SUPPLEMENTAL MATERIALS

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Primary antibodies

Monoclonal antibodies against Alix were generated in our lab as previously described (Pan et al., 2006). Monoclonal antibody against GST and polyclonal antibodies against fibronectin, α_5 integrin, and β_1 integrin were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). Polyclonal antibodies against transferrin receptor (TfR) were purchased from Abcam (Cambridge, MA). Rat monoclonal 9EG7 antibody and monoclonal antibodies against clathrin and tensin were purchased from BD Biosciences (San Jose, CA). Monoclonal antibodies against p53 and GSK3β, and polyclonal antibodies against actin were purchased from Sigma Aldrich (St. Louis, MO). BMA5, the functional blocking monoclonal antibody against mouse α_5/β_1 integrin, and Ab1950, monoclonal antibody against human α_5/β_1 integrin, were purchased from Chemicon (Temecula, CA). Functional blocking antibody 33B6, which blocks the function of human but not mouse β_1 integrin (Miyamoto et al., 2001), was a generous gift from Dr. Bradley McIntyre. Rabbit pre-immune serum and immune sera against GST-Alix were generated Department of Veterinary Sciences in M. D. Anderson Cancer Center.

Cell culture, siRNA transfection and examination of cell morphology

WI38 cells, a human diploid fetal lung fibroblast cell line, were maintained in Modified Eagle's medium (MEM) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). NIH/3T3 cells, an immortalized mouse embryo fibroblast cell line, were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

WI38 cells were transfected with Alix specific or GFP specific siRNA using Lipofectamine 2000 (Invitrogen). Culture plates might be treated with 20 μg/ml of a designated protein in PBS or PBS alone overnight at 4°C, washed twice with PBS and blocked with 1 mg/ml BSA for 1 hr before cell seeding. Fibronectin purified from human plasma was purchased from Sigma Aldrich. Cell morphology was examined under a phase contrast microscope, and images were taken with Olympus IX81 microscope (Olympus America Inc. Melville, NY) using Metamorph software (Molecular Devices Corp., Sunnyvale, CA).

Immuno-gold electron microscopy and indirect immunofluorescence microscopy

WI38 cells grown on glass coverslips for 48 hr were fixed for 1 hr with EM fixative [2% formalin (Ted Pella, Redding, CA) and 3% glutaraldehyde (Ted Pella) in 0.1 M sodium cacodylate (pH 7.3)] for immuno-gold electron microscopy. After fixation, cells on coverslips were washed twice for 15 min each time with 0.05% Tween-20 in PBS (PBST), blocked for 1 hr with 1% casein in PBS (BioRad, Philadelphia, PA), and then incubated at 4°C overnight with 1 μg/ml 3A9 antibody in blocking solution. Subsequently, cells were washed three times for 15 min each time with PBST, incubated overnight with a 1:50 dilution of 1-nm gold-conjugated goat anti-mouse IgG (Ted Pella) in blocking solution, and washed again three times for 15 min each time with PBST. Cells were then re-fixed for 20 min with 2% glutaraldehyde in PBST, washed three times for 15 min each time with molecular-grade water, and silver enhanced with Light Insensitive Silver Enhancer (Ted Pella) for 20 min. After enhancement, cells were washed three times for 10 min each time with molecular grade water and post-fixed again with 2% formalin and 3% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3). The post-fixed cells were

embedded in spurr resin (Polysciences, Inc., Warrington, PA) on coverslips, sectioned after the spur blocks were detached from coverslips, and analyzed by transmission electron microscopy.

For indirect immunofluorescence staining, cells grown on ethanol-cleaned 22 x 22 mm glass coverslips for at least 24 hr before fixation with EM fixative, 4% paraformaldehyde at room temperature or -20°C methanol. Indirect immunofluorescence staining of a specific protein was performed as previously described (Pan et al., 2006). For tensin staining, cells were fixed and permeabilized simultaneously before blocking (Volberg et al., 2001). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse and goat anti-rabbit IgGs, TRITC-conjugated phalloidin were purchased from Sigma Aldrich. After final wash of the cells after incubation with the secondary antibodies, cells were counterstained for nucleus with either propidium iodide (PI) or DAPI (Invitrogen).

