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Neurofibromin physically interacts with the N-terminal domain of Focal Adhesion Kinase

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Abstract

The NF1 gene that is altered in patients with type 1 neurofibromatosis encodes a neurofibromin (NF1) protein that functions as a tumor suppressor. In this report, we show for the first time physical interaction between neurofibromin and focal adhesion kinase (FAK), the protein that localizes at focal adhesions. We show that neurofibromin associates with the N-terminal domain of FAK, and that the C-terminal domain of neurofibromin directly interacts with FAK. Confocal microscopy demonstrates co-localization of NF1 and FAK in the cytoplasm, peri-nuclear and nuclear regions inside the cells. *Nf1*^{+/+} MEF cells expressed less cell growth during serum deprivation conditions, and adhered less on collagen and fibronectin-treated plates than *Nf1*^{-/-} MEF cells, associated with changes in actin and FAK staining. In addition, *Nf1*^{+/+} MEF cells detached more significantly than *Nf1*^{-/-} MEF cells by disruption of FAK signaling with the dominant-negative inhibitor of FAK, C-terminal domain of FAK, FAK-CD. Thus, the results demonstrate the novel interaction of neurofibromin and FAK and suggest their involvement in cell adhesion, cell growth, and other cellular events and pathways.

Keywords

Focal Adhesion Kinase; Neurofibromin; Neurofibromatosis; Protein interaction; Focal adhesions

Introduction

Neurofibromatosis 1 (NF1) is an autosomal dominant disorder predominantly characterized by nerve sheath tumors called neurofibromas [1]. The tumors are caused by loss of function of both alleles of the *NF1* gene (germline mutation and somatic mutation) in Schwann cells and can transform into malignant peripheral nerve sheath tumors (MPNSTs). Mutations in the *NF1* gene also predisposes to sarcomas, astrocytomas, juvenile myeloid leukemia and learning disabilities.

Neurofibromin is the 2,818 amino acid protein (est. 280 kDa) encoded by the *NF1* gene on chromosome 17q11.2 [2]. The gene spans 280kb of genomic DNA and contains 60 exons [3]. Neurofibromin is ubiquitously expressed at low levels with highest expression levels

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seen in neural crest derived tissues including neurons, Schwann cells and oligodendrocytes [4]. It is a tumor suppressor with a functional GTPase-activating protein domain that negatively regulates Ras [5]. Recent reports suggest that neurofibromin may play a critical role in other signaling pathways and cellular functions. For example, inactivating mutations of the *NF1* gene have been reported in non-NF1 related tumors without impaired regulation of ras [6–10]. Recently, neurofibromin was found to regulate mTor, the target of rapamycin [11], and to confer sensitivity to apoptosis through ras-independent pathways [12]. Furthermore, a nuclear localization sequence was recently identified in exon 43 of the protein [13] and was involved in neurofibromin shuttling between cytoplasm and nucleus, suggesting a possible novel role for the protein in the nucleus. The Akt signaling pathway is reportedly regulated by neurofibromin, in association with caveolin-1 [14]. Neurofibromin has also been suggested to associate with cytoskeletal and focal adhesion proteins such as actin filaments and microtubules [15,16] and to regulate cell adhesion, motility and cytoskeletal reorganization [14,16,17]. In addition, *Nf1* null mouse embryonic fibroblast (MEF) cells are highly motile and display a disorganized morphology with increased actin stress fibers [14], and knockdown of neurofibromin in HeLa and HT1080 cells with *NF1* siRNA increased paxillin localization at focal adhesions, leading to increased focal adhesion formation [16].

One of the main proteins localized at focal adhesions (contact sites of cells with extracellular matrix) is the Focal Adhesion Kinase (FAK), a 125kDa non-receptor tyrosine kinase that is encoded by the *PTK2* gene located on chromosome 8q24 in humans and chromosome 15 in mice [18,19]. FAK is a critical regulator of many cellular processes including adhesion, proliferation, cell spreading, motility and survival [20–24]. FAK has been shown to be overexpressed in many types of tumors [25–29] and to be up-regulated in the early stages of tumorigenesis [25,30]. The FAK protein contains an N-terminal domain with a primary autophosphorylation site (Tyr-397) that directly interacts with the Src homology 2 domain [31] and PI-3 kinase [22]; a central catalytic domain with two major sites of phosphorylation (Tyr-576/577) and a C-terminal domain containing two proline-rich segments and a focal adhesion-targeting subdomain that binds paxillin, talin, and other proteins [22,32]. The N-terminal domain of FAK associates with the epidermal growth factor receptor and platelet-derived growth factor receptor [33,34], with cytoplasmic tails of integrins [35] and with death receptor complex-binding protein, RIP [36]. The N-terminal domain of FAK also complexes with a protein inhibitor of activated STAT1 (PIAS1) [37], which causes FAK sumoylation and increases its autophosphorylation activity, suggesting a novel FAK role in signaling between the plasma membrane and the nucleus [37]. Recently, the N-terminus of FAK was shown to interact directly with the tumor suppressor p53 to enhance its degradation in the nucleus [38,39].

