in nuclear structures and implicated in transcription and RNA splicing (19-23). Thus, nuclear Pfn1 might function as an antitumor protein by regulating gene expression. We speculate that nuclear Pfn1 might partner with actin while executing these nuclear events. This is based on a prior study that actin-binding is essential for Pfn1 to suppress tumor growth in a breast cancer xenograft model (15). It is also consistent with the emerging role of nuclear actin as an important regulator of gene expression through association with all three RNA polymerases and chromatin remodeling complexes, as well as direct participation in transcription (34-37).

In contrast, cytoplasmic Pfn1 fails to inhibit tumor cell growth but instead rescues morphological and growth defects of PFN1 null cells. Although this finding comes as no surprise given the crucial role of F-actin integrity and dynamics, which are facilitated by Pfn1, in cell division and survival (38-42), it further supports our hypothesis that Pfn1’s opposing functions are compartmentally separated.

Functional effects of Ser-137 phosphorylation and the importance of PLP-binding – Our data that Ser-137 phosphorylation selectively disrupts Pfn1’s PLP-binding and acts as an off-switch for Pfn1’s antitumor effects suggest that finding the critical nuclear PLP-containing ligand(s) of Pfn1 will be the most straightforward way to unravel its antitumor mechanisms. Pfn1 has mostly been studied with regard to its cytoplasmic functions, and its nuclear PLP ligands have remained largely unexplored. Only two nuclear PLP proteins have been reported to interact with Pfn1 (transcriptional factor p42POP (22) and pre-mRNA splicing regulatory factor SMN (21)), neither of which has been linked to cancer. Thus, future efforts will be necessary to identify more nuclear PLP ligands of Pfn1 and to test their functional relationships.

Although Pfn1 inhibits tumor growth by influencing both proliferation and apoptosis, these two functions of Pfn1 appear to be differentially
regulated by Ser-137 phosphorylation. With regard to apoptosis, our data suggest that Ser-137 phosphorylation “toggles” Pfn1 between a pro-apoptotic and an anti-apoptotic state when tumor cells are triggered by certain \textit{in vivo} stimuli (Fig. 3). This implies that an unknown PLP-ligand is important for Pfn1’s effect on apoptosis (Fig. 7E). With regard to cell cycle arrest and proliferative inhibition by Pfn1, we find that both A/D substitutions at Ser-137 cause loss of function (Figs. 4 and 6). This could be due to complete inactivation of Pfn1. However, given clearly opposite effects of these point mutations in other aspects of Pfn1 function, we favor a model in which dynamic phosphorylation and dephosphorylation of Ser-137 may be required (Fig. 7E). The answer can only come from more definitive future studies.

Interestingly, data using PFN1 null chondrocytes suggest that Ser-137 phosphorylation also inhibits cytoplasmic Pfn1 function. This is consistent with the fact that interactions with several PLP-containing actin-regulatory factors (e.g. mDia, Ena/VASP, and N-WASP) are important for Pfn1’s optimal ability to promote actin polymerization (2,4).

Taken together, our data have uncovered the importance of nuclear localization and phosphorylation state of Ser-137 in tumor inhibition by Pfn1. They help reconcile the long-standing paradox regarding Pfn1’s opposing cellular functions and take us closer to understanding its molecular mechanisms.

REFERENCES

Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation


Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation


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FOOTNOTES
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FIGURE LEGENDS

FIGURE 1. Mimicking Ser-137 phosphorylation selectively inhibits PLP-binding of Pfn1. A, x-ray diffraction structure of human Pfn1 (middle, cyan) bound with actin (left, purple) and a 16aa PLP peptide derived from the vasodilator-stimulated phosphoprotein (right, green, in space-filling mode) (Protein Data Bank ID 2PAV). Ser-137 of Pfn1, indicated by the arrow, is located next to the PLP peptide, but far from actin. B, HEK293 cells were transfected with HA-tagged Pfn1 (wt, S137A or S137D), and lysates were bound to control or PLP-conjugated sepharose beads. Anti-HA antibody was used to detect HA-Pfn1 bound to the beads (pellet) and in the lysate (input) by Western blot. C, HEK293 cells transfected with pcDNA3 (vector) or Myc-tagged Pfn1 (wt, S137A and S137D) were fractionated and analyzed for F-actin/G-actin ratio by Western blot. Jasplakinolide was used to stabilize F-actin prior to fractionation as a control. Similar expression of all three Myc-Pfn1 proteins was confirmed by Western blotting using a Myc-tag antibody. D, NIH 3T3 cells were transfected with Pfn1 constructs (wt, S137A, S137D and Y59A) and subsequently co-stained for overexpressed Pfn1 with an anti-Pfn1 antibody, and F-actin with Rhodamine-Phalloidin. Arrowheads point to positive cells which expressed the indicated Pfn1 plasmids.

