

AFAP-110 Is Required for Actin Stress Fiber Formation and Cell Adhesion in MDA-MB-231 Breast Cancer Cells

ANDREA DORFLEUTNER,¹ CHRISTIAN STEHLIK,¹ JING ZHANG,^{2,3}
GARY E. GALLICK,^{2,3} AND DANIEL C. FLYNN^{1*}

¹The Mary Babb Randolph Cancer Center and the Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, West Virginia

²Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

³The Program in Cancer Biology, The University of Texas Graduate School of Biomedical Sciences, Houston, Texas

Regulation of actin organization and dynamics is a highly complex process that involves a number of actin-binding proteins, including capping, branching, severing, sequestering, and cross-linking proteins. The actin-binding and cross-linking protein AFAP-110 is expressed in normal myoepithelial cells. Screening of different breast epithelial cell lines revealed high expression levels of AFAP-110 in the human breast cancer cell lines MDA-MB-231 and MDA-MB-435. Knockdown of AFAP-110 expression in MDA-MB-231 cells does not result in any changes in cell proliferation but did result in a loss of actin stress fiber cross-linking and decreased adhesion to fibronectin. An inducible knockdown approach confirms that MDA-MB-231 breast cancer cells require AFAP-110 expression for stress fiber formation and adhesion. Thus, AFAP-110 may provide cytoskeletal tension through stress fiber formation, which is required for focal adhesion formation. Indeed, we could not detect any focal contacts or focal adhesions in AFAP-110 knockdown cells after adhesion to fibronectin. Although expression levels of crucial focal adhesion components were not influenced by AFAP-110 expression levels, treatment of AFAP-110 knockdown cells with LPA did not result in induction of actin stress fibers and focal adhesions. In summary, AFAP-110 plays an important role in MDA-MB-231 breast cancer cell adhesion possibly by regulating stress filament cross-linking which would promote focal adhesion formation.

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The actin filament associated protein of 110 kDa, AFAP-110, was first identified as a tyrosine-phosphorylated substrate and binding partner for the active form of the non-receptor tyrosine kinase Src, Src^{527F} (Reynolds et al., 1989; Flynn et al., 1993). AFAP-110 contains a functional actin-binding domain (Qian et al., 2000) and several protein-binding motifs, including two SH2- (Guappone et al., 1998) and one SH3- (Guappone and Flynn, 1997) binding motif, two plekstrin homology domains and a leucine zipper motif (Qian et al., 1998; Qian et al., 2004). Therefore, AFAP-110 is hypothesized to function as an adaptor molecule which links other proteins to the actin cytoskeleton. Activation of PKC α directs phosphorylation of AFAP-110 and enables it to activate cSrc, resulting in alteration of actin stress filament integrity and podosome formation (Gatesman et al., 2004). Thus, there is evidence to indicate a role for AFAP-110 in modulating cellular signals that regulate actin filament organization. However, it is unknown whether AFAP-110 has a direct role in modulating stress filament organization by virtue of its ability to directly bind actin filaments. There is evidence to support a possible role for AFAP-110 in directly modulating actin filament organization. In vitro studies demonstrate that AFAP-110 can exist as a multimer and promote actin filament cross-linking (Qian et al., 2004). Thus, AFAP-110 may play a direct role in modulating stress filament cross-linking.

The actin microfilament network is important in maintaining cell shape and function in eukaryotic cells. It has a multitude of roles in cellular processes such as cell adhesion, motility, cellular signaling, intracellular trafficking, and cytokinesis (Carraway and Carraway, 1995; Pantaloni et al., 2001; Bloom, 2006) and modulation of the cytoskeleton is primarily achieved by association of different actin-binding proteins (Bretscher, 1991). Cell adhesion to ECM substrates is mediated by

heterodimeric integrin receptors composed of α and β subunits (Hynes, 1992; Gomes et al., 2004) that can be intracellularly linked to actin stress filaments via cytoskeletal proteins such as talin, vinculin, and paxillin (Schoenwaelder and Burridge, 1999; Webb et al., 2002). These focal adhesion sites are linked to the termini of stress fibers which provide actomyosin-based contractility, which contributes to the assembly of stress fibers and focal adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Geiger and Bershadsky, 2002). Disruption of actin filaments completely inhibits all types of adhesive interactions and therefore plays an important role in cell adhesion (Haier et al., 1999; von Sengbusch et al., 2005; Conway et al., 2006). Thus efficient adhesion of cells to ECM components may be dependent on the assembly of microfilaments and signal transduction.

In this report, we sought to determine if AFAP-110 played a role in modulating cellular actin filament organization by using shRNA oligonucleotides to knock down expression and

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*Correspondence to: Daniel C. Flynn, The Mary Babb Randolph Cancer Center and the Department of Microbiology, Immunology, and Cell Biology, West Virginia University, Morgantown, WV 26505-9300. E-mail: dflynn@hsc.wvu.edu

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measure its effects on actin filament organization and cellular function. Our studies reveal that AFAP-110 is highly expressed in the human breast cancer cell line MDA-MB-231. AFAP-110 is necessary for stress fiber formation and for adhesion of these cells to fibronectin. In the absence of AFAP-110 expression, stress filament organization was lost, and focal complexes and focal adhesions were not detected, even after stimulation with the Rho activator lysophosphatidic acid (LPA).

Materials and Methods

Cell culture and reagents

The MDA-MB-231 cell line and other breast cancer cell lines (T47D, ZR75-1, MCF7, MCF10A, MDA-MB-435) were a kind gift from Dr. Jeannine Strobl (Virginia College of Osteopathic Medicine) and were cultured in appropriate medium as recommended by the American Type Culture Collection (Rockville, MD). MDA-MB-231 cell lines were grown in DMEM (Mediatech, Herndon, VA), 10% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2 mM L-glutamine (Gibco, Grand Island, NY), and penicillin/streptomycin (Mediatech) at 37°C with 5% CO₂. For adhesion assays plates were coated with 10 µg/ml fibronectin from BD Biosciences (San Jose, CA). LPA was obtained from Sigma (St. Louis, MO) and was used at 10 µM for 3 min on cells to induce stress fiber and focal adhesion formation. The following antibodies were used for Western blotting or immunofluorescence: paxillin (BD Biosciences), AFAP-110 (first described by Flynn, 1993), actin (MP Biomedicals, Solon, OH), GAPDH (Abcam, Cambridge, MA), α5 and β1 integrin (Chemicon/Millipore, Billerica, MA), Rhodamine-Phalloidin (Sigma). Secondary antibodies for immunofluorescence were obtained from Invitrogen (Carlsbad, CA).

Generation of stable AFAP-110 knockdown cell lines

The knockdown cell lines were generated with the Invitrogen BLOCK-iT Inducible HI RNAi Entry Vector Kit. Briefly, target sequences were identified by RNAiDesigner from Invitrogen and oligos (bp 1098–1117 of human AFAP-110) were cloned into the pENTR/H1/TO vector. Control shRNA was directed against lacZ. MDA-MB-231 cell lines were transfected with Lipofectamine Plus (Invitrogen). After selection control and AFAP-110 knock down clones were picked and screened by Western blotting for AFAP-110 expression. To obtain an inducible knock down cell line clone #1 was stably transfected with a tetRepressor construct (pQC-TS-IN from Clontech, Mountain View, CA) and inducible shRNA expression was tested by Western blot when treated with 1 µg/ml doxycycline (Dox) in a timecourse experiment over 15 days. All further experiments, where Dox treatment is indicated, were completed within 15–30 days of Dox treatment and AFAP-110 expression levels were controlled by Western blotting prior to the experiment.

