

**FAAP, a novel murine protein, is involved in cell adhesion through regulating vinculin-paxillin association**

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**TABLE OF CONTENTS**

1. Abstract
2. Introduction
3. Materials and methods
  - 3.1. Cell culture and antibodies
  - 3.2. Construction of expression plasmid
  - 3.3. Transfection and immunofluorescence assay
  - 3.4. Immunoprecipitation and Western blotting
  - 3.5. Cell spreading assays
  - 3.6. Quantification of cell adhesion dynamics
4. Results
  - 4.1. FAAP restrains HeLa cells from spreading
  - 4.2. FAAP inhibits cell adhesion translocation, but not cell adhesion incorporation
  - 4.3. FAAP mediates interaction of adhesion molecules
  - 4.4. FAAP induces dephosphorylation of ERK
5. Discussion
6. Acknowledgement
7. References

**1. ABSTRACT**

Focal adhesion associated protein (FAAP), encoded by murine *D10Wsu52e* gene, is highly homologous to human HSPC117, which interacts with vinculin and talin. HeLa cells transfected with FAAP exhibited normal adhesion incorporation but showed impaired cell spreading, and restrained focal adhesion translocation. Moreover, FAAP facilitated vinculin-paxillin association, decreased interaction of paxillin-focal adhesion kinase and inhibited the phosphorylation of extracellular signal-regulated kinase. Together, these results suggest that FAAP, by virtue of modulating interaction of adhesion molecules, regulates cell adhesion dynamics.

**2. INTRODUCTION**

*Mus musculus D10Wsu52e* gene encodes a novel, conserved and ubiquitous protein of 505 amino acids, which we named as focal adhesion associated protein (FAAP) (GenBank accession No. AY424364) (1). This gene is a member of function-unknown protein family, UPF0027, which exist in various species. The overall amino acid sequence of FAAP shares more than 99% identity to *H. sapiens* hypothetical protein HSPC117 (<http://www.ncbi.nlm.nih.gov>). FAAP protein and its homologues are ubiquitously expressed in various cells of mouse and other mammals (1). Interestingly, this protein is highly expressed in many tumor cell lines (1, 2). HSPC117

## FAAP associates with focal adhesion protein

is found in a large RNase-sensitive granule as a binding partner of conventional kinesin (KIF5) that transports RNA in dendrites (3). HSPC117 is also present in protein complexes, such as the TNF- $\alpha$  mRNA 3' AU-rich element binding complexes (4) and osmotic response element binding protein KIAA0827 (5). This protein is also found in spreading initiation center (SIC), a structure that appears only during the early stages of cell spreading. HSPC117 can bind to talin and vinculin, but not paxillin (6). Together these features suggest that HSPC117 and its homologues can regulate cell adhesion by an unknown mechanism.

Cell adhesion is involved in special sub-cellular structures. These structures contain a large number of cytoskeletal proteins, kinases, phosphatases and many enzymes, including vinculin, paxillin, focal adhesion kinase (FAK), Src-family kinases and mitogen-activated protein kinase (MAPK). These molecules are regulated through changes in expression and/or activation level (7, 8, 9, 10). Vinculin is a cytoskeletal protein which signals through involving in activation of multiple proteins, which include p130 Crk-associated substrate (Cas), Crk-II adaptor proteins (11, 12), extracellular signal-regulated kinase (ERK) (13, 14), phosphatidylinositol-3 kinase (PI-3K) (7, 15), paxillin (16) and FAK (17). It regulates adhesion structures by modulating the recruitment of signaling molecules to special subcellular locations (18, 19). Paxillin interacts with many cytoskeletal proteins and signaling molecules, such as vinculin, tubulin, Src, Csk and FAK. These interactions facilitate an effective cooperation of these partners (16). FAK activity affects multiple cellular processes through regulating many protein complexes and the effects can be inhibited by negative regulator of FAK activity, FAK-related nonkinase (FRNK) (20). COOH-terminus of FAK contains a focal adhesion targeting (FAT) region that binds paxillin (21, 22). The interaction of FAK and paxillin is crucial for many signaling cascades associated with many vital cellular processes (16, 23). The FAT region and vinculin tail share the same conserved binding sites on paxillin, which encompasses repeats of leucine-rich sequences named LD motifs (16, 24, 25). And the vinculin has been reported to control cell survival and motility through decreasing paxillin-FAK interaction and subsequent ERK1/2 activation (26).