We have shown that an analogous C-terminal FAK fragment construct, called FRNK or FAK-CD, can regulate FAK function in a dominant negative way by decreasing FAK phosphorylation and displacing FAK from the focal adhesion [40,41]. Overexpression of FAK-CD in cancer cells leads to cell rounding, detachment, reduction of invasion, and apoptosis [32,42–45]. FAK has been shown to suppress both transformation-associated apoptosis [41] and anoikis (detachment-induced apoptosis) of epithelial cells [46], promoting survival in cells subjected to apoptotic signals. In addition, constitutively active forms of FAK prevented anoikis and stimulated transformation of epithelial cells, resulting in anchorage-independent growth and tumor formation in nude mice [46].

Because neurofibromin has been suggested to be important in many FAK signaling pathways such as focal adhesion formation, cell motility, and cytoskeletal reorganization [16], and several other reports suggesting indirect link of FAK and NF1 [14],[47], we analyzed whether FAK and neurofibromin can interact directly. First, we show that

neurofibromin is expressed in many types of cancer cells. Then we show that neurofibromin interacts directly with FAK and mapped this interaction to the N-terminal domain of FAK by immunoprecipitation and pull-down assays. We show that FAK and neurofibromin co-localize by confocal microscopy. In addition, *Nf1*^{+/+} MEF cells expressed significantly less cell growth in low-serum conditions and adhered significantly less than *Nf1*^{-/-} on collagen I and fibronectin-treated plates, associated with changes of actin and FAK cellular distribution. We show that the dominant-negative FAK protein, adenoviral FAK-CD caused more detachment of the *Nf1*^{+/+} MEF cells than in *Nf1*^{-/-} MEF cells. Thus, this is the first report demonstrating direct interaction between FAK and neurofibromin that can play an important role in cellular signaling.

Materials and Methods

Cells and cell culture

The normal mouse embryonic fibroblast *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells and normal Schwann cells (pn97.4) and tumor Schwann cells (SNF02.2, SNF94.3, SNF96.2) were kindly provided by Dr. Margaret Wallace at the University of Florida, Gainesville and were maintained in DMEM plus 10% fetal bovine serum and 1 µg/ml penicillin/streptomycin. The SV40 Immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells were kindly provided by Dr. R. Stein, at the Tel Aviv University in Israel and were maintained in DMEM plus 10% fetal bovine serum and 1 µg/ml penicillin/streptomycin. BT474 (human breast cancer) cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 1 µg/ml penicillin/streptomycin and 5 µg/ml insulin.

DNA Constructs

The N-terminal, Central/Kinase and C-terminal domains of FAK were generated by PCR with the gene-specific primers (available upon request). All sequences were analyzed by automatic sequencing in both directions (University of Florida Sequencing Facility). For generation of GST-fusion proteins, the C-terminal fragment of neurofibromin (aa 2205 – 2785) and FAK fragments (N-terminal (aa 1–423), kinase domain (aa 416–676), and C-terminal domain (aa 677–1052) were cloned into the pGEX-4T1 GST vector (*Amersham Biosciences*).

Antibodies and Reagents

Monoclonal anti-FAK (4.47) antibody specific to the N-terminal domain of FAK was obtained from *Upstate Biotechnology, Inc.*, and *BD Transduction Laboratories*, respectively. Polyclonal antibody for the C-terminal domain of FAK, C-20, was obtained from Santa Cruz Biotechnology Inc. (*Santa Cruz, CA*). Polyclonal antibody for the C-terminal domain of neurofibromin was obtained from *Santa Cruz Biotechnology Inc.* Monoclonal antibody for the C-terminal domain of neurofibromin was obtained from *Abnova*. HA-tagged antibody was obtained from *Cell Signaling Inc.* The recombinant purified GPF expressed in baculoviral expression vector system was kindly provided by Dr. S. Haskill. The recombinant purified FAK expressed in baculoviral expression vector system was generated and described in [38]. Polyclonal antibody for neurofibromin (SC-67) was obtained from *Santa Cruz*. Monoclonal antibody for neurofibromin C-terminus was obtained from *Abnova* (H00004763-Q01).

Immunoprecipitation and Western Blotting

Cells were washed twice with cold 1x PBS and lysed on ice for 30 min in a buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% Triton-X, 0.5% NaDOC, 0.1% SDS, 5 mM EDTA, 50 mM NaF, 1 mM NaVO₃, 10% glycerol, and protease inhibitors: 10

μg/ml leupeptin, 10 μg/ml phenyl methyl sulfonyl fluoride, 1 μg/ml aprotinin. The lysates were cleared by centrifugation at 10,000 rpm for 30 min at 4 °C. Protein concentrations were determined using Bio-Rad kit. The cleared lysates with equivalent amount of protein were incubated with 5 μl of primary antibody for 1 h at 4 °C and 25 μl of protein A/G-agarose beads (*Oncogene Research Products Inc.*). The precipitates were washed with the lysis buffer three times and re-suspended in 30 μl of 2x Laemmli buffer. The boiled samples were loaded on Ready SDS-10% polyacrylamide gels (Bio-Rad) and used for Western blot analysis with the protein-specific antibody. The blots were stripped in a stripping solution (Bio-Rad) at 37 °C for 15 min and then re-probed with the primary antibody for checking equal loading of proteins. Immunoblots were developed with chemiluminescence Renaissance reagent (*Perkin Elmer Life Sciences*).