Immunoblotting

Cells were lysed for 20 min on ice in lysis buffer (10 mM Na₂PO₄ (pH 7.2), 150 mM NaCl, 1% NP40, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA, 5 µg/ml aprotinin, 2 µg/ml leupeptin, 1 mM PMSF) and cleared by centrifugation at 4°C for 10 min at 12,000g. Protein concentrations were determined with a BCA Protein Assay Kit (Pierce, Rockford, IL) according to the manufacturers' protocol. Equal protein amounts (100 µg) were used for SDS–polyacrylamide gel electrophoresis and subsequent immunoblotting. Resolved proteins were transferred by semidry electroblotting to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P from Millipore, Billerica, MA). Proteins were detected by incubation with primary antibodies as indicated in a solution of 5% (w/v) powdered milk, 0.05% (v/v) Tween 20 in PBS, followed by incubation with a 1:3000 dilution of secondary donkey anti-mouse or donkey anti-rabbit horseradish peroxidase-

conjugated antibodies (GE Healthcare Bio-Sciences, Piscataway, NJ). Peroxidase-conjugated secondary antibodies were visualized by electrogenerated chemiluminescence.

Proliferation assay

Cells (2×10^4) were seeded into 96-well microtiter plates and incubated for 16, 40, 65, and 90 h before they were fixed in 3.7% formaldehyde, rinsed with phosphate-buffered saline (PBS), and stained with 1% crystal violet in 2% ethanol. Stained cells were lysed in 10% acetic acid and OD₆₀₀ was determined in a plate reader. The amount of dye that was taken up by the cells correlates to the cell number.

Adhesion assay

Quantitative cell adhesion assays were carried out in non-tissue culture treated 96-well microtiter plates (Evergreen Scientific, Los Angeles, CA) which were coated with 10 µg/ml fibronectin for 1 h at room temperature. Fibronectin coated and uncoated control wells were blocked for 1 h with 5% bovine serum albumin (BSA) at 37°C. Cells were split 1 day prior to the experiment to achieve a subconfluent culture. Briefly, cells were collected with $0.5 \times$ trypsin, quenched with soybean trypsin inhibitor (Calbiochem), and 2×10^4 cells in 100 µl medium were added to each well and incubated for 40 min at 37°C. Unattached cells were taken off and stained with trypan blue to exclude cell death as a reason for cells to not attach. Wells were washed three times with PBS and to exclude non-specific adhesion, BSA-blocked wells were controlled to not show any attached cells. Attached cells were fixed in 3.7% formaldehyde, rinsed with PBS, and stained with 1% crystal violet in 2% ethanol. Pictures were taken on a Leica microscope which is connected to a CCD camera before cells were lysed in 10% acetic acid and OD₆₀₀ was determined in a plate reader.

Electric cell-substrate impedance sensing (ECIS)

Cells were prepared as for the adhesion assay but plated into 1 cm² chambers with 10 ECIS-electrodes (Applied Biophysics, Troy, NY) that were precoated with 10 µg/ml fibronectin. The machine measures impedance at 40 kHz frequency over the time period of 1 h. If cells are covering the small electrode discs (0.25 mm²) the current flow is blocked which leads to increased impedance. Data were exported to Excel for calculation of slopes. This method is described by the manufacturer for use in cell adhesion at www.biophysics.com and has been referenced by several groups (Wegener et al., 2000; Helfer et al., 2006).

Immunofluorescence

Cells were seeded on 10 µg/ml fibronectin-coated coverslips for 0.5, 2.5 h or grown for 2 days. Unattached cells were rinsed off with PBS and attached cells were fixed in 3.7% formaldehyde, permeabilized with 0.2% Triton X-100 and stained for actin with TRITC-phalloidin, for AFAP-110 with F1 polyclonal antibody (Flynn et al., 1993) and paxillin with monoclonal antibody. Secondary antibodies were used at a 1:200 dilution. Coverslips were mounted on slides with Fluoromount-G (Southern Biotech, Birmingham, AL) and pictures were taken with a Zeiss LSM 510 microscope. Images were imported into Photoshop 7.0 (Adobe) for processing.

Results

AFAP-110 expression in human breast epithelial cell lines

Actin filaments provide the basic infrastructure for maintaining cell morphology and cellular physiological functions such as adhesion, motility, exocytosis, endocytosis, and cell division. A panel of six breast epithelial cell lines was screened for AFAP-110 expression in order to find a model system to investigate the cellular function of AFAP-110. Figure 1 shows

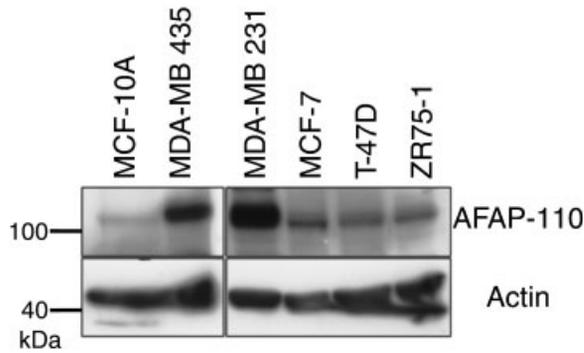


Fig. 1. AFAP-110 expression in human normal and cancer breast epithelial cell lines. Equal amounts of cell lysates (100 μ g) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using a polyclonal AFAP-110 antibody (upper part). The positions to which protein standards of known molecular mass migrated to on the SDS-polyacrylamide gel are indicated on the left. Membranes were probed for actin as a loading control (lower part).

very low expression of AFAP-110 in a normal breast epithelial cell line (MCF-10A) and low AFAP-110 expression in the tumorigenic breast cancer cell lines MCF-7, T-47D, and ZR75-1. However, AFAP-110 expression was high in the invasive breast cancer cell lines MDA-MB-231 and MDA-MB-435. The actin-binding and cross-linking protein, AFAP-110, is strongly expressed in the myometrium layer of normal breast ducts (Baisden et al., 2001b). MDA-MB-231 cells are thought to have been derived from myoepithelium. Thus, we chose this cell line as an appropriate model system for this study.

AFAP-110 knockdown in MDA-MB-231 cells leads to loss of actin stress fibers

To investigate the role of AFAP-110 in the MDA-MB-231 breast cancer cell line, endogenous AFAP-110 expression levels were knocked down by short hairpin RNA (shRNA) technique. Plasmid constructs that contain sense and antisense sequences from a target gene, which are connected by a loop sequence, are expressed in mammalian cells. The transcribed shRNA is transported from the nucleus into the cytoplasm, where the hairpin is cleaved off by Dicer generating a small interfering RNA (siRNA). Incorporation of siRNA into the RISC complex leads to activation of the complex and subsequently to sequence-specific mRNA degradation and reduced protein expression.