Given the similarities between FAAP and HSPC117, FAAP is likely to have a functional role in cell adhesion. The data provided here are consistent with the idea that FAAP participates in cell adhesion dynamics by regulating the interaction of the adhesion molecules, vinculin, paxillin and FAK.

## 3. MATERIALS AND METHODS

### 3.1. Cell culture and antibodies

The HeLa cells were cultured in high glucose DMEM (Life Technologies Inc., USA), supplemented with 10% (v/v) fetal bovine serum (Gibco, USA) in an incubator at 37°C with 5% (v/v) CO<sub>2</sub> and 95% humidity. The antibodies against hemagglutinin (HA) tag, FAK, paxillin and vinculin were from Santa Cruz (USA). The anti-actin

antibody was purchased from Sigma (USA). The anti-HSPC117 antibody was kindly provided by Professor Hirokawa from the University of Tokyo (3). All conjugated secondary antibodies were from Vector Laboratories (USA).

### 3.2. Construction of expression plasmid

The sense and antisense strands of HA tag sequence were annealed and the double stranded oligonucleotide was cloned into pIRES-EGFP vector (Clontech, USA) at KpnI/SmaI (New England Biolabs, Fermentas, USA) sites to construct HA-pIRES-EGFP. The FAAP coding sequence was amplified by PCR from mouse brain RT-PCR products and FAAP-pIRES-EGFP construct was made from the PCR products by cloning them at EcoRI/KpnI sites into HA-pIRES-EGFP vector. The sequence of all constructs was confirmed by sequencing.

### 3.3. Transfection and immunofluorescence assay

The plasmid DNA was prepared using the Wizard Plus SV Minipreps DNA Purification System (Promega, USA). Cells were plated onto dishes (Costar, USA) at required density and were transfected using the LipofectAMINE2000 Reagent (Life Technologies Inc., USA) according to the manufacturer's instructions.

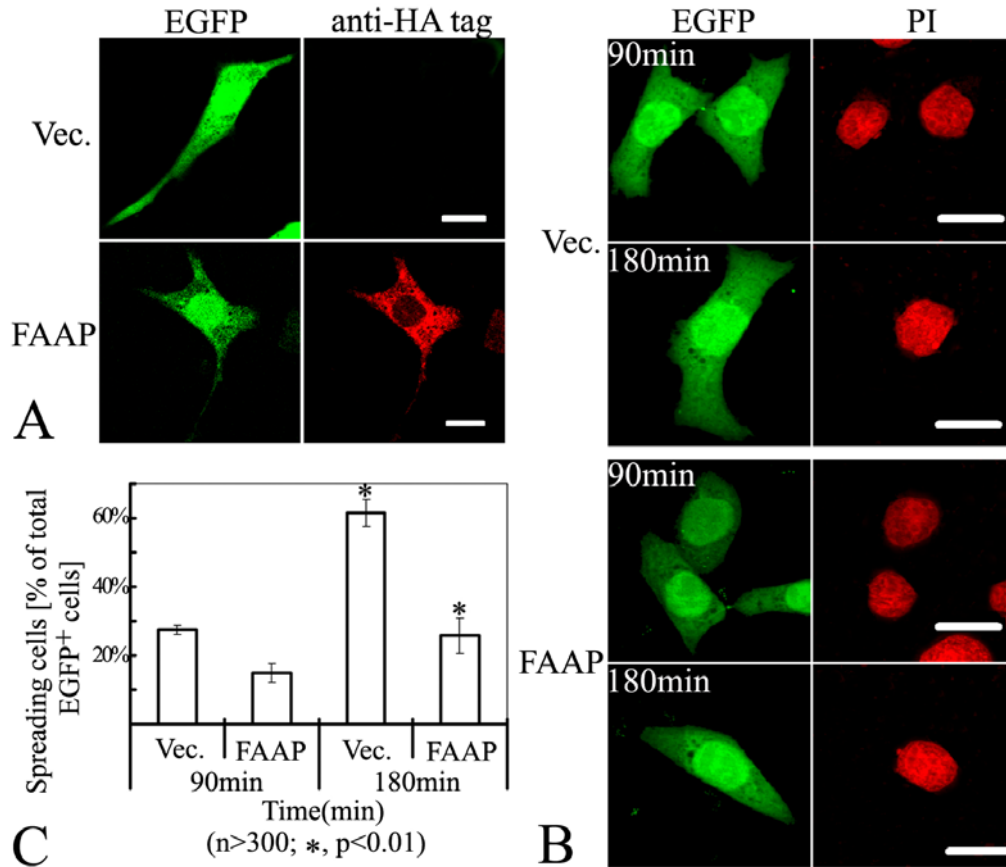
Immunofluorescence assays were performed as described previously (6). HeLa cells were transfected with FAAP-pIRES-EGFP or pIRES-EGFP plasmids and expressed Enhanced Green Fluorescence Protein (FAAP expressed EGFP<sup>+</sup> cells or control EGFP<sup>+</sup> cells) for 24 hr. Cell monolayers were washed in PBS, fixed in 4% paraformaldehyde and 0.05% Triton X-100 at room temperature for 10 min and washed with PBS for three times. These were followed by a blocking with 5% BSA at 37 °C for 30 min, incubation with a primary antibody at 37°C for 1 hr and washing with PBS for three times. Then the cells were incubated with fluorescence dyes conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA) at 37 °C for 1 hr and also washed with PBS for three times. Data were acquired by confocal laser scanning microscopy (Leica, Germany).