Immunostaining

Cells were fixed on coverslips with 4% paraformaldehyde in 1x PBS for 10 min and permeabilized with 0.2% Triton X-100 for 5 min on ice. Cells were blocked with 25% normal goat serum in 1x PBS for 30 min, washed in 1x PBS, and incubated with primary antibody diluted 1:200 in 25% goat serum in 1x PBS for 1h. Cells were washed in 1x PBS three times and a FITC or Rhodamine-conjugated secondary antibody (1:400 dilution in 25% goat serum) was applied to the coverslip for 1h. After washing three times with 1x PBS, cells were stained with Hoechst 33342 for detecting nuclei. After mounting, cells were examined under a fluorescent Zeiss microscope or Leica microscope.

Expression of Recombinant GST Fusion Proteins

GST fusion proteins containing different domains of FAK (GST-FAK-NT, FAK-Kinase, and FAK-CD) and C-terminal domain of neurofibromin (GST-NF1-CD) were engineered by PCR. The fusion proteins were expressed in *Escherichia Coli* bacteria by incubation with 0.2mM isopropyl β-D-galactopyranoside for 6 h at 37 °C. The bacteria were lysed by sonication, and the fusion proteins were purified with glutathione-agarose beads as described in [38]. Isolated GST-FAK-NT, -Kinase domain and FAK-CD were confirmed by Western blot analysis with FAK-domain specific antibodies [38].

Pull-down Assay

For the pull-down binding assay, cell lysates (0.5mg) or purified recombinant baculoviral protein (0.1 μg) were precleared with 10 μg of GST immobilized on glutathione-agarose beads by rocking for 1 h at 4 °C. The washed pre-cleared lysates were incubated with 2–4 μg of GST fusion protein immobilized on the glutathione-agarose beads for 1 h at 4 °C and then washed three times with lysis buffer. Equal amounts of GST fusion proteins (2μg) were used for the binding assay. Bound proteins were boiled in 2x Laemmli buffer and analyzed by Western blotting.

Phage display assay

Phage display assay was performed with the purified N-terminal domain of FAK protein (1–422 aa) using the 7 amino acid phage library consisting of $>2 \times 10^9$ sequences (New England Biolabs) according to New England Biolabs protocol.

Apoptosis Assay

Cells treated with Ad FAK-CD were collected and fixed in 3.7% formaldehyde in 1x PBS solution. Detection of apoptosis was done with Hoechst 33342 method as previously described. Briefly, Hoechst 33342 in 1x PBS solution (1μg/ml) was added to the fixed cells for 10 min, and cells were washed twice with 1x PBS, spread evenly on the slide, and examined under a fluorescent microscope. The percentage of apoptotic cells was calculated

on a blind basis as a ratio of apoptotic cells with fragmented nuclei to the total number of non-apoptotic cells with large non-fragmented, non-condensed nuclei in three independent experiments in several fields with the fluorescent microscope. More than 300 cells were analyzed for each experimental sample.

Adhesion Assay

96 well plates were coated with 1–10 µg /ml of collagen I, fibronectin or poly-L-lysine at 37°C for 1.5 hr and 1% BSA as control. The plate wells were washed with 1xPBS and blocked with 1% BSA at 37°C for 30 min. The blocking solution was aspirated and 20,000 cells in the medium with 0.1% BSA were added per well and incubated in a CO₂ incubator at 37°C for 1 hour. The plate wells were then washed multiple times with 1% BSA in 1xPBS, fixed in 3.7% paraformaldehyde for 10 min at room temperature (R.T.), stained with 2% crystal violet (in 2% ethanol) for 10 minutes at R.T., washed 4 times with 1xPBS and allowed to dry for 30 minutes. The absorbed dye was dissolved with 2% SDS, and absorbance read at 562 nm on an Elisa plate reader to determine adhesion.

Confocal Microscopy

Immunostained slides were optically sectioned under a confocal laser inverted Leica microscope, as described [38]. Images were captured per channel and merged with Leica CS software.

Adenovirus Transfection

The adenoviral constructs Ad LacZ, Ad GFP, and Ad FAK-CD are previously described in [41]. Cells were plated and allowed to grow for 24 h and then infected with Ad LacZ (or Ad GFP) or Ad FAK-CD for various time points at a concentration of 300ffu/cell. Expression of HA-tagged FAK-CD was detected by Western blotting with anti-HA monoclonal antibody.

Cell Growth Assay

MEF cells were starved for 24h, split in to a density of 5×10^4 cells/6-cm plate in triplicate, and then stimulated with 0.5% and 2.5% serum. After indicated time, cells were trypsinized and counted.

Statistical Analysis

Statistical analysis was performed with Student's t-test. A p-value of $p < 0.05$ was considered significant.

RESULTS

Neurofibromin and FAK are both expressed in normal and most tumor cells

We analyzed neurofibromin and FAK expression levels in many cancer cell lines including Malignant Peripheral Nerve Sheath Tumors (MPNST) (SNF02.2, SNF94.3, SNF96.2), neuroblastoma (BE, AS), melanoma (A375, C8161), pancreatic tumors (Panc-1, Panc-2.03, Panc-L3.6p1, Mia Paca), breast cancer (BT474, MCF7, MDA-MB 231), normal human Schwann cells (pn97.4), Human Embryonic Kidney (HEK 293) cells, human cervical cancer (HeLa) cells, and isogenic normal mouse embryonic fibroblast (MEF) FAK^{+/+} and FAK^{-/-} and *Nf1*^{+/+} and *Nf1*^{-/-} cells (Figures 1 A and B). The results showed variable expression of both neurofibromin and FAK in many cancer types. Importantly, the results demonstrate that both FAK and neurofibromin are expressed in many types of tumor cells.