Stable knockdown cell lines targeting a control (*lacZ*) mRNA or AFAP-110 mRNA were generated. AFAP-110 expression levels of the stable clones were analyzed by Western blotting. Figure 2A shows AFAP-110 expression levels of MDA-MB-231 cells (lane 1) and of some selected clones with control knockdown (sh-ctrl #14 and #16) or with AFAP-110 knockdown (sh-AFAP #1, #2, and #4). Clone #1 had the strongest knockdown (5% of MDA-MB-231 cells as determined by densitometry scan) followed by clone #4 (6% of MDA-MB-231 cells). There were no apparent morphological differences in the presence or absence of AFAP-110 based on phase contrast microscopy analysis (data not shown) and proliferation rates also showed no dramatic changes (Fig. 2B). Subsequently, the consequence of AFAP-110 knockdown on actin filament integrity was investigated by immunofluorescence. Figure 2C demonstrates that unlike AFAP-110-expressing cells, upon knockdown of AFAP-110,

phalloidin was unable to decorate stress filaments, while cortical actin staining was still detectable. The same observations were made in the other two knockdown clones, however to a lesser extent in clone #2 which retained 20% of AFAP-110 expression compared to MDA-MB-231 cells (data not shown). These data indicate a loss of stress filament integrity in the absence of AFAP-110.

AFAP-110 knockdown in MDA-MB-231 cells leads to decreased adhesion and spreading on fibronectin

During tissue culture passaging of the cells we observed that cells lacking AFAP-110 expression always detached faster and easier than MDA-MB-231 and control knockdown clones. We hypothesized that AFAP-110 may contribute to adhesion of cells to extracellular matrix and therefore an adhesion assay was performed. The results (Fig. 3A) demonstrated a correlation of AFAP-110 expression with the ability of cells to attach and spread on fibronectin. Figure 3A shows fixed cells after 40 min adhesion to fibronectin. However, after extended periods of time (16 h) AFAP-110 knockdown cells did completely adhere (data not shown) and there was no evidence for changes in cell death. Quantification (Fig. 3B) revealed that AFAP-110-expressing cells adhere about 2.5-fold better than AFAP-110 knockdown cells after 40 min of adhesion to fibronectin. To verify this result, we used electric cell-substrate impedance sensing (ECIS) where cells are plated in wells containing 10 electrodes. The current and voltage measurements across the electrode are used to calculate the impedance which changes if cells are covering the electrode and cell membranes are blocking the current flow. With this method cell adhesion and spreading can be monitored in a quantifiable manner over time. In general, AFAP-110-expressing cells showed a fast increase in impedance which is associated with efficient cell adhesion and spreading, while AFAP-110 knockdown cell lines had a much slower increase in impedance (Fig. 3C). However, the control knockdown clone #16 had surprisingly low impedance readings, which we can only attribute to clonal variation resulting in decreased cell spreading in this particular clone. Overall, the impedance changes correlated with AFAP-110 expression levels and can be expressed as the slope of the curves which is presented in Figure 3D. These data indicate that AFAP-110 expression is also important for cell adhesion and spreading on fibronectin.

Expression of a tetracycline repressor in AFAP-110 knockdown cells rescues AFAP-110 expression and confirms that the loss of actin stress fibers is associated with AFAP-110 expression in MDA-MB-231 cells

To rule out clonal differences between MDA-MB-231, control, and AFAP-110 knockdown cell lines, inducible expression of AFAP-110-directed shRNA was generated by introducing a tetracycline repressor plasmid into MDA-MB-231 sh-AFAP #1 clone, which had the strongest knockdown. Expression of the tetracycline repressor will prevent shRNA expression and therefore restore AFAP-110 expression in the knockdown clone. After treatment with the tetracycline homologue, Dox, shRNA expression was enabled and AFAP-110 expression was knocked down again. Figure 4A shows a Western blot of MDA-MB-231 cells (lane 1) and MDA-MB-231 sh-AFAP #1 tetR containing the tetracycline repressor (lane 2). AFAP-110 expression was almost completely restored (compare lane 1 and 2), while increasing time of Dox treatment (lanes 3–6) suppressed AFAP-110 expression almost to the level of the original MDA-MB-231 sh-AFAP #1 clone (compare lane 6 and 7). As a loading control, the samples were also probed with anti-GAPDH. To confirm the effect of AFAP-110 expression on actin stress fiber formation MDA-MB-231 sh-AFAP #1 tetR cells containing the tetracycline repressor were either left