### 3.4. Immunoprecipitation and Western blotting

The immunoprecipitations were performed as described previously (6). About 2×10<sup>6</sup> HeLa cells expressing FAAP-IRES-EGFP and the control cells were cultured for 24 hr or 48 hr, washed in PBS and lysed in an ice-cold lysis buffer (20mM HEPES, pH 7.4, 5mM MgCl<sub>2</sub>, 100mM KCl, 5 mM NaCl, 1% Triton X-100), supplemented with 1×protease inhibitor cocktail (Sigma, USA) for 10 min on ice. 400 $\mu$ l of the lysate was cleaned by centrifugation for 10 min at 12 000 xg and added to 4  $\mu$ g of primary antibody or non-immune IgG as a control. The mixtures were incubated at 4 °C for 2 hr and followed by incubation with 30  $\mu$ l a protein-A agrose slurry (Sigma, USA) at 4 °C for 4 hr under gentle agitation. Beads were washed three times with lysis buffer. Immunoprecipitates were separated in SDS-PAGE gel and analyzed by Western blotting.

After electrophoresis, proteins were transferred to

## FAAP associates with focal adhesion protein



**Figure 1.** Cell spreading assays. HeLa cells expressing pIRES-EGFP (Vec.) or FAAP-pIRES-EGFP (FAAP) were used in cell spreading assays. (A) Immunofluorescence images of transfected cells stained with anti-HA antibodies showing FAAP in the cytoplasm (Bar: 20 $\mu$ m). (B) Cell spreading assays of the transfected cells recorded at 90 and 180 min. Cells were labeled with EGFP. Nuclei were stained with propidium iodide (Bar: 20  $\mu$ m). (C) The percentages of spread cells to the total number cells were determined in three independent experiments (mean  $\pm$  s.e.; n>300).

Immobilon membrane (Amersham Biosciences, USA). After blocking with 5% BSA at 37 °C for 30 min, the Western blots were incubated with a primary antibody at 4 °C for 12 hr and washed with TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for five times. Then the Western blots were incubated with horseradish peroxidase-conjugated IgG at 37 °C for 1 hr and also washed with TBST for five times. Bands were developed by an enhanced chemiluminescence (ECL; Pierce, Rockford, IL), according to the manufacturer's specifications.