FAK and neurofibromin proteins interact *in vivo*

To determine whether FAK and neurofibromin (NF1) interact *in vivo*, we performed co-immunoprecipitation of FAK and NF1 in mouse embryonic fibroblasts (MEF) cells (Figure 2A). First we immunoprecipitated endogenous FAK protein in *Nf1*^{+/+} (Figure 2A, left panels) and *Nf1*^{-/-} MEF cells (Figure 2A, middle panels) and performed Western blot with neurofibromin antibody. Immunoprecipitation of FAK detected neurofibromin in complex with FAK in *Nf1*^{+/+} cells (Figure 2A, left panels), while it did not detect in control *Nf1*^{-/-} MEF cells (Figure 2A, middle panels). In addition, we immunoprecipitated neurofibromin and detected FAK in complex with the neurofibromin in *Nf1*^{+/+} MEF cells (Figure 2A, right panels). FAK and neurofibromin binding was not detected in control immunoprecipitations without antibody (Figure 2A). Thus, neurofibromin and FAK interact in the *Nf1*^{+/+} mouse embryonic fibroblast (MEF) cells *in vivo*.

To demonstrate interaction of FAK and neurofibromin in human cell lines, we performed co-immunoprecipitation of FAK and neurofibromin in human BT474 breast cancer cells (Figure 2B, left panels) and in human normal Schwann cells (pn97.4) and Schwann-derived MPNST cells (SNF02.2, SNF94.3) (Figure 2B, right panels). Immunoprecipitation of FAK detected neurofibromin in complex with FAK in all human cell lines (Figure 2B). Thus, endogenous FAK and neurofibromin interact *in vivo*.

Neurofibromin interacts with the N-terminal domain of FAK *in Vitro*

To identify which domain of FAK interacts with neurofibromin, we performed pull-down assay with the N-terminal, FAK-NT; Central, FAK kinase domain, and C-terminal, FAK-CD domains of FAK shown on (Figure 3A) using cell extracts of normal (pn97.4) Schwann cells (Figure 3B, upper panel). The Coomassie stained GST-FAK domain proteins are shown on (Figure 3B, lower panel). Neurofibromin was pulled down in a complex with the FAK-NT protein (Figure 3B), while it did not interact with control GST protein or the kinase, GST-FAK-Kinase and C-terminal domains of FAK, GST-FAK-CD proteins (Figure 3B). These results demonstrate that neurofibromin interacts with the N-terminal domain of FAK.

C-terminal domain of neurofibromin directly interacts with FAK *in Vitro*

Since we demonstrated interaction between the N-terminal domain of FAK (FAK-NT) and neurofibromin, we performed phage display assay with the purified FAK-NT protein using a degenerate 7 amino acid phage library consisting of $>2 \times 10^9$ peptide sequences (New England Biolabs). We identified a 7 amino acid peptide HSTPPKM with 71% homology (5 out of 7 a.a.; homologous amino acids are underlined) to neurofibromin peptide inside the nuclear localization sequence of neurofibromin (*NF1*-NLS) KRQEMESGITTPPKMMRR (2534 – 2550 a.a.) in the C-terminal domain of neurofibromin (not shown) that additionally supports our data on interaction of neurofibromin with the N-terminal domain of FAK. The C-terminal domain of neurofibromin has been suggested to have additional functions independent from the NF1 GAP-domain [1,13,47,48].

To determine that C-terminal domain of neurofibromin directly interacts with FAK, we generated the GST-NF1, containing the C-terminal part of neurofibromin, called GST-NF1-CD. To determine whether NF1 directly interacts with FAK, we expressed a GST-NF1-CD protein (C-terminal aa 2205 – 2785) (Figure 4A), containing a fragment of the C-terminal domain of neurofibromin and performed a pull-down assay with the baculoviral purified FAK (Figure 4B, left panel). As a control for non-specific binding, we used baculoviral purified GFP (Figure 4B, left panel). For pull-down-assay, we used GST-NF1-CD protein; GST-Paxillin (a known binding partner of FAK) used as a positive control for binding with FAK and GST protein, used as a negative control for non-specific binding (Figure 4B, right

panels). All GST proteins were analyzed by Coomassie staining and confirmed by Western blotting with specific antibodies (Figure 4B, right panels). The pull-down assay showed binding of FAK with GST-NF1-CD protein and GST-Paxillin protein, but not with GST protein (Figure 4C, left panel). To check specificity of GST-NF1 binding with FAK, we used purified recombinant baculoviral GFP protein in the same pull-down assay with GST, GST-NF1-CD and GST-paxillin (Figure 4C, right panel). We did not detect binding of control GFP protein (Figure 4C, right panel) with the GST-NF1-CD protein compared with the FAK protein (Figure 4C, left panel), indicating the specificity of FAK and neurofibromin binding. Thus, the C-terminal domain of neurofibromin directly interacts with FAK *in vitro*.

To determine whether GST-NF1-CD binds to endogenous FAK protein and show specificity of the binding we performed the same pull-down assay using FAK^{+/+} and FAK^{-/-} MEF cell extracts (Figure 4D). The pull-down assay showed binding of GST-NF1-CD with endogenous FAK protein in FAK^{+/+} MEF cells, but not in FAK^{-/-} MEF cells (Figure 4D). Thus, C-terminal domain of NF1 specifically interacts with endogenous FAK.