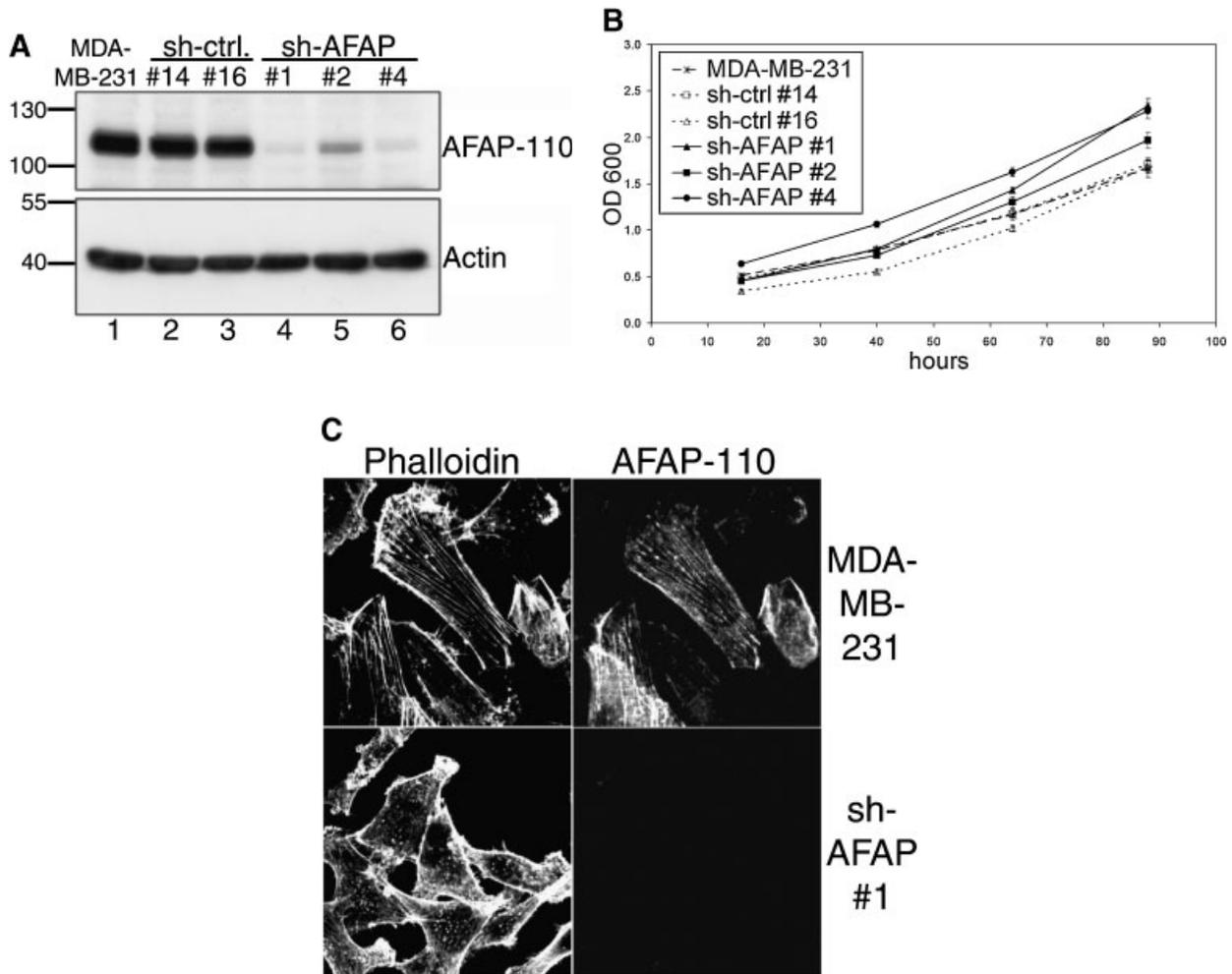


Fig. 2. AFAP-110 knockdown in the MDA-MB-231 breast cancer cell line. **A:** Immunoblotting was performed on stable cell lines expressing sh-RNA constructs. Protein lysates (100 μ g) of cells expressing sh-RNA that targets either lacZ as control (sh-ctrl) or AFAP-110 (sh-AFAP) were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotted with AFAP-110 antibody (upper part). The two-lacZ control clones #14 and #16 (lanes 2–3) have similar AFAP-110 expression as the original MDA-MB-231 cell line (lane 1). However, in clones #1, #2, and #4 of AFAP-110 knockdown cell lines (lanes 4–6) AFAP-110 expression is reduced. Membranes were probed for actin as a loading control (lower part) and the position of the protein standards are indicated on the left. **B:** Graphs show the result of one representative out of three independently performed proliferation assays of MDA-MB-231 (star symbol), sh-ctrl (open symbols), and sh-AFAP (solid symbols) cell lines. Equal amounts of cells were seeded in 96-well plates and fixed after different time points. Cells were stained with crystal violet, lysed in 10% acetic acid, and OD₆₀₀ was measured. There are no significant differences in proliferation of the cells in the presence or absence of AFAP-110 expression. **C:** Immunofluorescence staining of wild-type MDA-MB-231 cells expressing AFAP-110 compared to AFAP-110 knockdown cell lines. Forty eight hours after plating of cells onto fibronectin-coated glass coverslips, they were fixed and stained for AFAP-110 with polyclonal AFAP-110 antibody and F-actin with phalloidin, as described in “Materials and Methods.” Phalloidin staining revealed a clear deficiency in the ability of stress fiber formation in the absence of AFAP-110 expression. This observation was uniform throughout all analyzed cells.

untreated, or treated with Dox, and subsequently used for immunofluorescence staining with AFAP-110 and actin. Results are shown in Figure 4B and confirm the previous observations that indicate that AFAP-110 expression is associated with efficient actin stress fiber formation.

Doxycycline-regulated AFAP-110 expression levels correlate to adhesion and spreading on fibronectin

To confirm the previously observed effect of AFAP-110 expression on adhesion and spreading on fibronectin, adhesion assays were performed with the rescued and Dox-treated MDA-MB-231 sh-AFAP #1 tetR cell lines. Figure 5A shows that cells with high AFAP-110 expression levels (left side) adhere

better than cells with low AFAP-110 expression levels (right side). Quantification (Fig. 5B) confirmed that there was a dramatic difference in cell adhesion in the presence or absence of AFAP-110 expression. Cells without AFAP-110 expression adhere about 2.7-fold less. The importance of AFAP-110 in adhesion to fibronectin was also tested by using Dox inducible expression of AFAP-110 in an ECIS experiment.

Figure 5C follows cell adhesion and spreading of MDA-MB-231 cells and the tetracycline repressor expressing MDA-MB-231 sh-AFAP #1 for 60 min. AFAP-110-expressing cells (MDA-MB-231 and sh-AFAP #1 tetR) adhere and spread faster than cells with low AFAP-110 expression levels (sh-AFAP #1 tetR + Dox) which is represented by the steepness of the curve. The slope can be calculated which is shown in Figure 5D.