### 3.5. Cell spreading assays

The cell spreading assays were performed as described (27). Briefly, FAAP expressed EGFP<sup>+</sup> cells or control EGFP<sup>+</sup> cells were cultured for 24 hr after transfection and they were detached from culture substrate by 1 mM EDTA and plated onto cover slips coated with 1  $\mu$ g/ml fibronectin in 6-well plates at a density 5.0 $\times$ 10<sup>5</sup> cells/well. FAAP expressed EGFP<sup>+</sup> cells or control EGFP<sup>+</sup> cells were imaged with confocal microscopy and analyzed with TCS SP2 (Leica, Germany) software. Cells were considered as spreading if the cytoplasmic area was at least twice the nuclear surface area. Spreading assays were

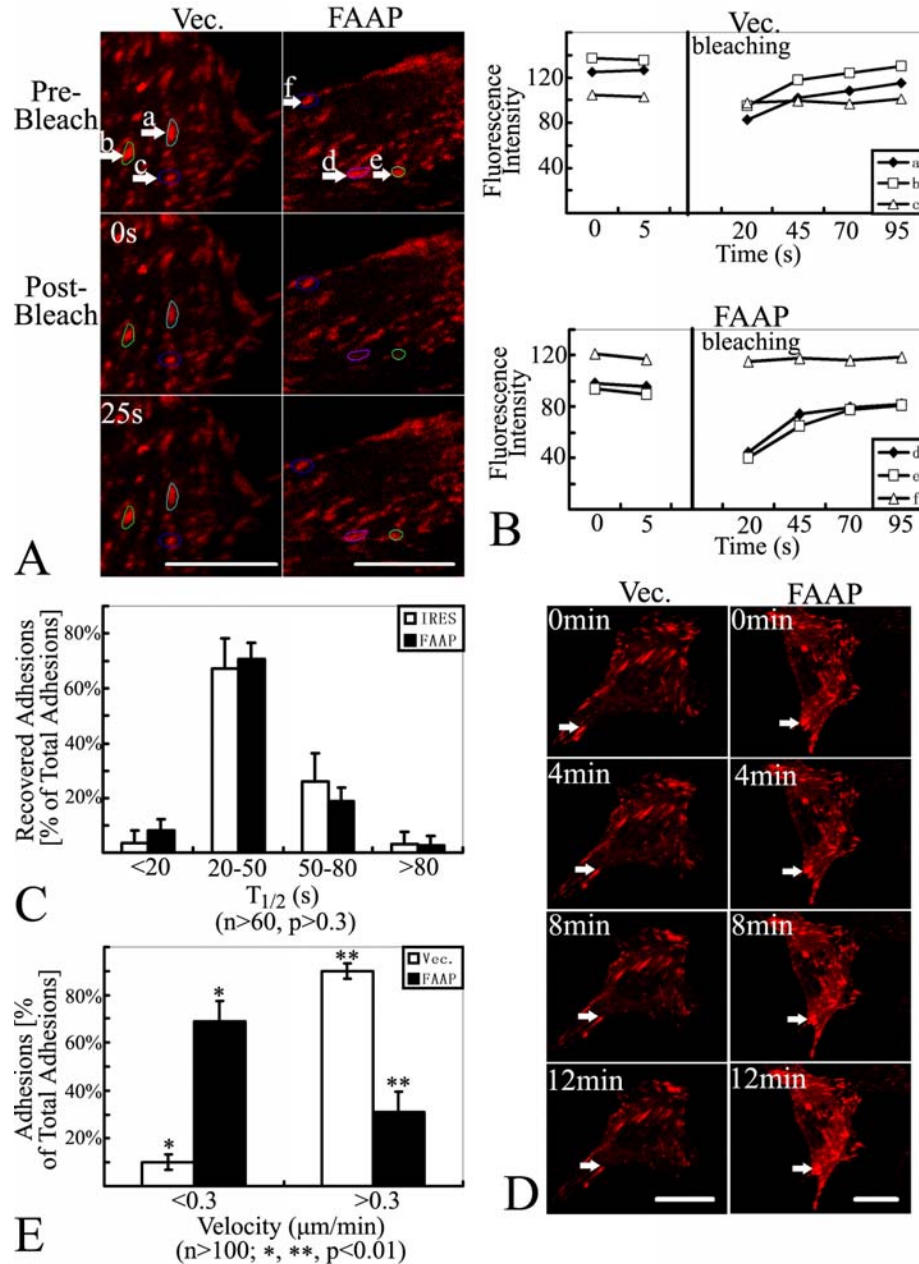
performed for three times and over 100 cells were scored each time.