Neurofibromin co-localizes with FAK

To determine co-localization and intracellular distribution of FAK and neurofibromin, we performed immunostaining of normal human Schwann cells (pn97.4) *Nf1*^{+/+} and MPNST Schwann cells (SNF96.2) lacking neurofibromin expression, *Nf1*^{-/-} (Figure 5A). Neurofibromin was detected mostly in the cytoplasm, while FAK was detected in the cytoplasm and at the focal adhesions (Figure 5A). FAK co-localized with neurofibromin in the cytoplasmic, peri-nuclear and nuclear regions (Figure 5A, lower panel) in pn97.4, *Nf1*^{+/+} cells. There were no NF1 expression and co-localization in control SNF96.2, *Nf1*^{-/-} cells (Figure 5A, upper panel). To precisely determine co-localization, we performed confocal microscopy analysis of BT474 breast cancer cells (Figure 5B, upper panel) and pn97.4 (Figure 5B, lower panel). Confocal microscopy detected co-localization in the cytoplasmic, peri-nuclear and nuclear regions in both cell types (Figure 5B). These results demonstrate co-localization between neurofibromin and FAK proteins.

Nf1^{+/+} MEF cells express significantly less cell growth during serum deprivation and adhere significantly less than *Nf1*^{-/-} MEF cells on Collagen-I and fibronectin

To characterize and compare *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells, we examined SV40 immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cell growth during nutrient deprivation. Cells were serum starved for 24h and then stimulated with 0.5% or 2.5% serum for 24, 48 and 72h (Figure 6A). There was significantly less growth in *Nf1*^{+/+} MEF cells stimulated with 0.5% serum (Figure 6A, upper left panel) or 2.5% serum (Figure 6A, upper right panel) compared to *Nf1*^{-/-} MEF cells stimulated with the same serum (Figure 6A). The same results were obtained with normal primary MEF fibroblasts (not shown). Thus, neurofibromin significantly decreases cell growth in *Nf1*^{+/+} cells compared to *Nf1*^{-/-} MEF cells during serum deprivation.

Because neurofibromin has been suggested to regulate focal adhesion formation [16], we examined the adhesion of SV40 immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF on Collagen-I, Laminin, Fibronectin and control poly-L-lysine or BSA (Figure 6A, lower left panel). *Nf1*^{+/+} MEF cells adhered significantly less than *Nf1*^{-/-} MEF cells on Collagen-I and Fibronectin (Figure 6A, lower left panel). There was no significant difference on Laminin or control poly-L-lysine-treated plates (Figure 6A, lower left panel). The same results were obtained on normal primary MEF fibroblasts, where NF^{+/+} cells adhered significantly less than NF^{-/-} cells (Figure 6A, lower right panel). There were also striking differences in the morphology and FAK staining in *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells on collagen (Figure 6B, upper panels). FAK was localized at the leading edge of elongated *Nf1*^{-/-} cells with more

spread morphology compared to *Nf1*^{+/+} MEF cells (Figure 6B, upper panels). In addition, *Nf1*^{+/+} MEF cells grown on Collagen-I displayed fewer actin stress fibers (Figure 6B, lower panels) than *Nf1*^{-/-} MEF cells. Thus, *Nf1*^{+/+} MEF adhered less on Collagen I and Fibronectin compared to *Nf1*^{-/-} cells, and expressed changes in FAK and actin distribution, suggesting the role of neurofibromin in cell adhesion and cytoskeletal and focal adhesion functions.

Dominant negative FAK inhibitor, FAK-CD causes significantly more detachment in *Nf1*^{+/+} MEF cells than in *Nf1*^{-/-} MEF cells

To inhibit FAK signaling in both cell lines, we used our model system in which the transduction of an adenoviral C-terminal domain of FAK, FAK-CD (dominant-negative FAK inhibitor) into the cells causes inhibition of FAK and displacement of FAK from the focal adhesions [41]. We applied this model to test the effect of FAK attenuation on isogenic *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells (Figure 7). We studied focal adhesion formation of cells treated with Ad-FAK-CD for 16h and 24h (Figure 7A, upper panels). FAK was displaced by FAK-CD from focal adhesions in *Nf1*^{+/+} MEF cells at 16h and at 24h, but was not displaced from the focal adhesions in *Nf1*^{-/-} MEF cells (Figure 7A, lower panels). No displacement of FAK from focal adhesion was detected with control Ad-LacZ at 24 h in both *Nf1*^{-/-} and *Nf1*^{+/+} MEF cells (Figure 7A, right panels). Next, we looked at cell detachment and apoptosis induced by treatment with Ad-FAK-CD and control Ad-LacZ in *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells (Figure 7B). *Nf1*^{+/+} MEF cells showed significantly higher levels of detachment (78.9%) compared with *Nf1*^{-/-} MEF cells (17.7%) after 24h treatment with Ad-FAK-CD (Figure 7B). No detachment was observed with control Ad-LacZ in both cell lines (Figure 7B). There was not significant level of Ad-FAK-CD-induced apoptosis in both *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells (Figure 7B). FAK-CD caused complete degradation of FAK in both *Nf1*^{+/+} MEF cells, while FAK was present in *Nf1*^{-/-} MEF cells (Figure 7C). Control Ad-LacZ did not cause degradation of FAK compared with Ad-FAK-CD in *Nf1*^{+/+} cells (Figure 7C). Both cell lines showed efficient expression of HA-tagged FAK-CD, detected by Western blotting with HA-antibody (Figure 7C). Increased detachment and cell rounding was observed in *Nf1*^{+/+} cells compared with *Nf1*^{-/-} MEF cells in response to Ad-FAK-CD are shown in Figure 7D. Thus, inhibition of FAK with FAKCD caused more detachment of *Nf1*^{+/+} MEF cells, suggesting that FAK inhibits neurofibromin functions critical for cytoskeletal and focal adhesion formation.