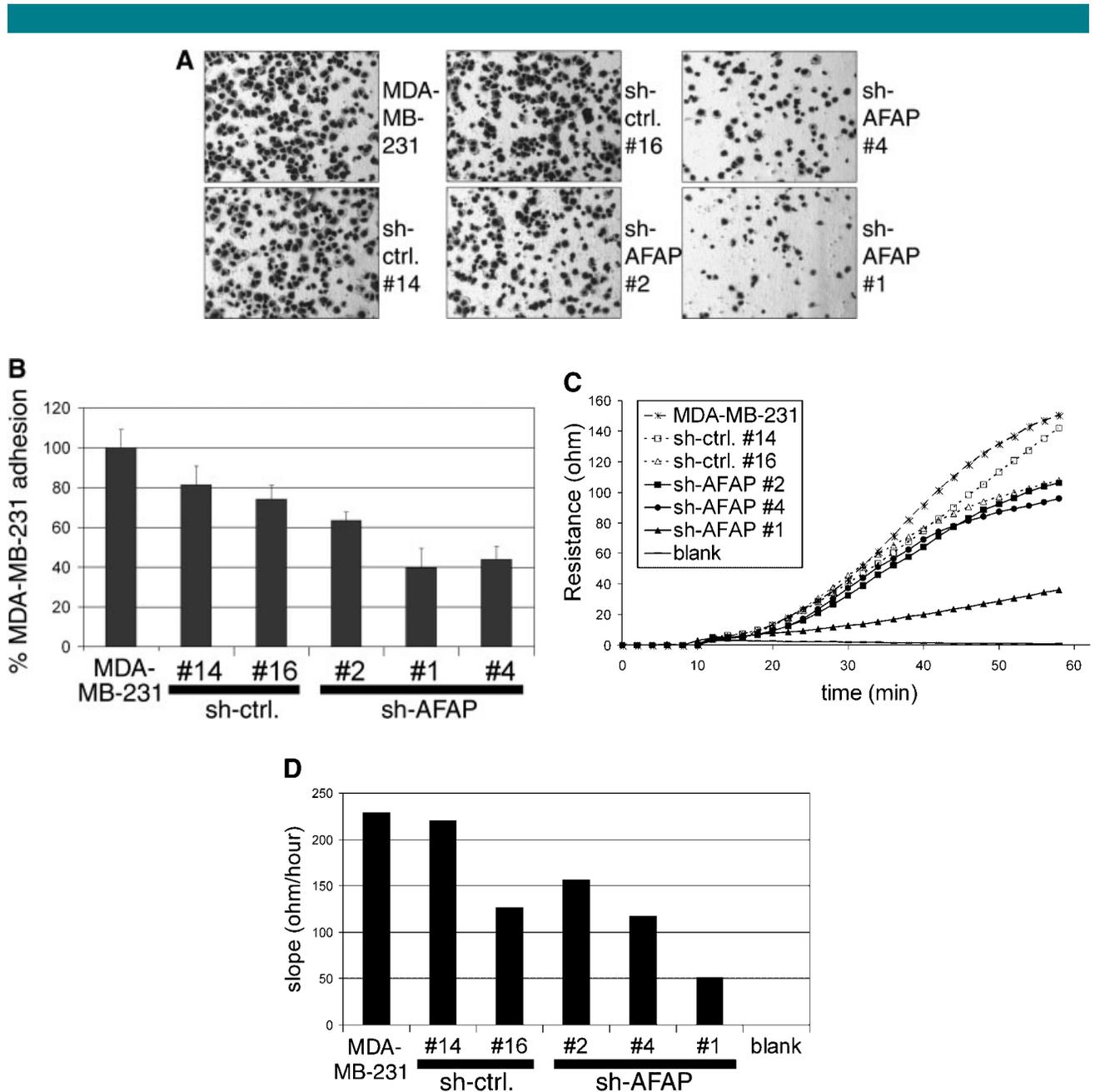


Fig. 3. Effects of AFAP-110 expression on cell adhesion and spreading. **A:** Breast cancer cells with high, intermediate, or low AFAP-110 expression levels were used for a cell adhesion assay. Equal number of cells (2×10^4) were plated on fibronectin-coated wells of a 96-well plate and after 40 min adhesion live unattached cells (as tested by trypan blue exclusion) were rinsed off and attached cells were fixed in 3.7% formaldehyde. Cells were stained with crystal violet and pictures were taken. High AFAP-110 expression (left column) correlated with strong adhesion and spreading on fibronectin while decreasing amounts of AFAP-110 expression (intermediate expression in middle column and low expression in right column) correlated with fewer adhered and spread cells. **B:** Quantification of cell adhesion to fibronectin. Crystal violet stained cells from **A** were lysed in 10% acetic acid and OD_{600} was measured. Columns show the average of eight wells and are expressed as % adhesion compared to MDA-MB-231 cells. **C:** ECIS analysis measures cell adhesion and cell spreading over time. Increased resistance (measured in ohm) correlates with increased amount of cells attaching and spreading and is plotted against time. Graphs show the result of one representative out of three independently performed assays of MDA-MB-231 (star symbol), sh-ctrl (open symbols), and sh-AFAP (solid symbols) cell lines. **D:** ECIS results are displayed as the slopes calculated from the linear part of the graphs from **3C**.

Focal complex and focal adhesion formation is dependent on AFAP-110 expression

There are a number of mechanisms by which AFAP-110 could regulate breast cancer cell adhesion. One possible consequence of loss of stress filament organization is reduced cytoskeletal tension which can impede focal contact formation, adhesion,

and cell spreading (Chrzanowska-Wodnicka and Burridge, 1996). To visualize the process of cell adhesion and focal complex/ focal adhesion formation in AFAP-110-expressing compared to AFAP-110 knockdown cell lines, cells were seeded on fibronectin-coated coverslips and immunofluorescence staining was performed. We find that MDA-MB-231 cells show focal complexes after 30 min adhesion

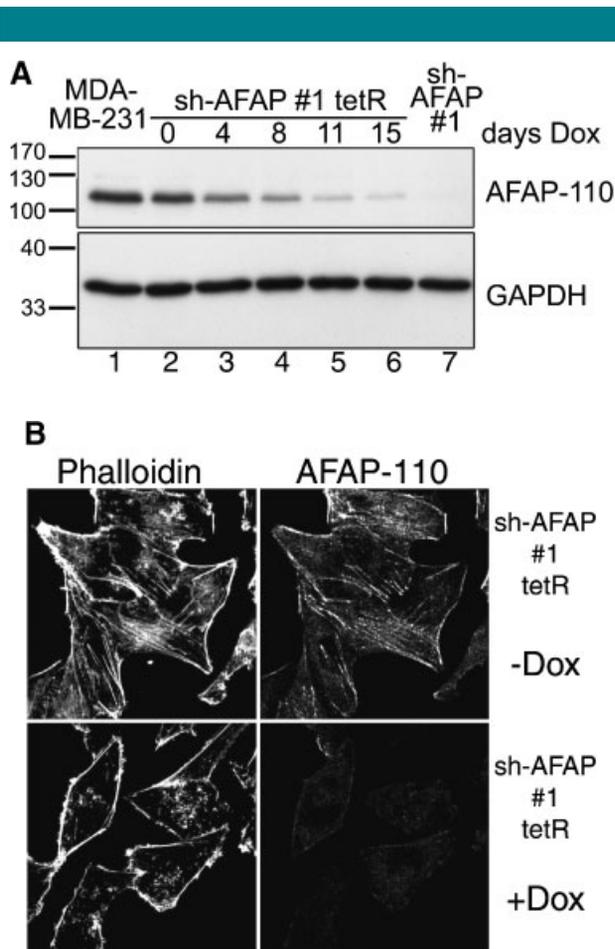


Fig. 4. Inducible expression of AFAP-110 sh-RNA affects actin stress fiber formation. **A:** Immunoblotting of MDA-MB-231 cells (lane 1) and derivative clones expressing AFAP-110 sh-RNA (sh-AFAP #1—lane 7) or AFAP-110 sh-RNA plus a tetracycline repressor (sh-AFAP #1 tetR—lanes 2–6) in the presence (lanes 3–6) or absence (lane 2) of doxycycline (Dox). Increased time of Dox treatment reduces AFAP-110 expression (upper part). Membranes were probed for GAPDH as a loading control (lower part). **B:** Immunofluorescence staining of cells with inducible AFAP-110 sh-RNA expression. Forty eight hours after plating of cells onto fibronectin-coated glass coverslips they were fixed and stained for AFAP-110 and F-actin, as described in “Materials and Methods.” F-actin is stained with phalloidin and reveals a clear deficiency in the ability of stress fiber formation in the presence of Dox along with the absence of AFAP-110 expression.

to fibronectin, which was assessed by paxillin staining (Fig. 6A, part a–i). Focal complexes appear as small round areas in the cell periphery, are positive for paxillin staining and are pointed out by a white arrowhead in Figure 6A (part b). We also observed strong cross-linking of cortical actin (part a), which is also where AFAP-110 is localized (part c). In the absence of AFAP-110 the cortical actin was still apparent (part d and g), but there were no pronounced focal complexes (part e and h). After 2.5 h of adhesion to fibronectin, wild type MDA-MB-231 cells form strong actin stress fibers and focal adhesions are localized at the ends of actin stress fibers (Fig. 6A, part j–r). Focal adhesions are also positive for paxillin staining but have a different shape, are more elongated than focal contacts and form later, after adhesion is established. Focal adhesions are pointed out by white arrowheads in Figure 6A, part k, after 2.5 h of attachment to fibronectin. However, in AFAP-110 knockdown cell lines almost no stress fiber formation was

detected after 2.5 h of adhesion to fibronectin (part m and p) and no focal adhesions were found (part n and q). Therefore, we conclude that AFAP-110 plays an important role in actin filament cross-linking which is required for focal complex and focal adhesion formation during cancer cell adhesion.