FIGURE 2. Mimicking Pfn1 phosphorylation on Ser-137 promotes tumor growth in vivo. A, western blot showing stable overexpression of exogenous Pfn1 (wt, S137A and S137D) over endogenous Pfn1 in MDA-MB-231 cells. GUS, a bacterial reporter gene encoding β-glucuronidase as the negative control. β-actin was blotted as the loading control. B-E, stable MDA-MB-231 cells were injected into the mammary fat pads of 5-week old female NOD/SCID mice (10^6 cells per side, five mice per group). Tumor cells were monitored one day after injection and weekly thereafter using bioluminescence imaging (BLI). Caliper measurements started 4 weeks after injection. B, representative BLI images showing tumor burden at four weeks post-injection. C, time course of tumor growth measured by BLI. Total photon flux (photons/sec/binning 2 imaging acquisition is shown for each data point. Data are mean ± S.E.M. of ten ROI (region of interest covering each entire tumor) values for each group (two tumors per mouse, five mice per group). D, time course of tumor volume increase measured by caliper. Data are
mean ± SEM of ten tumors within each group (two tumors per mouse, five mice per group). P values were based on two-tailed unpaired t-test (Pfn1 vs. Gus tumors). E, end-point tumor weights at resection. P values were based on one-way ANOVA and multiple comparisons using the Tukey method. *, p<0.05; **, p<0.01; ***, p<0.001, ****, p<0.0001.

FIGURE 3. Mimicking Pfn1 phosphorylation on Ser-137 inhibits tumor cell apoptosis in vivo. A-B, frozen tumors from the xenografted mice were analyzed for cleaved PARP (c-PARP) and cleaved caspase-3 (c-casp-3) by Western blot. Protein bands were quantified based on densitometry and normalized against β-actin levels. For simplicity, c-PARP and c-Casp-3 levels within the GUS tumor were arbitrarily set at 1, and those in Pfn1-expressing tumors were calculated accordingly. Tumors from two mice within each group were analyzed (which showed similar results) one of which is shown here. C, immunohistochemical analysis of formalin-fixed and paraffin-embedded tumors for c-Casp-3. Nuclei were counterstained with hematoxylin. Shown are representative images at different magnifications. D, stable MDA-MB-231 cells grown in 2D cultures in the absence or presence of 50µM etoposide (24hr) were analyzed for c-PARP and c-casp-3 levels by Western blot. Etoposide was used as a positive experimental control to induce apoptosis. β-actin was blotted as the loading control.

FIGURE 4. Preventing or mimicking Ser-137 phosphorylation both inhibits cell cycle arrest by Pfn1. A, frozen xenograft tumors were analyzed for geminin by Western blot. Protein bands were quantified based on densitometry and normalized against β-actin levels. Relative geminin levels in the Pfn1-expressing tumors over the Gus control tumors were shown. B-C, stable MDA-MB-231 cells expressing Gus or Pfn1 (wt, S137A or S137D) grown in 2D cultures were serum starved for 48hrs and subsequently release for 24hrs. They were labeled with propidium iodide, and analyzed for DNA content using flow cytometry. A representative experiment was shown in (B). Data in (C) are mean ± SEM of four independent experiments. D, stable MDA-MB-231 cells expressing Gus or Pfn1 (wt, S137A or S137D) were seeded on plastic and grown for 7 days. They were quantified by MTT labeling on Day 1 and Day 7, and the Day 7/Day 1 ratios of Pfn1-expressing cells were normalized against that of Gus control cells to give rise to their relative proliferation rates (Y-axis: positive % values for increase and negative % values for decrease). Data are mean ± SEM of five independent experiments. P values for C and D were based on one-way ANOVA analysis and multiple comparisons using the Tukey method. *, p<0.05; **, p<0.01; ***, p<0.001.

FIGURE 5. Pfn1 fused to nuclear localization sequence (NLS) or nuclear export sequence (NES) maintains actin and PLP-binding abilities. A, subcellular localization of YFP-NLS-Pfn1 and YFP-NES-Pfn1 in stable MDA-MB-231 cells. B, relative expression levels of YFP-NLS-Pfn1 and YFP-NES-Pfn1 in stable MDA-MB-231 cells by Western blot. C, MDA-MB-231 stable cell lysates expressing YFP, NLS-Pfn1 or NES-Pfn1 lacking or containing S137 mutations were immunoprecipitated with an anti-GFP antibody, and analyzed for actin-binding by Western blot. D, same cell lysates from (C) were mixed with PLP-conjugated beads and analyzed for the binding of NLS or NES-tagged Pfn1 by Western blotting using a GFP antibody.

FIGURE 6. Cell cycle arrest and growth inhibition by Pfn1 requires nuclear localization. A, stable MDA-MB-231 cells were seeded in 2D cultures, and quantified by MTT assay at Day 1 and Day 7. Relative growth rates of Pfn1 fusion-expressing cells over those expressing YFP control were calculated as described in Figure 4D. Data are mean ± SEM of three separate experiments. P values were based on one-way ANOVA analysis and multiple comparisons using the Tukey method. ***, p<0.001; ****, p<0.0001. B, DNA content analysis of stable MDA-MB-231 cells blocked by double thymidine and released for various lengths of time (3, 6, 12 and 26hrs). Arrowheads indicated obvious changes in cell cycle profiles caused by NLS-Pfn1(wt) expression, which have been confirmed by three independent experiments. C, stable MDA-MB-231 cells were grown in 0.4% soft agar for 3 weeks, and stained with
crystal violet. Shown are representative images from one of three independent experiments which generated data of similar trends. 