Discussion

In this study, we demonstrated that neurofibromin is expressed in many types of cancers in addition to the cancer cells of nervous system. We demonstrated direct interaction between neurofibromin and Focal Adhesion Kinase. Using immunoprecipitation, we demonstrated association of FAK with neurofibromin in *Nf1*^{+/+} MEF cells and BT474 cancer cells. We also detected binding of neurofibromin and FAK in normal and tumor Schwann cells. We showed co-localization of neurofibromin and FAK in cytoplasm, perinuclear and nuclear regions. We demonstrated direct interaction between FAK and the C-terminal domain of neurofibromin *in vitro*. We also demonstrated interaction between neurofibromin and the N-terminal domain of FAK (FAK-NT). Interestingly, we performed phage display assay with the purified FAK-NT protein using a degenerate 7 amino acid phage library consisting of >2 × 10⁹ sequences (New England Biolabs) and identified a 7 amino acid peptide with 71% (5 out of 7 a.a.) homology to the nuclear localization sequence of neurofibromin that additionally supports our data on interaction of neurofibromin with the N-terminal domain of FAK (data not shown). Interestingly, the neurofibromin nuclear localization sequence (NF1-NLS) that is located in exon 43 (2534 – 2550 a.a.) is present inside the cloned C-terminal fragment of neurofibromin that was used to demonstrate direct interaction with full

length FAK. This neurofibromin fragment also contains a tyrosine kinase recognition site (2549 – 2556a.a.) next to the *NF1*-NLS, suggesting its potential role in intracellular functions and protein shuttling.

We showed that *Nf1*^{+/+} MEF cells have decreased cell growth under nutrient deprivation and that these cells adhered significantly less than *Nf1*^{-/-} MEF cells on Collagen-I and Fibronectin. We also demonstrated that the dominant negative FAK protein, FAK-CD caused significant detachment and degradation of FAK in *Nf1*^{+/+} MEF cells but not in *Nf1*^{+/+} MEF cells. These results suggest that neurofibromin is important in cell adhesion and in modulating FAK survival signaling in the cell.

The N-terminal domain of FAK and FERM domain promote tumor cell proliferation and survival by binding to and enhancing p53 degradation in the nucleus [39]. It is interesting that FAK was only degraded by FAK-CD in the presence of neurofibromin in *Nf1*^{+/+} MEF cells, and that neurofibromin co-localizes with FAK around the nucleus. This may be a mechanism by which neurofibromin modulates FAK shuttling in to the nucleus and recruitment to the focal adhesions. By binding with FAK in the peri-nuclear region, neurofibromin may inhibit FAK transportation into the nucleus or recruitment to the focal adhesions or it may enhance FAK degradation via the ubiquitin-proteasome pathway that was recently reported to regulate neurofibromin levels in the cell [49].

The data demonstrating that *Nf1*^{+/+} cells adhered less to Collagen I and Fibronectin I than *Nf1*^{-/-} cells and cells expressed differences in actin and FAK staining suggest that NF1 can regulate cytoskeletal reorganization consistent with data of Ozawa et al [16]. The authors demonstrated that neurofibromin affected cell adhesion and motility by reorganizing actin dynamics. NF1-depleted cells had increased focal adhesion, paxillin (focal adhesion protein and binding partner of FAK) staining and increased actin stress fiber formation compared with control cells [16]. We also showed that *Nf1*^{-/-} cells had morphology of motile elongated cells with FAK distribution on the leading edge consistent with data on role of neurofibromin in motility [16]. The data show that NF1 and FAK complex can play role in cytoskeletal and focal adhesion functions.

Inhibition of FAK with FAK-CD demonstrated significantly higher detachment of *Nf1*^{+/+} cells, suggesting that FAK can sequester NF1 from growth inhibitory functioning, and once FAK is inhibited by FAK-CD, NF1 is released for cellular functioning. When FAK-CD expressed in the *Nf1*^{+/+} cells, FAK was displaced from focal adhesions and completely degraded, thus preventing formation of complex with NF1 protein and causing increased cell detachment. The data are consistent with our proposed model of FAK sequestering of pro-apoptotic proteins, such as RIP [36] and p53 [38], [50], suggesting a novel survival cellular mechanism in carcinogenesis, where FAK inhibits pro-apoptotic proteins in addition to integrating pro-survival signaling from integrins and growth factor receptors [50]. Interestingly, that *Nf1*^{-/-} cells expressed increased resistance to down-regulation of FAK in response to Ad-FAK-CD that is important for developing FAK-targeted therapy.

Thus, in this report we have demonstrated an expression of neurofibromin in many types of cancer cells. We have shown a novel interaction of neurofibromin and FAK *in vitro* and *in vivo*, their co-localization and distribution inside the cells, and demonstrate that inhibition of FAK has differential effect on adhesion in *Nf1*^{-/-} and *Nf1*^{+/+} cells, suggesting that FAK and NF1 interaction can play significant role in the intracellular signaling and carcinogenesis.