AFAP-110 expression does not influence protein levels of major adhesion molecules

Another possible mechanism for reduced cell adhesion is that loss of AFAP-110 expression may have altered the expression levels of integrins that bind fibronectin or the actin-binding proteins that link stress filaments to integrins. To determine whether AFAP-110 expression influenced expression levels of the fibronectin receptor ($\alpha 5 \beta 1$ integrin) and other proteins that are essential for the formation of focal adhesions, a Western blot was performed. Previous data ruled out differences in actin expression since we show in Figure 2A that if equal amounts of protein are loaded there is no difference in actin expression levels in the presence or absence of AFAP-110. Figure 6B shows that there were also no differences in $\alpha 5$ integrin, $\beta 1$ integrin, paxillin, and vinculin expression. Thus, some of the components that are important for cellular adhesion to fibronectin are retained in the AFAP-110 knockdown cell lines.

LPA does not induce stress fiber and focal adhesion formation in the absence of AFAP-110

Focal adhesion formation is necessary for efficient cell attachment and cell spreading and can be induced by growth factors or phospholipids, such as LPA stimulation (Hall, 1998). Therefore the ability of AFAP-110 to influence actin cross-linking and focal adhesion formation in response to LPA was investigated. Cells were plated on fibronectin overnight before treatment with or without LPA. Stress fibers in untreated MDA-MB-231 cells after 24 h adhesion to fibronectin (Fig. 2C) or after 2.5 h adhesion to fibronectin (Fig. 6A part j) appeared very similar and have been shown earlier in the article. Focal adhesions of untreated MDA-MB-231 cells after 24 h attachment to fibronectin (data not shown) were comparable to those that were formed after 2.5 h of attachment to fibronectin as shown in Figure 6A part k. However, treatment with LPA increased actin cross-linking into stress fibers (compare Fig. 6A part j with Fig. 6C) and also showed much more pronounced focal adhesions (compare Fig. 6A part k with Fig. 6C). Figure 6C shows the localization of AFAP-110 to actin stress fibers and particularly strong staining at the end of stress fibers where they transition into focal adhesions (see arrowhead in Fig. 6C). AFAP-110 did not co-localize with paxillin to focal adhesion sites (see merged image in Fig. 6C). However, in the AFAP-110 knockdown cell line sh-AFAP#1 LPA was unable to induce actin stress fiber formation and there was no apparent focal adhesion formation (Fig. 6C). Similar observations were also made with the other two AFAP knockdown cell line clones sh-AFAP #2 and #4 (data not shown). Thus, AFAP-110 is responsible for actin stress fiber and subsequent focal adhesion formation in MDA-MB-231 breast cancer cell adhesion to fibronectin and after LPA stimulation.

Discussion

The goal of this study was to determine whether AFAP-110 had the capacity to regulate cellular actin filament integrity. AFAP-110 is a 110 kDa actin-binding and cross-linking protein. Expression of AFAP-110 has been described in various cell types, including fibroblasts, epithelial and endothelial cells (Kanner et al., 1991; Baisden et al., 2001a). Analysis of human breast epithelial cell lines revealed strong AFAP-110 expression in MDA-MB-231 and MDA-MB-435 breast cancer cell lines as