D, parental MDA-MB-231 cells were subjected to double thymidine (2mM) block or treated with aphidicolin (5µg/ml), SN-38 (200nM), and etoposide (50µM) for 24hours. Cells were fractionated and Pfn1 in the cytoplasmic and nuclear fractions were analyzed by Western blot. Tubulin and Rb were used as cytoplasmic and nuclear markers.

FIGURE 7. Cytoplasmic Pfn1 rescues cellular defects of PFN1 null chondrocytes. A, Equal numbers of MDA-MB-231 cells were infected with lentiviral shRNAs containing a scrambled sequence (control) or two different Pfn1-targeting sequences (#1 and #2). Cells images were taken at day 7 post-infection. B, Western blot analysis of shRNA infected MDA-MB-231 cells for total Pfn1. Actin was blotted as the loading control. C, Pfn1(-/-) chondrocytes infected with YFP, YFP-NLS-Pfn1 or YFP-NES-Pfn1 viruses were seeded in 2D cultures and quantified by MTT assay at Day 1 and Day 4. Relative growth rates of Pfn1 infected cells over YFP infected control cells were calculated as described in Figure 5C. Data are mean ± SEM of three separate experiments. P values were based on one-way ANOVA analysis and multiple comparisons using the Tukey’s method. *, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001. D, morphological differences of virus infected Pfn1(-/-) chondrocytes grown on plastic. E, A Spatial Confinement model to explain Pfn1’s opposing cellular functions and the role of Ser-137 phosphorylation. Pfn1 acts as a tumor suppressor in the nucleus by augmenting apoptosis and blocking cell cycle, whereas it acts in the cytoplasm to support proliferation and survival by facilitating actin polymerization and remodeling. In the nucleus, the pro-apoptotic activity of Pfn1 depends on interaction with actin and an unknown PLP protein. Ser-137 phosphorylation blocks PLP binding, and converts Pfn1 from an apoptotic enhancer to an inhibitor. As for cell cycle inhibition, dynamic association/dissociation of Pfn1 with a different PLP protein might be required (dashed line). In the cytoplasm, Pfn1 interacts with actin and multiple PLP-containing proteins (most are actin-regulatory proteins such as mDia, Ena/VASP, and N-WASP) to support proliferation and survival. Ser-137 phosphorylation inhibits these activities by disrupting Pfn1 binding to PLP.
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

Figure 1

A

B

C

D
Figure 3

Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

A

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C

Gus | Pfn1(wt) | Pfn1(S137A) | Pfn1(S137D) |
2.5x

H/E, cleaved Casp3

20x
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

Figure 4

Panel A: Western blot analysis showing the normalized level of Geminin and β-actin in different samples.

Panel B: Flow cytometry analysis showing the FL2-A distribution for different samples.

Panel C: Graph showing the cell cycle distribution in different conditions.

Panel D: Relative proliferation vs. control graph with statistical significance indicated.

* p=0.003
** One-way ANOVA
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

Figure 5

A

YFP-NLS-Pfn1

wt  S137A  S137D

YFP-NES-Pfn1

wt  S137A  S137D

B

Pfn1 fusions

Pfn1 (endogenous)

C

YFP

NLS-Pfn1(wt)

NLS-Pfn1(S137A)

NLS-Pfn1(S137D)

 actin

Pfn1 fusions

YFP

IP: anti-GFP

WB: anti-actin and anti-GFP

D

Pfn1 fusions

Input

PLP pull-down

Pfn1 fusions

WB: anti-GFP
Inhibition of Nuclear Pfn1 by Ser-137 Phosphorylation

Figure 6
Figure 7

**A**

control shRNA  |  Pfn1 shRNA #1  |  Pfn1 shRNA #2

**B**

Control shRNA  |  Pfn1 shRNA #1  |  Pfn1 shRNA #2

- Pfn1
- actin

**C**

*PFN1(−/−)* chondrocytes

One-way ANOVA: p < 0.0001

Relative Proliferation vs. Control

- Pfn1 (wt)
- NLS-Pfn1 (S137A)
- NES-Pfn1 (S137D)
- NLS-Pfn1 (wt)
- NES-Pfn1 (S137A)
- NES-Pfn1 (S137D)

**D**

YFP-NLS-Pfn1

- wt
- S137A
- S137D

- Pfn1(wt)

**E**

Diagram showing the relationship between Pfn1, nuclear and cytoplasmic processes, including:

- Tumor inhibition
- Cell Cycle
- Apoptosis
- Proliferation
- Survival
Subcellular Localization and Ser-137 Phosphorylation Regulate Tumor-Suppressive Activity of Profilin-1

Marc I. Diamond, Shirong Cai, Aaron Boudreau, Clifton J. Carey, Jr., Nicholas Lyle, Rohit V. Pappu, S. Joshua Swamidass, Mina Bissell, Helen Piwnica-Worms and Jieya Shao

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