Immunoblotting and immunoprecipitation

Preparation of crude cell lysates, immunoblotting and immunoprecipitation were all performed as previously described (Pan et al., 2006). 3A9 anti-Alix antibody was routinely used for immunoblotting, and mixed 1A12, 1A3, and 3A9 antibodies were routinely used for Alix immunoprecipitation.

Fractionation of the conditioned medium from WI38 cell cultures

Sub-confluent 100-mm cultures of WI38 cells were switched to fresh culture medium supplemented with 0.1% fetal bovine serum and maintained under this condition for 2 days. The conditioned medium was then collected from these cultures and fractionated by consecutive centrifugations at 1000 *g* for 10 min, at 10,000 *g* for 30 min and at 100,000 *g* for 1 hr with pellets

collected at each step. The pelleted proteins from different steps were dissolved in SDS-PAGE sample buffer. The unpelleted proteins from the last step of the centrifugation were precipitated by 10% trichloroacetic acid (TCA) on ice for 1 hr and pelleted down by centrifugation at 10,000 *g* for 15 min. After the pellets were washed twice with –20°C acetone, the pelleted proteins were dissolved in 8 M urea and samples were mixed with SDS-PAGE sample buffer. The 100,000 *g* pellets of the conditioned medium were also dissolved in RIPA buffer (1% NP-40, 0.5% DOC, 0.1% SDS and 150 mM NaCl in 20 mM Tris-HCl, pH7.4), and samples were fractionated by gel filtration through a 40-ml Superose 6 (Amersham Biosciences, Piscataway, NJ) column pre-equilibrated in TBS (150 mM NaCl in 50 mM Tris, pH 7.4) with 1-ml fractions collected. Proteins in the collected fractions were precipitated with TCA and processed for immunoblotting.

Protein identification by mass spectrometry

A mixture of 1A3, 3A9 and 1A12 anti-Alix antibodies were covalently linked to Affi-prep protein A beads (Bio-Rad, Hercules, CA) through dimethylpimelimidate catalysed reaction (Schneider et al, 1982). Immunoprecipitation of Alix from WI38 cell lysates prepared in RIPA buffer, SDS-PAGE, silver staining, tryptic digestion and protein identification by mass spectrometry were all performed as previously described (Pan et al., 2006).

REFERENCES FOR MATERIALS AND METHODS

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LEGENGS FOR SUPPLEMENTAL FIGURES

Figure S1. Sequence homology between Alix and adhesin. (A) Schematic illustration of the structural features between Alix and adhesin. (B) Sequence alignment between Alix and adhesin by program BLAST TWO SEQUENCES. The + signs indicate similar amino acid residues. The underlined sequences indicate the Src phosphorylation consensus sequence. The boxed sequences indicate the functional domain of extracellular Alix.

Figure S2. Characterization of anti-Alix antibodies. (A) Schematic illustration of Alix fragments generated as GST-tagged recombinant proteins. (B) Immunoblots of similar amounts