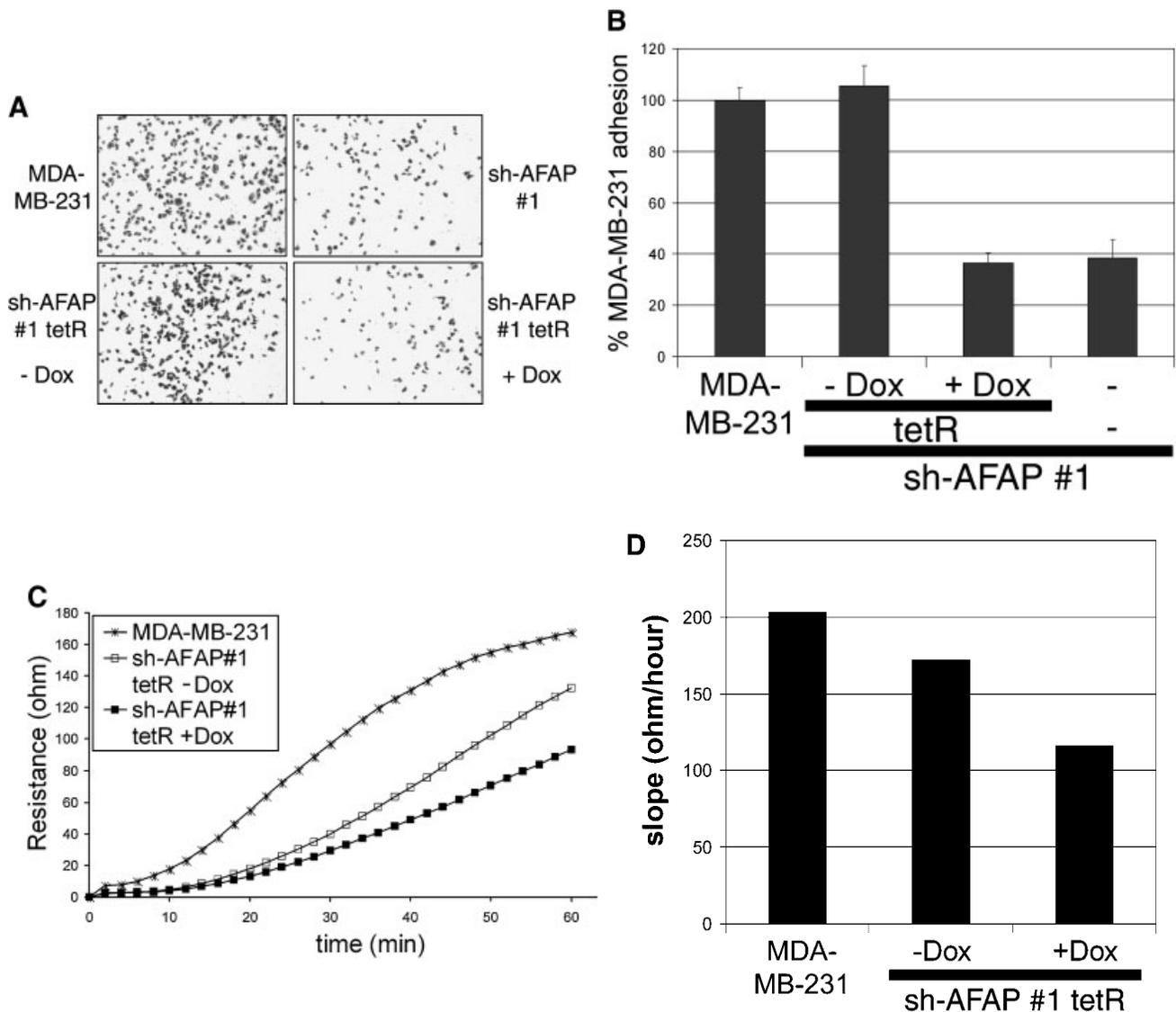


Fig. 5. Effects of inducible AFAP-110 sh-RNA expression on cell adhesion and spreading. **A:** A cell adhesion assay was performed on MDA-MB-231 and AFAP-110 knockdown (sh-AFAP #1) cell lines as well as cell lines with Dox inducible AFAP-110 sh-RNA expression (sh-AFAP #1 tetR). In the presence of AFAP-110 expression (left column) many cells are attached and well spread on fibronectin after 40 min. In the absence of AFAP-110 expression (right column) only few cells attached and spread on fibronectin after 40 min. Cells are visualized by crystal violet staining before pictures were taken. **B:** Quantification of cell adhesion assay from 5A. Crystal violet stain was released from cells with 10% acetic acid and OD₆₀₀ was measured. Columns show the average of eight wells and are expressed as % adhesion compared to MDA-MB-231 cells. **C:** ECIS analysis of breast cancer cells with inducible AFAP-110 sh-RNA expression measures cell adhesion and cell spreading over time. Increased resistance (measured in ohm) correlates with increased cell attachment and spreading and is plotted against time. **D:** ECIS results are displayed as the slopes, calculated from the linear part of the graphs from 5C.

determined by immunoblotting. We chose to use the MDA-MB-231 cell line as a model system to further study the functional role of AFAP-110. A stable knockdown cell line was generated and we further rescued AFAP-110 expression and generated a cell line where AFAP-110 expression could be switched on or off in the absence or presence of Dox, respectively. This model system provided us with a clearer basis for analyzing the functional consequences of AFAP-110 expression than comparisons between existing cell lines from heterogeneous sources or overexpression studies.

We initially observed that AFAP-110 expression was not associated with any differences in MDA-MB-231 cell proliferation rates. Since AFAP-110 is an actin-binding protein

that can cross-link actin filaments *in vitro* (Qian et al., 2004), we examined the effect of loss of AFAP-110 expression on the actin cytoskeleton. Actin-binding/bundling proteins are believed to mediate interactions between actin filaments to form both orthogonal networks and ordered bundles in order to provide mechanical support to the cytoplasm and to reinforce cellular protrusions. Orthogonal networks and bundles appear in specialized subcellular complexes, including lamellipodia, filopodia, stress fibers, and focal adhesions. Stress fibers in adherent epithelial cells and fibroblasts contain α -actinin, fascin, and tensin (Burridge and Chrzanowska-Wodnicka, 1996), which are proteins shown to have actin cross-linking/bundling function *in vitro*. The fact that several actin cross-linking/

bundling proteins co-localize to subcellular organelles suggests that these proteins complement each other and/or have overlapping functions (Cant et al., 1998; Rivero et al., 1999; Tseng et al., 2002). The relative tolerance of cells to null mutation of genes that code for a single cross-linking protein suggests that the functions of actin cross-linking proteins are likely redundant (Witke et al., 1992; Rivero et al., 1996a; Rivero et al., 1996b). Since AFAP-110 has the capacity to cross-link actin filaments in vitro (Qian et al., 2004), we studied if AFAP-110 has unique or redundant functions in MDA-MB-231 breast epithelial cells. In AFAP-110 containing cells immunofluorescence staining shows that F-actin consists of cortical actin and actin stress fibers and AFAP-110 co-localized to these structures. However, knockdown of AFAP-110 protein in MDA-MB-231 cells results in the loss of stress fiber organization. Therefore, we conclude that the expression of the actin-binding and cross-linking protein AFAP-110 has

unique functions in this cell line and is required for maintenance of steady-state actin stress fiber formation.

It had been suggested that stress fibers not only provide mechanical support for the cell but also contract and cause isometric tension which contributes to cell adhesion and focal adhesion assembly (Burrige, 1981). The adhesive interactions between a cell and its surrounding ECM, is characterized by specialized sites of adhesion, called focal adhesions. They consist of aggregated ECM receptors (integrins) that span the plasma membrane, interacting on the outside with ECM components and on the inside with stress fibers. Integrins that mediate adhesion will aggregate into focal adhesions when contractility is stimulated and, conversely, will disperse from focal adhesions when contractility is inhibited (Chrzanowska-Wodnicka and Burrige, 1996). Consistently, we find that loss of AFAP-110 expression and subsequent loss of actin stress fibers also resulted in a deficiency in cell adhesion by traditional