### 3.6. Quantification of cell adhesion dynamics

HeLa cells were co-transfected with paxillin-pDsRed2 (provided by Dr Horwitz) (8) and FAAP-pIRES-EGFP plasmids (FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells), or paxillin-pDsRed2 and pIRES-EGFP plasmid served as control (control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells). After 24 hr, the cells were harvested with 1mM EDTA and plated on coverslips coated with 1  $\mu$ g/ml fibronectin at a density of 2.0 $\times$ 10<sup>5</sup> cells/dish for over than 2 hr. The cells were maintained at 37°C and pH 7.4 throughout the observation period, imaged over time using confocal microscopy and analyzed by TCS SP2 software.

For adhesion translocation assays, the individual adhesions tracks were marked with DsRed2-tagged paxillin molecules in EGFP<sup>+</sup> cells. The velocities for paxillin movement of adhesions were analyzed. The average velocities and the proportions of the number of adhesions in different velocity sections were scored from 100 adhesions in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells or control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells respectively. The assays were carried out three times.

## FAAP associates with focal adhesion protein

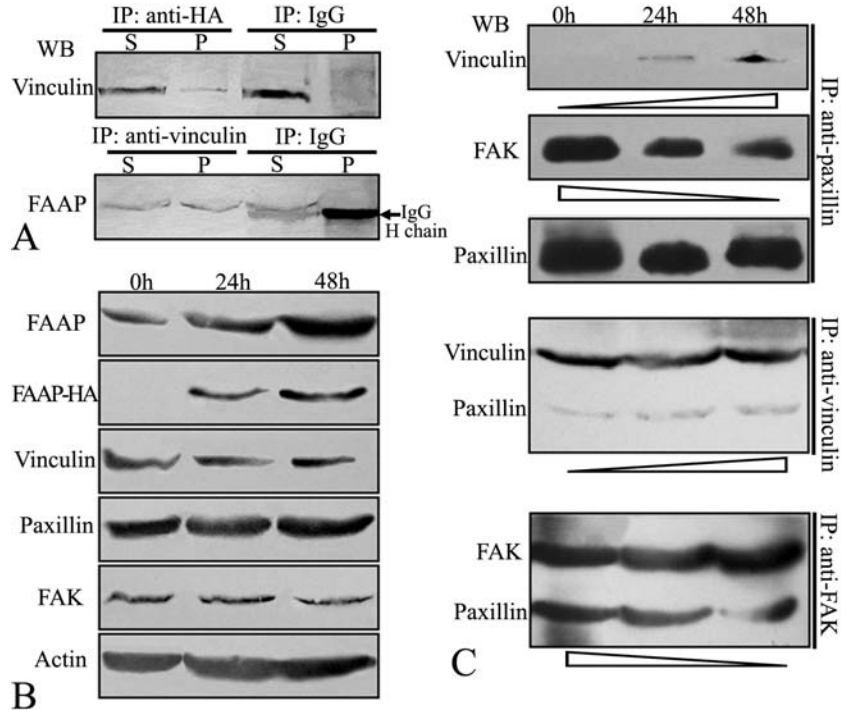


**Figure 2.** Adhesion-site dynamics. In HeLa cells co-expressing pDsRed2-paxillin and FAAP-pIRES-EGFP (FAAP) or co-expressing pDsRed2-paxillin and pIRES-EGFP (Vec.), stable adhesions were analyzed by confocal microscopy. (A) FRAP images showed the incorporation of DsRed2-paxillin fluorescence intensity in transfected cells on adhesion sites a, b, c (Vec.) or d, e, f (FAAP) sites. The sites c and f were FRAP experiment controls (Bar: 10  $\mu\text{m}$ ). (B) The diagrams show changes of DsRed2 fluorescence intensity before and after photobleaching (upper panel, a, b, c) and (lower panel, d, e, f) sites. (C) The half times of fluorescence recovery after photobleaching on each adhesion site are from three independent experiments. The proportion of the number of recovered adhesions to the total adhesions were measured at each time point in three independent experiments (mean  $\pm$  s.e.; n>60). (D) The dynamics of DsRed2-paxillin translocation on individual adhesion in both vector (Vec.) and FAAP (FAAP) transfected cells (t = 0-12min, Bar: 20 $\mu\text{m}$ ). (E) Quantification of the retrograde movement speed and the average velocity of each adhesion. The proportion of the number of recovered adhesions to the total adhesions were separated into two different velocity groups (lower or higher than 0.3 $\mu\text{m}/\text{min}$ ) in three independent experiments (mean  $\pm$  s.e.; n>100).