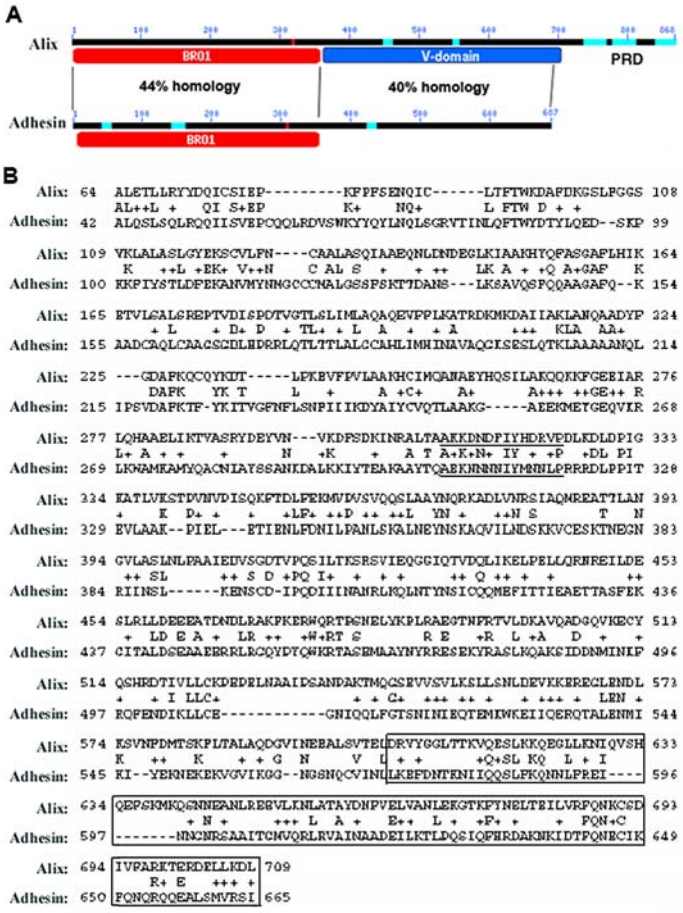
of indicated Alix fragments with 3A9 antibody. (C) Immunoblots of similar amounts of indicated Alix fragments with 1A3 antibody.

Figure S3. Comparison of WI38 and NIH/3T3 cells in morphology, fibronectin assembly and extracellular Alix. (A). Phase contrast images of WI38 and NIH/3T3 cell cultures at low (upper panel) and high cell densities (lower panel). (B) Indirect immunofluorescence staining of WI38 and NIH/3T3 cell cultures with anti-fibronectin antibodies and FITC-conjugated secondary antibodies. (C) Phase contrast images of NIH/3T3 cells grown on fibronectin-coated substrata at low and high densities. (D) Lysates of WI38 and NIH/3T3 cells were immunoblotted with anti-Alix and anti-actin antibodies. (E) Protein extracts of biotinylated cultures of WI38 and NIH/3T3 cells were immunoprecipitated with mouse IgG (mIgG) or anti-Alix antibodies, and the immunoprecipitates were immunoblotted with anti-Alix antibodies (left panel) or probed with streptavidin (right panel).

Figure S4. Alix-coating promotes spreading and integrin-mediated signaling pathway of NIH/3T3 cells. (A) NIH/3T3 cells were seeded onto GST or GST-Alix coated substrata, and relative cell attachments were determined at 1, 2, and 6 hr after cell seeding. Error bars represent standard deviations from triplets of each group. (B) NIH/3T3 cells were seeded onto GST or GST-Alix coated substrata, and the percentage of spread cells were determined at 1, 2, and 6 hr after cell seeding. Error bars represent standard deviations from triplets of each group. (C) NIH/3T3 cells grown on GST- or GST-Alix-coated substratum for indicated times were fixed and immunostained with anti-fibronectin antibodies and FITC-conjugated secondary antibodies.

Figure S5. Alix knockdown inhibits 9EG7 epitope-positive integrin-mediated cell adhesions. At 2 hr after control and Alix knockdown WI38 cells were seeded on coverslips, cells were fixed and immunostained with 9EG7 antibody (upper panel) or antibodies recognizing total $\alpha_5\beta_1$ integrin (lower panel). Arrows indicate focal adhesions.

Figure S6. PDI is a potential binding partner of extracellular Alix. (A) Lysates of biotinylated NIH/3T3 cells were incubated with GST or GSTAlixMB, and proteins eluted from washed beads were stained with Ponceau S or blotted with streptavidin. The asterisk indicates a protein of ~65 kDa that was both pulled down by GST-AlixMB and detected by streptavidin. (B) Lysates of biotinylated WI38 cells were immunoprecipitated with mouse IgG (mIgG) or anti-Alix antibodies, and proteins eluted from washed immunoprecipitates were blotted with streptavidin and immunoblotted with anti-Alix antibodies. The asterisk indicates a protein of ~65 kDa in the Alix immunocomplex that was detected by streptavidin and not by anti-Alix antibodies. HC and LC represent heavy and light chains of the precipitating antibodies, respectively. (C) WI38 cell lysates were immunoprecipitated with covalently linked mIgG or anti-Alix antibodies, and eluted proteins from washed immunocomplexes were resolved by SDS-PAGE and stained with silver. The asterisk indicates a protein of ~65 kDa that was specifically precipitated by anti-Alix antibodies. (D) Sequence alignment of a peptide sequence from the 65-kDa protein with PDI, which is also the beta subunit of prolyl 4-hydroxylase.