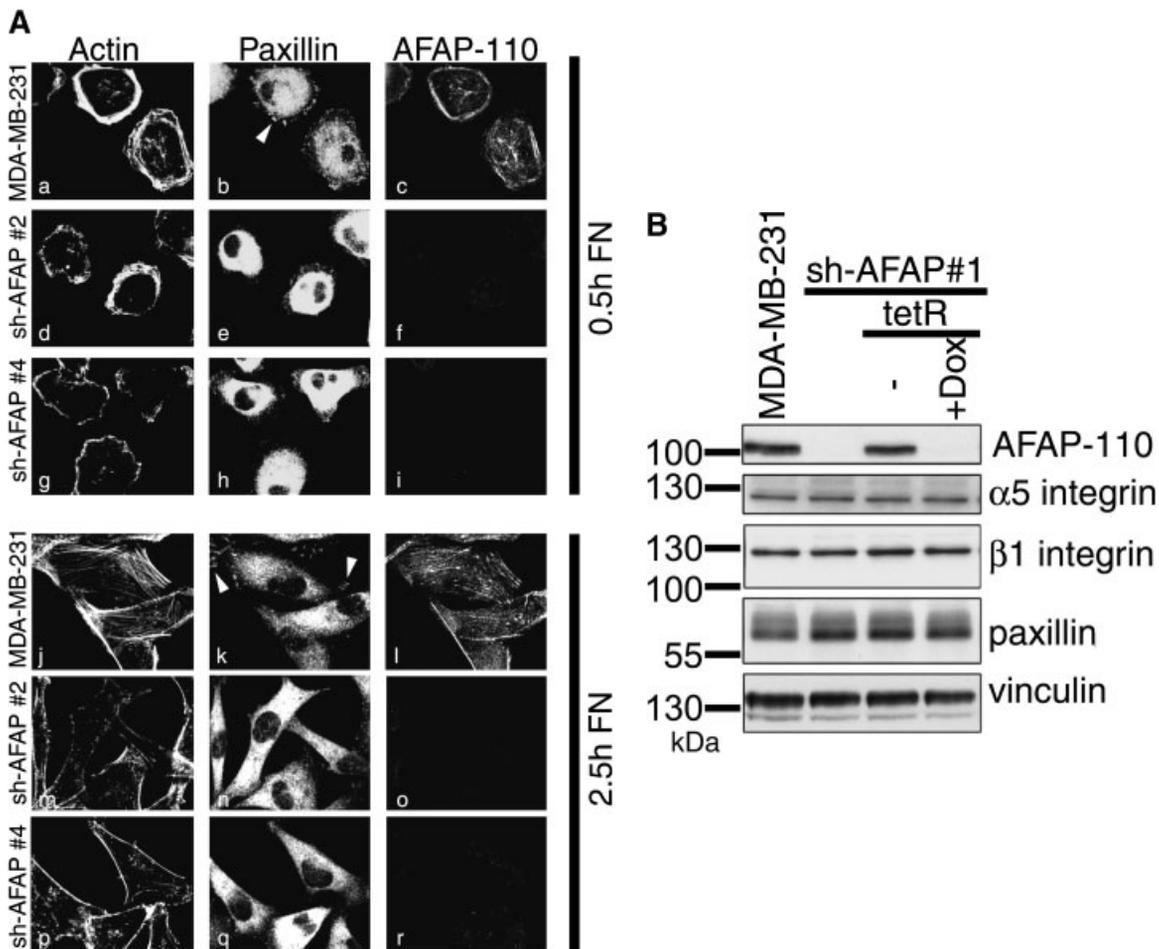


Fig. 6. AFAP-110 expression regulates focal complex and focal adhesion formation independent of protein expression levels of crucial focal complex/adhesion components. **A:** Immunofluorescence staining of AFAP-110 expressing (MDA-MB-231) or AFAP-110 knockdown (sh-AFAP #2 and #4) cell lines adhering to fibronectin for 0.5 h (top part) or 2.5 h (bottom part). F-actin (parts a,d,g,j,m,p), Paxillin (parts b,e,h,k,n,q), and AFAP-110 (blue—parts c,f,i,l,o,r) triple staining was performed as described in “Materials and Methods.” The arrowheads point out paxillin-rich focal complexes after 0.5 h adhesion (part b) or paxillin-rich focal adhesions after 2.5 h (part k) adhesion to fibronectin. **B:** Expression levels of the fibronectin receptor $\alpha 5\beta 1$, paxillin, and vinculin are not altered in the presence or absence of AFAP-110. Equal protein amounts of cell lysates were subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting with antibodies against AFAP-110, $\alpha 5$ and $\beta 1$ integrin, paxillin, and vinculin. **C:** LPA-induced focal adhesion formation requires AFAP-110 expression/stress fiber formation. Immunofluorescence staining of AFAP-110 expressing (MDA-MB-231) or AFAP-110 knockdown (sh-AFAP #1) cell lines after treatment with LPA was performed as described in “Materials and Methods.” Boxed areas of the top row parts are magnified $3\times$ (bottom parts) to show sites of focal adhesion formation which is pointed out by an arrowhead. Actin (red), Paxillin (green), AFAP-110 (blue), and merged pictures are shown.

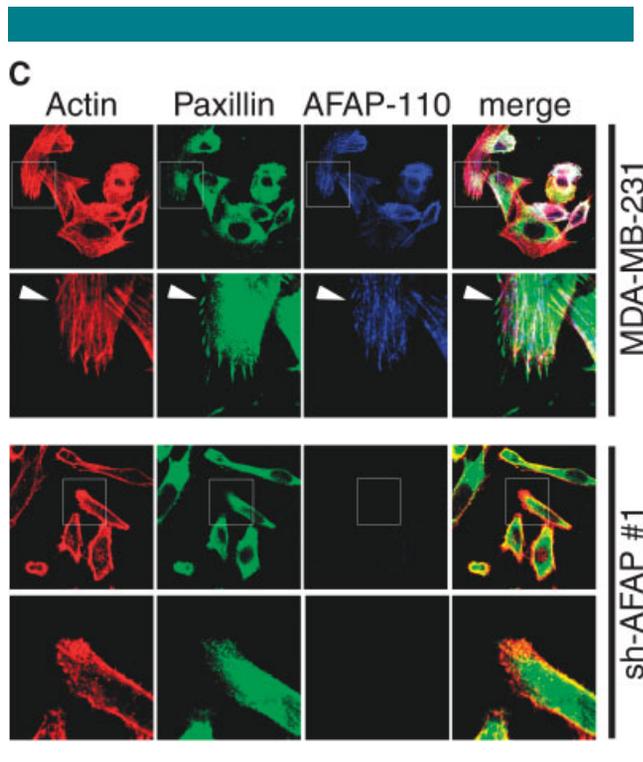


Fig. 6. (Continued)

adhesion assays and ECIS experiments. In addition, loss of AFAP-110 expression also correlated with an inability of MDA-MB-231 cells to form focal complexes and focal adhesions on fibronectin. We conclude that without the formation of stress fibers, contractility can not be applied to integrins and therefore, like treatment of cells with agents that inhibit contractility, the absence of AFAP-110 will lead to disassembly of both stress fibers and focal adhesions.

A major regulator of focal adhesions and stress fibers is the small GTP-binding protein Rho (Ridley and Hall, 1994; Takai et al., 1995) which can be activated by growth factors, neuropeptides (e.g., bombesin) or lipids (e.g., LPA). We find that LPA treatment can enhance stress fiber and focal adhesion formation in MDA-MB-231 breast cancer cells. However, in AFAP-110 knockdown cell lines LPA was unable to induce Rho-mediated stress fiber and focal adhesion formation. A more detailed study about the involvement of the Rho signaling cascade in AFAP-110 mediated regulation of stress filament cross-linking is underway.

AFAP-110 is thought to function as actin binding as well as cell-signaling protein due to its domain composition. It has an actin-binding domain which is necessary for AFAP-110 colocalization with actin (Qian et al., 2000) and it has several domains involved in protein-protein (SH2 and SH3-binding domains) or protein-lipid (PH domain) interactions which could influence cell signaling. Upon cell attachment to fibronectin, integrin receptor clustering triggers a series of sequential phosphorylation events, with one of the primary events being FAK autophosphorylation at tyrosine 397 to recruit and activate Src family kinases. AFAP-110 is a substrate and binding partner of cSrc (Reynolds et al., 1989; Flynn et al., 1993) and can also function as a cSrc-activating protein (Gatesman et al., 2004). It is currently unclear if cSrc mediates AFAP-110 phosphorylation or if AFAP-110 can activate cSrc in response to integrin engagement. Interestingly, loss of cSrc expression does correlate with deficits in cell adhesion (Kaplan

et al., 1995; Parsons and Parsons, 1997) displaying a similar phenotype as AFAP-110 knockdown. It is further unclear if integrin engagement can trigger a conformational change of AFAP-110 (maybe through phosphorylation or binding to another protein) which will result in a change of its actin cross-linking properties. For example, it is known that PKC activation is required to organize actin stress fibers and focal contacts (Woods and Couchman, 1992; Defilippi et al., 1997) following adhesion to matrix proteins and PKC-mediated phosphorylation of AFAP-110 has been shown to influence the ability of AFAP-110 to cross-link actin (Qian et al., 2002). Thus, it is possible that fibronectin-integrin engagement may cause a change in the actin cross-linking properties of AFAP-110 or that AFAP-110 may activate cSrc and regulate cell signals that control adhesion. Further studies will investigate this signaling pathway and its relationship to cell adhesion.

In summary, we describe in this study for the first time a physiological function of AFAP-110 in cell adhesion. In particular, we demonstrate that AFAP-110 knockdown cell lines lose their ability to form actin stress fibers and have decreased adhesion to fibronectin due to their inability to efficiently form focal complexes and focal adhesions upon attachment and spreading.

Acknowledgments

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