Fluorescence recovery after photobleaching (FRAP) analysis were carried out on the individual adhesions that remained stable for more than 5 min by

bleaching the fluorescence of DsRed2-tagged paxillin molecules in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells or control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells. The dynamics of the

## FAAP associates with focal adhesion protein



**Figure 3.** Interaction dynamics of paxillin-FAAP and paxillin-vinculin. Lysates from HeLa cells transfected with FAAP-pIRES-EGFP for 0 hr, 24 hr and 48 hr were used for immunoprecipitations. IP, immunoprecipitation; WB, Western blotting. (A) Immunoprecipitations of cell lysates from 48 hr transfected cells were performed with FAAP or vinculin antibodies respectively to identify FAAP-vinculin interaction. S, suspension; P, pellet. (B) Western blotting of cell lysates showed that increasing FAAP expression did not affect the expression of vinculin, paxillin, and FAK. (C) Immunoprecipitations of cells lysates were performed with paxillin, vinculin or FAK antibodies respectively. The data showed that increasing FAAP expression decreased paxillin-FAK, but increased paxillin-vinculin association. The immunoprecipitation proteins bands, IP-paxillin, IP-vinculin, and IP-FAK were quantification of the bands by intensity measurements. Results shown were representative of three independent experiments.

fluorescence intensities were quantified and the half time of individual adhesions fluorescence recovery after photobleaching were scored. The average recovery times and the proportions of the number of adhesions in different recovery time sections were quantified from about 60 adhesions in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells or control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells from three independent experiments respectively.

## 4. RESULTS

### 4.1. FAAP restrains HeLa cells from spreading

Immunofluorescent images of FAAP expressed EGFP<sup>+</sup> cells stained with anti-HA antibodies showed FAAP localizing in the cytoplasm whereas control EGFP<sup>+</sup> cells showed only EGFP fluorescence (Figure 1A). In addition, the level of FAAP in transfected cells increased with time (Figure 3B). HeLa cells that expressed various levels of FAAP were obtained by manipulation of transfection time.

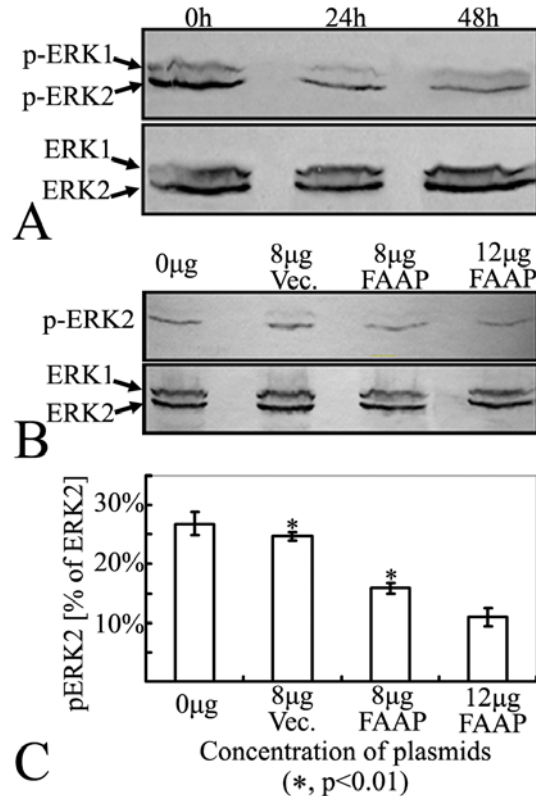
Cell spreading assays were performed in FAAP expressed EGFP<sup>+</sup> cells or control EGFP<sup>+</sup> cells. Cells expressing FAAP exhibited lower spreading cell area than cells that expressed only EGFP (Figure 1B). The ratio of

spreading cells in FAAP expressed EGFP<sup>+</sup> cells was markedly less than that of control EGFP<sup>+</sup> cells (Figure 1C), suggesting that FAAP plays a role in restraining cell spreading.