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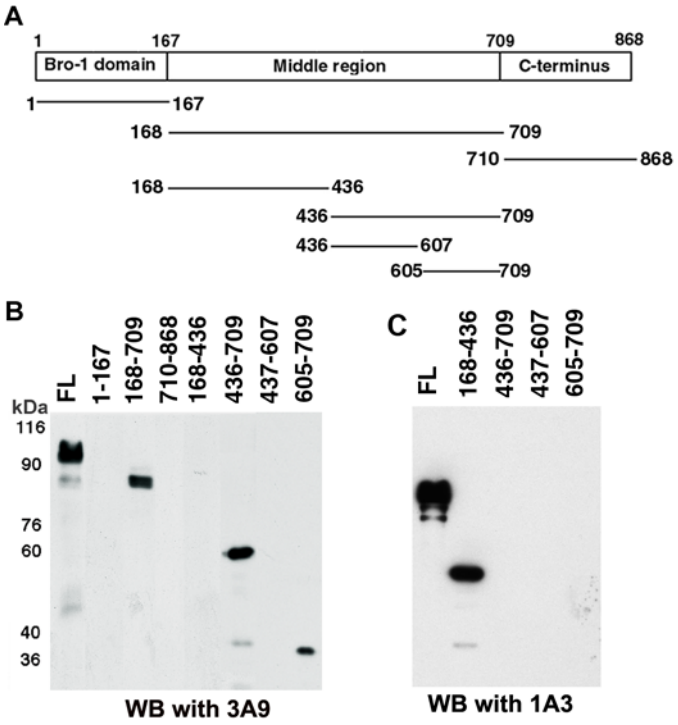
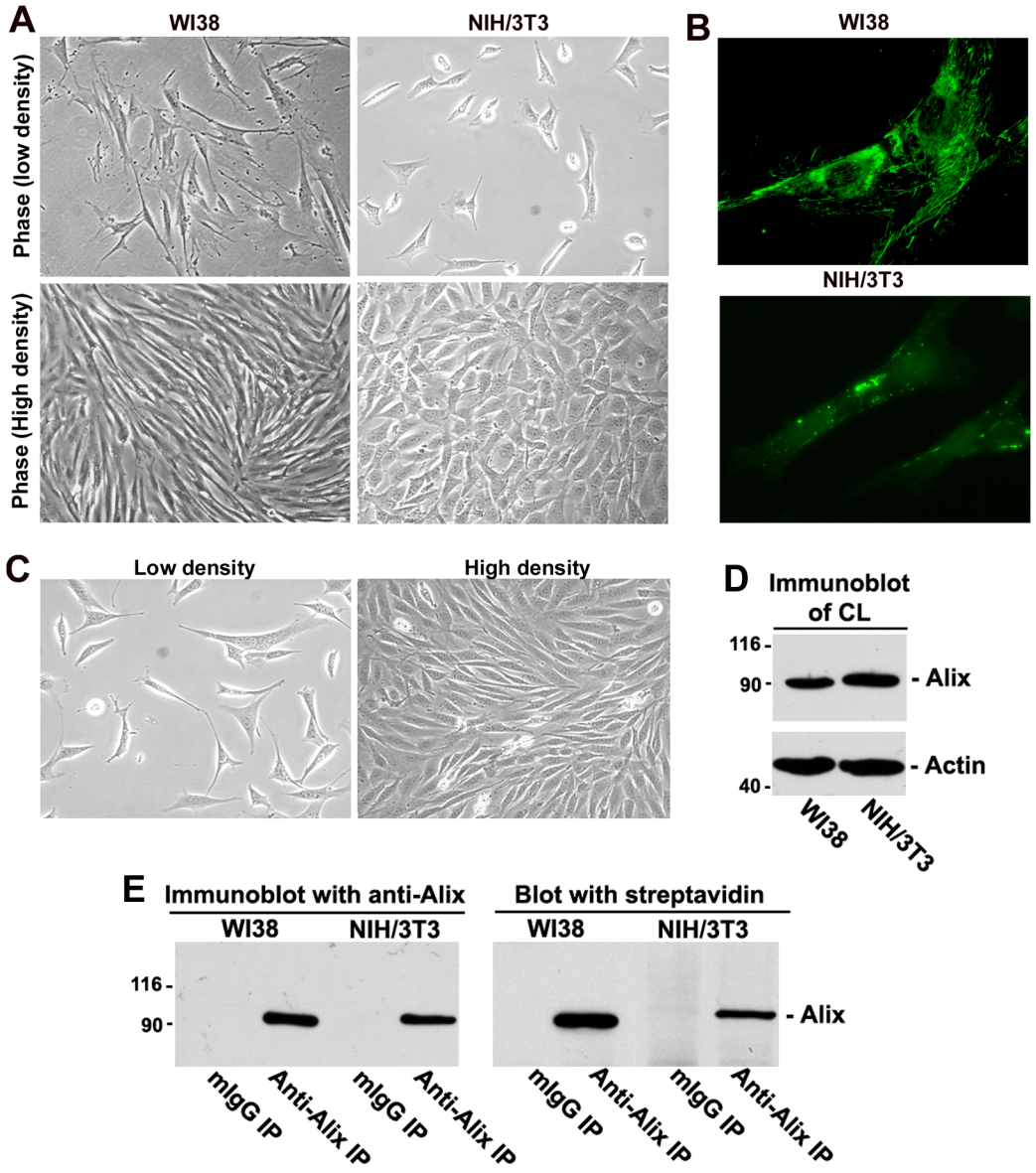