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Abbreviations

FAK	Focal Adhesion Kinase
FAK-CD	C-terminal domain of FAK
NF1	Neurofibromin
MEF	mouse embryonic fibroblasts
R.T.	room temperature

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Figure 1. Expression of neurofibromin and FAK in different cancer cell lines

Western blotting analysis of neurofibromin and FAK expression was performed in normal Schwann cells (pn97.4), Malignant Peripheral Nerve Sheath Tumors (MPNST) (SNF02.2, SNF94.3, SNF96.2), breast cancer cells BT474, human cervical cancer HELA cells, mouse embryonic fibroblast (MEF) cells (FAK^{+/+} and FAK^{-/-}, Nf1^{+/+} and Nf1^{-/-} (upper panel), and in pancreatic tumors (Panc-1, Mia Paca, Panc-L3.6p1, Panc-2.03), HEK 293 cells, neuroblastoma (BE, AS), melanoma (A375, C8161) and breast cancer MCF7, MDA-MB231 cells (lower panel).

A **B** **Figure 2.**

Figure 2A. FAK and neurofibromin interact in *Nf1*^{+/+} cells *in vivo*. Left and middle panels: Immunoprecipitation of FAK was performed with anti-FAK C20 antibody followed by Western blotting with neurofibromin antibody in *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells.

Immunoprecipitation without antibody was used as a control (-Ab). Then membrane was stripped and Western blotting with FAK antibody was performed. FAK and NF1 interact in *Nf1*^{+/+}, but not in *Nf1*^{-/-} MEF cells. Right panels: **Immunoprecipitation with NF1 antibody detects interaction with FAK in *Nf1*^{+/+} MEF cells.** Immunoprecipitation was performed with NF1 antibody, and then Western blotting with FAK antibody. Then membrane was stripped and Western blotting was performed with NF1 antibody. FAK and NF1 interacted *in vivo* in NF^{+/+} cells.

Figure 2B. FAK and neurofibromin interact in breast cancer BT474 cells and in Human Schwann cells *in vivo* Immunoprecipitation with FAK antibody detected interaction with NF1 in BT474 (left panels) and in normal pn97.4 and Malignant Peripheral Nerve Sheath Tumors (MPNST) SNF02.2 and SNF94.3 Schwann cells (right panels). Immunoprecipitation was performed as above.

Figure 3A

Pull-down with normal Schwann cells

Figure 3. NF1 interacts with the N-terminal domain of FAK

A, The scheme of the GST FAK fusion proteins used for pull-down assay to demonstrate interaction of NF1 with FAK: GST-N-terminal FAK-NT; Central, FAK-Kinase and C-terminal FAK-CD domains. The GST proteins are marked by arrows. Upper panel: The three FAK domains (FAK-NT, FAK-Kinase and FAK-CD) were used in a pull-down assay with normal Schwann cells to identify which domain of FAK interacts with neurofibromin. After pull-down assay, Western blotting with the NF1 antibody was performed. The NF1 antibody detected of NF1 protein. Two bands of NF1 protein in normal Schwann cells were detected at high resolution gel electrophoresis conditions. Pull-down assay demonstrates interaction of NF1 with the N-terminal domain, but not with the Kinase or C-terminal

domains of FAK. Lower panel: Coomassie staining of GST-FAK domain proteins. The GST-FAK protein domains were analyzed by Coomassie staining (lower panel, GST-proteins are marked by asterisks).

Fig.4a

Fig 4B

Figure 4. Direct interaction between FAK and neurofibromin *in vitro*

A) The scheme of neurofibromin domains structure. The scheme demonstrates the location of the recombinant C-terminal domain of NF1, GST-NF1-CD fusion protein (2205-2785 aa) inside neurofibromin protein, containing the CSRD (cysteine/serine Rich domain), TBD (tubulin binding domain), GRD (GAP-Related domain), Sec14 (sec14 domain) and NF1-NLS (nuclear localization signal). **B) C-terminal domain of NF1 and FAK proteins directly interact *in vitro*.** Left panel: Baculoviral purified FAK and GFP proteins were used for pull-down assay. Coomassie staining shows the fusion proteins that were confirmed by Western blotting with GFP and FAK antibodies [38]. Right panel: Coomassie staining and Western blotting analysis of GST, GST-NF1-CD and GST-paxillin

fusion proteins (Materials and Methods) used in pull-down assay. Asterisks mark GST-fusion proteins. Western blotting with NF1 antibody and paxillin antibody confirmed NF1 and paxillin proteins. **C). Pull-down with baculoviral FAK demonstrated direct binding with the C-terminal domain of NF1 protein.** Left panel: Pull-down assay was performed with baculoviral FAK protein and GST, GST-NF1-CD and GST-paxillin. C-terminal domain of NF1 directly binds FAK protein. No binding of FAK with the negative control GST protein is detected, while it was detected with the positive control GST-paxillin protein. Right panel: **Pull-down with baculoviral GFP protein did not detect binding with FAK.** The same pull-down as with baculoviral FAK protein was performed with baculoviral GFP protein. No binding of GST-NF1-CD was detected with GFP protein. **D) Interaction of neurofibromin with FAK in FAK^{+/+}, but not in FAK^{-/-} MEF cells.** Pull-down assay of GST-NF1-CD protein and demonstrates interaction between GST-NF1-CD with endogenous FAK protein in FAK^{+/+}, but not in FAK^{-/-} MEF cell extracts.