### 4.2. FAAP inhibits cell adhesion translocation, but not cell adhesion incorporation

The focal adhesion protein, paxillin, was fused with DsRed2 protein to mark individual focal adhesion (8). The effect of FAAP on the adhesion incorporation rates was examined using FRAP to bleach individual adhesions and then the re-incorporation rates of DsRed2-paxillin fluorescence intensity in both FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells and control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells were measured. The fluorescence intensity dynamics of DsRed2-paxillin remained unchanged in both FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells and control cells (Figure 2A, B). The half-times of individual adhesion fluorescence recovery after photobleaching in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells were similar to that of control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells ( $T_{1/2}$ : control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells:  $38.5 \pm 15.7$ s; FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells:  $35.4 \pm 15.0$ s). The FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells revealed comparable percentages with control cells in different recovery half-time groups (Figure 2C). Hence,

## FAAP associates with focal adhesion protein



**Figure 4.** Identification of ERK dephosphorylation. (A) Cell lysates from HeLa cells transfected with FAAP showed increasing FAAP expression did not affect ERK level, but induced a dephosphorylation of ERK. (B) Western blotting of the total lysates of HeLa cells transfected with various concentrations of plasmid per 60 mm dish showed an ERK dephosphorylation by FAAP. Results shown in A and B were representatives of three independent experiments. (C) The degree of phosphorylation were quantified as p-ERK2 to total ERK2 from experiment B (mean  $\pm$  s.e.; n=3).

FAAP appears not to interfere with the incorporation and exchange rates of paxillin in individual stable adhesion site.

We quantified the translocation rates of paxillin on individual adhesion in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells and control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells to investigate whether FAAP affects adhesion translocation. Microscopic videos revealed that the retrograde sliding of individual adhesion was significantly inhibited in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells compared with control EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells (Figure 2D). For quantification, we estimated the average speeds of adhesion-site movement towards the cell center, and separated them into low (lower than 0.3µm/min) and high (higher than 0.3µm/min) speed groups. Significantly more adhesion-site movement was in the low speed group than in the high speed group in FAAP expressed EGFP<sup>+</sup>/DsRed2<sup>+</sup> cells compared to control cells (Figure 2E). This reflected a function of prominent stabilization of adhesion sites by FAAP protein.

### 4.3. FAAP mediates interaction of adhesion molecules.

We used immunoprecipitation of cell lysates to reveal the molecular mechanism of impaired cell spreading in FAAP expressed cells. We demonstrated that FAAP could associate with vinculin in FAAP expressed cells

(Figure 3A). However, Western blotting of lysates from FAAP expressed cells did not show apparent changes in the levels of paxillin, vinculin, or FAK though FAAP expression increased (Figure 3B). But in FAAP expressed cells, the binding between paxillin and FAK decreased, while the binding between paxillin and vinculin was enhanced as FAAP expression increased (Figure 3C). This suggests that FAAP facilitates association of vinculin and paxillin but inhibits paxillin-FAK interaction.

### 4.4. FAAP induces dephosphorylation of ERK

It is known that paxillin-FAK interaction affects FAK activation, while ERK is the target of FAK-Src signaling pathway (26). Therefore, we determined whether FAAP interferes with ERK activity through paxillin-FAK interaction signaling. Western blotting showed that the total level of ERK1/2 did not change, while as the levels of FAAP increased, their phosphorylation levels decreased (Figure 4A). Furthermore, further increase in FAAP expression levels by transfecting cells with higher concentrations of FAAP-pIRES-EGFP, a reverse effect was observed, i.e., the higher the level of FAAP expression, the lower the ERK phosphorylation (Figure 4B,C). Thus, FAAP induces dephosphorylation and/or reduce phosphorylation of ERK to interfere with the activation of MAPK family proteins.