Figure S2. Recognition region of monoclonal anti-Alix antibodies



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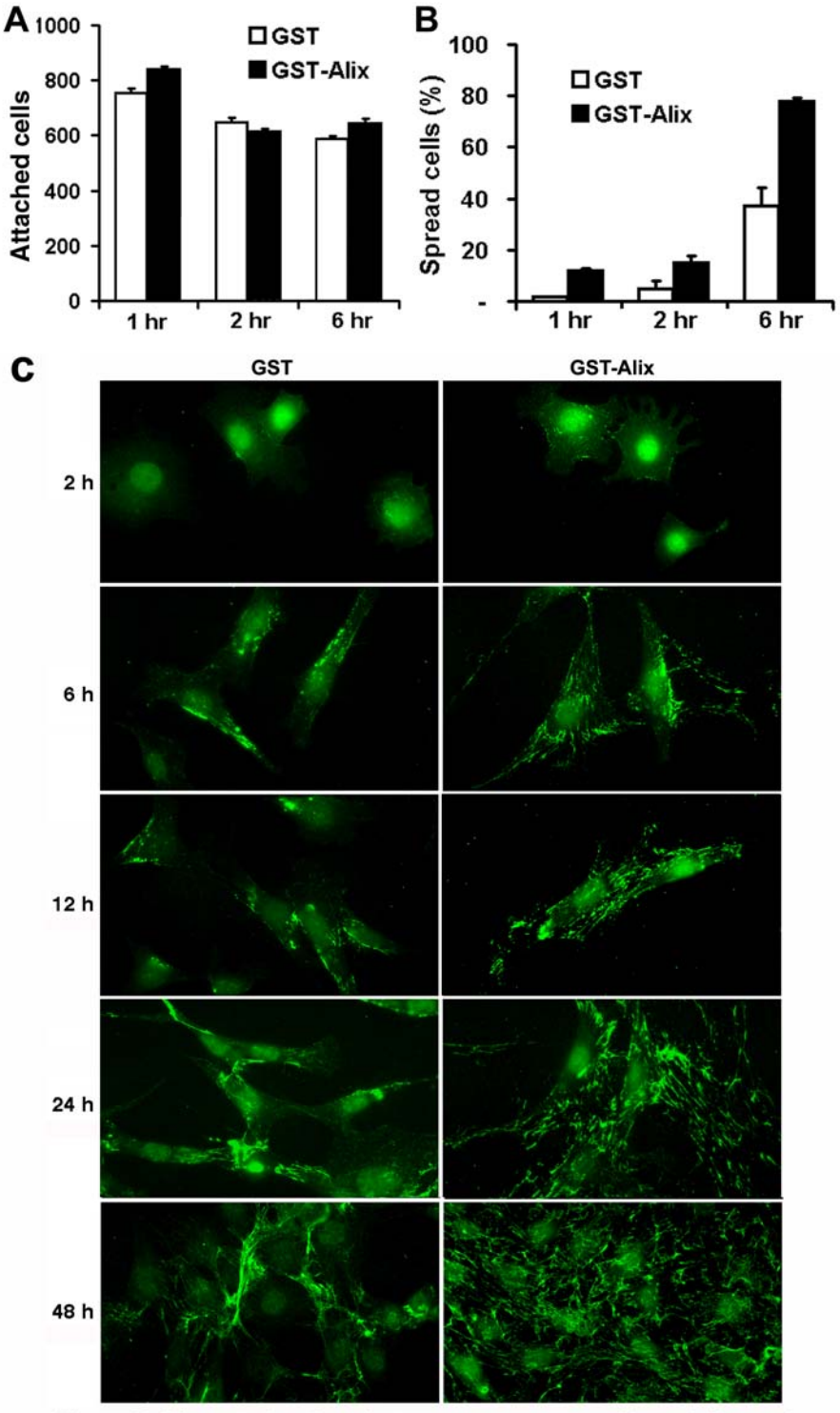