Figure 5A

A

Figure 5B

B

Figure 5. Co-localization of neurofibromin with FAK

A) Immunocytochemical and fluorescence microscopy analysis of FAK and neurofibromin localization in normal Schwann cells (pn97.4) (lower panel) and tumor Schwann cells (SNF96.2) lacking neurofibromin expression) (upper panel). FAK and neurofibromin co-localized in the cytoplasmic, peri-nuclear and nuclear regions in pn97.4 (*Nf1*^{+/+}) cells. No co-localization is detected in SNF96.2 (*Nf1*^{-/-}) cells. B) Confocal microscopy detects interaction of NF1 and FAK in breast cancer BT474 cells and pn97.4 Schwann cells. FAK and neurofibromin co-localization was observed in the cytoplasmic, peri-nuclear and nuclear regions in both cell lines.

Fig.6A

Fig 6B

Figure 6.

Figure 6 A. *Nf1*^{+/+} MEF cells expressed significantly less cell growth at serum deprivation conditions and adhered less on Collagen I and Fibronectin compared with *Nf1*^{-/-} MEF cells. SV40 immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells were counted on hemocytometer at 0, 24, 48 and 72 hours after plating in medium with 0.5% FBS (upper left panel) or 2.5% FBS (upper right panel). *Nf1*^{+/+} MEF cells demonstrated significantly less cell growth at 0.5% and 2.5% serum versus *Nf1*^{-/-} MEF cells. Lower left panel: **Adhesion of SV40 immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells on BSA, Collagen I, Laminin, Fibronectin, and Poly-L lysine.** SV40 immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells were plated on BSA, Collagen I, Fibronectin, Laminin and Poly-L-lysine-coated plates, and adhesion assay was performed as described in Materials and Methods. Immortalized *Nf1*^{+/+} cells had significantly less adhesion on Collagen I and Fibronectin than *Nf1*^{-/-} cells. Lower right panel: **Adhesion of normal primary *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells on BSA, Collagen I, Laminin, Fibronectin, and Poly-L lysine** The same experiment as with immortalized MEF cells (above) was performed with primary MEF fibroblasts. Primary *Nf1*^{+/+} cells had significantly less adhesion than *Nf1*^{-/-} cells.

Figure 6B. FAK and actin staining in *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells. Upper panel. FAK immunostaining was performed on immortalized *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells, plated on Collagen I. *Nf1*^{-/-} and *Nf1*^{+/+} MEF cells had different cell morphology and FAK localization. *Nf1*^{-/-} cells had more elongated and more spread morphology and increased FAK staining at the leading edge of cells compared with less spread *Nf1*^{+/+} cells. Lower panel. ***Nf1*^{-/-} MEF cells expressed increased actin staining compared with *Nf1*^{+/+} cells.** Actin staining was performed with FITC-conjugated Phalloidin. Actin staining was increased in *Nf1*^{-/-} cells compared with *Nf1*^{+/+} MEF cells.

Figure 7.

A. Dominant-negative FAK inhibitor, Ad-FAK-CD causes displacement of FAK from focal adhesions in *Nf1*^{+/+} cells compared to *Nf1*^{-/-} cells. *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells were infected with Ad FAK-CD (300 FFU/cell) and Ad LacZ for 16 h and 24 h and cells were immunostained with anti-FAK 4.47 antibody to analyze focal adhesion formation (white arrowheads). Ad FAK-CD displaced FAK from focal adhesions in *Nf1*^{+/+} MEF cells but not in *Nf1*^{-/-} MEF cells at 16h and at 24h. No displacement of FAK from focal adhesion was detected with control Ad-LacZ at 16 h (not shown) and at 24 h. Focal adhesion marked by white arrowheads.

B. Detachment and apoptosis analysis of *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells treated for 24 h with Ad FAK-CD. Detachment and apoptosis were detected, as described in Materials and Methods. *Nf1*^{+/+} cells had significantly more detachment in response to Ad-FAK-CD than *Nf1*^{-/-} MEF cells. **C) FAK is displaced from focal adhesions by FAK-CD in *Nf1*^{+/+} cells compared with *Nf1*^{-/-} cells.** Western blotting analysis of Ad-LacZ and Ad-FAK-CD-treated *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells at 24 h. Western blotting with NF1, FAK, HA-tag and beta-Actin antibodies were performed. Western blotting with NF1 antibody shows NF1 expression in *Nf1*^{+/+} and absence in *Nf1*^{-/-} cells. Ad-FAK-CD caused degradation of FAK in *Nf1*^{+/+} cells, while Ad-LacZ did not cause this effect on FAK. NF1^{-/-} cells did not have FAK degradation in response to Ad-FAK-CD. Western blotting with HA-tag antibody demonstrates that both cell lines effectively express HA-tagged FAK-CD. Western blotting with beta-actin show equal protein loading. **D) Images under microscope of *Nf1*^{+/+} and *Nf1*^{-/-} MEF cells treated with Ad LacZ and Ad FAK-CD for 24 h.** Ad-FAK-CD caused more detachment and cell rounding in *Nf1*^{+/+} cells compared with *Nf1*^{-/-} MEF cells.