## **5. DISCUSSION**

This study provides the evidence that FAAP impairs cell spreading not by affecting focal adhesion incorporation, rather by reducing focal adhesion translocation. In cell adhesion dynamics, the effective interaction of vinculin and paxillin affects cell spreading by acting as a scaffold, recruitment of several proteins to special locations (29, 30) and by increasing the residence lifetime of proteins at adhesion sites (31). They regulate adhesion structures (18) by modulating the recruitment of signaling molecules to special sub-cellular locations (19). A cell spreading defect was observed in vinculin lipid binding domain mutants. These mutants decreased adhesion movement and stabilized initial adhesion through increasing vinculin-paxillin association (28). The findings reported here on FAAP are consistent with these mechanisms of action. FAAP facilitates vinculin-paxillin association and stabilizes the initial adhesion. The impairment in function of FAAP decreases paxillin-FAK interaction and facilitates vinculin-paxillin association and consequently delays cell spreading.

The FAT regions of FAK and vinculin tail domain share the same paxillin LD motifs. The FAT region interacts with two distinct subdomains on paxillin, LD2 and LD4 motifs (21), while the vinculin tail domain binds paxillin LD1, LD2, and LD4 motifs (16, 24, 25). Thus, vinculin can interfere with FAK-paxillin interaction. This is supported by previous reports that vinculin oligomers block FAK binding to LD2 and LD4 motifs on the same paxillin molecules (18). Also, vinculin null cells are rescued with vinculin and exhibit a decrease in paxillin-FAK interaction (26). Our results demonstrated that FAAP does interfere with paxillin-FAK interaction, while it enhanced vinculin-paxillin association. Therefore, FAAP effects on vinculin-paxillin and paxillin-FAK interaction are antagonistic.

Furthermore, vinculin has been demonstrated to affect the ERK activity through modulation of paxillin-FAK interaction (26). Paxillin-FAK interaction leads to paxillin phosphorylation at Y31 and Y118 (16). Vinculin null cells exhibit an increase in paxillin-FAK interaction and an increase in phosphorylation levels of FAK Y397, paxillin Y118 and ERK1/2. In vinculin null cells, all these increases can be reversed when vinculin or its tail domain is replenished. Expression of a paxillin Y31FY118F mutant in the vinculin null cells induces a substantial reduction in ERK1/2 activity (26). As shown here, the reduction of paxillin-FAK interaction in cells that over-express FAAP is consistent with a diminished phosphorylation of ERK 1/2 in these cells.

Cell spreading also relies on non-receptor tyrosine kinases, including FAK. The vinculin-deficient fibroblasts show an increased FAK activity, a less-stable focal adhesion, reduced cell spreading and locomotion (32). These changes differ from severe spreading defect which appear in cells that over-express FAAP. Impairment by FAAP results from changes in initial adhesion stabilization, and not from adhesion deficiency, or adhesion dynamic

affected by increasing FAK activity. The tyrosine kinase activity negatively regulates the vinculin-paxillin association in adhesion sites, affects cytoskeletal protein functions, and leads to an increase in cell spreading (31). The impairment of adhesion dynamics by FAAP is consistent with such effects.

The regulation of cell adhesion dynamics is a fundamental process in cell-cell interaction in structural integrity of tissues and in morphogenesis. Cell adhesion is controlled by the vinculin-paxillin and paxillin-FAK interaction which are responsive to multiple molecules that reside in signaling complexes. The data shown here confirm that FAAP by regulating vinculin-paxillin and paxillin-FAK interactions, is a regulator in cell adhesion dynamics.

## **6. ACKNOWLEDGEMENTS**

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## FAAP associates with focal adhesion protein

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## **FAAP associates with focal adhesion protein**

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**Abbreviations:** FAAP: focal adhesion associated protein; FAK: focal adhesion kinase; ERK: extracellular signal-regulated kinase; MAPK: mitogen-activated protein kinase; FRNK: FAK-relative nonkinase; FAT: FAK's COOH-terminal contains focal adhesion targeting region; HA: hemagglutinin; FRAP: fluorescence recovery after photobleaching

**Key Words:** D10Wsu52e gene, FAAP, Cell Adhesion, Vinculin, Paxillin, FAK

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