Figure S4. Alix coated on the substratum promotes spreading and fibronectin assembly of NIH/3T3 cells

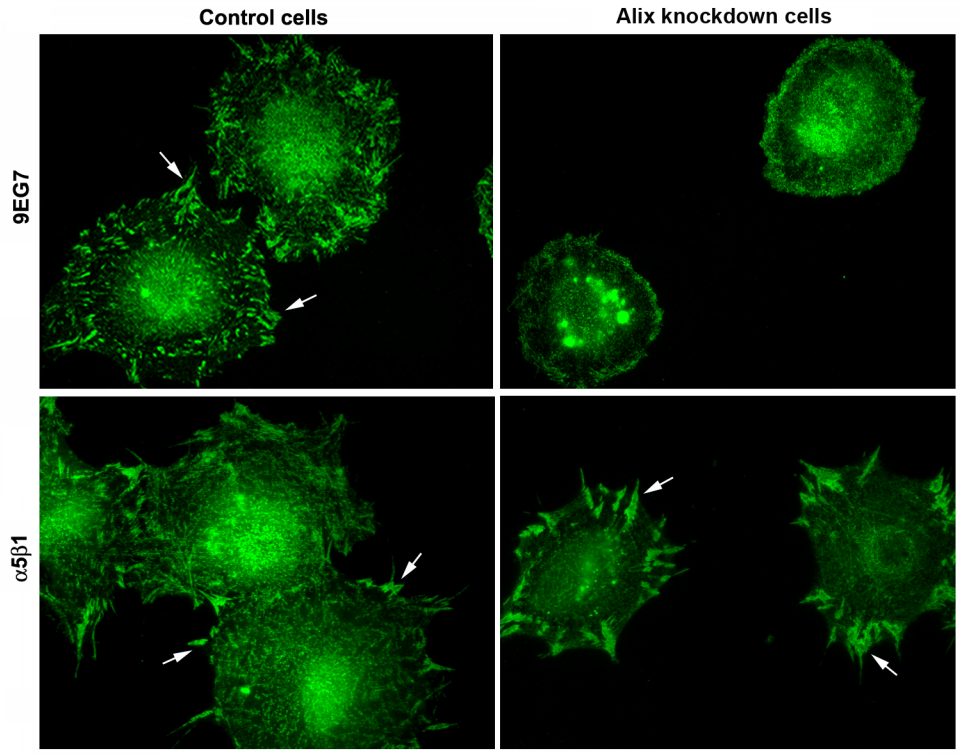
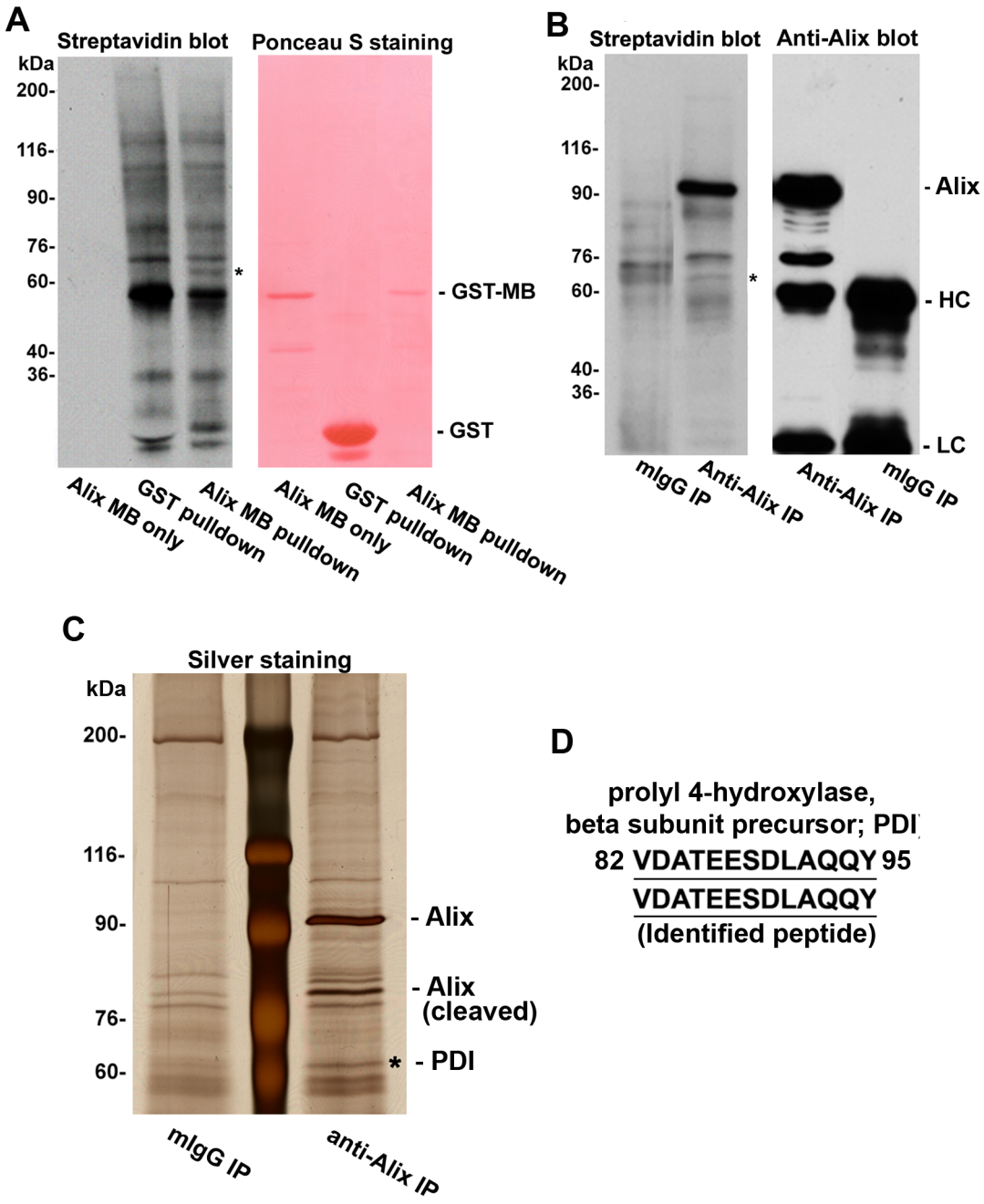


Figure S5. Alix knockdown inhibits 9EG7 epitope-positive integrin-mediated cell adhesions.



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