Mis-localization or Reduced Expression of Arf GTPase-activating Protein ASAP1 Inhibits Cell Spreading and Migration by Influencing Arf1 GTPase Cycling


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The abbreviations used: Arf, ADP-ribosylation factor; ASAP1, Arf-GAP containing SH3, Ankyrin repeats and PH domain; CD2AP, CD2-associated protein; ECM, extracellular matrix; FAK, focal adhesion kinase; FN, fibronectin; GAP, GTPase-activating protein; GST, glutathione S-transferase; mAb, monoclonal antibody; PH, pleckstrin homology; REF, rat embryo fibroblasts; SH3, Src homology 3; siRNA, small interfering RNA
Summary

ADP-Ribosylation Factor (Arf) family of small GTP-binding proteins plays a central role in membrane trafficking and cytoskeletal remodeling. ASAP1 (Arf-GAP containing SH3, Ankyrin repeats and PH domain) is a phospholipid-dependent Arf GTPase-activating protein (Arf-GAP) that binds to protein tyrosine kinases Src and Focal Adhesion Kinase (FAK). Using affinity chromatography and mass spectrometry (MS), we identified the adaptor protein CD2-associated protein (CD2AP) as a candidate binding partner of ASAP1. Both co-immunoprecipitation and GST pulldown experiments confirmed that CD2AP stably interacts with ASAP1 through its N-terminal SH3 domains. Using a mis-localization strategy, we show that sequestration of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein (the three N-terminal SH3 domains of CD2AP fused to Listeria monocytogenes ActA mitochondria-targeting sequence) inhibited REF52 cell spreading and migration in response to fibronectin stimulation. Using an alternative strategy we show that suppressing ASAP1 expression with siRNA duplexes also significantly retarded cell spreading and inhibited cell migration. Furthermore, abrogation of ASAP1 function using either siRNAs or mis-localization approaches caused an increase of GTP loading on Arf1 and loss of paxillin from adhesions. These results taken together with our previous observations that overexpression of ASAP1 inhibits cell spreading and alters paxillin localization to adhesions (Mol. Biol. Cell, 13, 2147-2156, 2002) suggest that the recruitment of certain adhesion components such as paxillin requires dynamic GTP/GDP turnover of Arf1 GTPase.
Introduction

Cell migration is a cyclic process comprised of four basic steps, adhesion, attachment, translocation and retraction of the rear (1,2). Among the many events that are regulated during the migratory cycle, coordinated regulation of the actin cytoskeleton, formation, turnover and maturation of adhesion structures, coordinated translocation of intracellular vesicles have been studied as key events in the process. Of increasing importance is elucidating how membrane internalized from the cell surface is trafficked to the leading edge of migrating cells to fulfill the requirement of surface area increase as a result of cell front extension (3). An understanding of the coordination between membrane traffic, cell adhesion and actin re-organization has been facilitated by the identification and characterization of Arfs (ADP Ribosylation Factors), a family of small GTP-binding proteins. The Arf family members were originally identified as co-factors for Cholera toxin-catalyzed ADP Ribosylation of Gs (4). The primary physiological function of Arfs, however, is to regulate endocytosis and vesicle trafficking by controlling the interaction of coat proteins with intracellular organelle membranes (5-7). More recently, Arfs have also been implicated in the regulation of cytoskeletal remodeling (8-11), although the mechanisms by which they act are poorly understood. Arf1, the prototypic member of this family, is reported to mediate the recruitment of paxillin to focal adhesions and to facilitate Rho-stimulated stress fiber formation in Swiss 3T3 fibroblasts (9). Arf6, the least conserved member of Arf family, cycles between plasma membrane and endocytic compartments, depending on its nucleotide status (12). Both the
constitutively active and the dominant negative forms of Arf6 cause pronounced cell morphology changes when overexpressed in cells (8,10). It has been shown that Arf6 functions primarily through the activation of lipid-modifying enzymes such as phosphatidylinositol 4-phosphate 5-kinase (PIP5-kinase) and the modulation of actin cytoskeleton (11,13). Arf6 also plays a role in clathrin-mediated endocytosis by recruiting Nm23-H1, a nucleoside diphosphate (NDP) kinase, which provides a source of GTP for dynamin-dependent fission of coated vesicles (14). The dual functions of Arf GTPases in both vesicle trafficking and actin cytoskeletal remodeling point to these proteins being candidates for the coordinators that regulate critical steps during cell migration.

Evidence for Arfs as regulators of adhesion also comes from the identification of proteins with Arf-GAP homology that bind to focal adhesion components. Paxillin Kinase Linker (PKL), also known as GIT (G protein-coupled receptor interacting target)2/CAT (Cool-associated, tyrosine-phosphorylated)2, binds directly to the LD4 domain of paxillin and the Guanine Nucleotide exchange Factor (GEF) PIX (PAK-interacting exchange factor) and thus mediates the association of paxillin with a complex composed of PAK (p21-activated kinase), Nck and PIX (15). A PKL-related Arf-GAP, GIT1, binds to paxillin and FAK and its overexpression causes a loss of paxillin from focal adhesions (16). The inhibition of paxillin localization to adhesions is also observed upon overexpressing another distantly-related Arf-GAP ASAP1 (Arf-GAP containing SH3 domain, Ankyrin repeats and PH domain) (17). ASAP1 is found is
peri-nuclear regions, membrane ruffles and focal adhesions (17-19). ASAP1 binds to a proline-rich motif of FAK through its C-terminal SH3 domain (17) and overexpression of ASAP1 inhibits cell spreading and PDGF-induced dorsal ruffles (17,19). An ASAP1-related protein PAPα (Pyk2 C terminus-Associated Protein α)/KIAA0400 interacts with the FAK-related protein tyrosine kinase Pyk2 (20) and paxillin (21). Overexpression of PAPα inhibits paxillin recruitment to focal contacts (21), a phenotype common to overexpression of GIT1 and ASAP1. The phenotypes caused by these Arf-GAPs are likely due to the downregulation of Arfs activity. However, the multi-domain nature of ASAP1 type Arf-GAPs suggests that they may possess other functions in addition to modulating Arf activity in vivo.

Here we show a requirement for ASAP1, a focal adhesion-enriched Arf-GAP, in integrin signaling. We identified CD2AP/CMS (Cas ligand with multiple SH3 domains), an SH3 domain-containing adaptor protein that interacts with ASAP1 via its N-terminal SH3 domains. Using a mis-localization strategy, we show that sequestration of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein (the three N-terminal SH3 domains of CD2AP fused to Listeria monocytogenes ActA mitochondria-targeting sequence) (22) inhibited REF52 cell spreading and migration in response to FN stimulation. Using an alternative strategy we show that suppressing ASAP1 expression with siRNAs also significantly retarded cell spreading and inhibited cell migration. Furthermore, abrogation of ASAP1 function with either siRNAs or mis-localization approaches resulted in an increase of GTP loading on Arf1 and loss of
paxillin from adhesions. These results taken together with our previous observations
that overexpression of ASAP1 inhibits cell spreading and disturbs paxillin localization to
adhesions (17) indicate that the recruitment of certain adhesion components such as
paxillin requires dynamic turnover of Arf1 GTPase. In addition, under conditions in
which paxillin recruitment to cell adhesions was compromised, tyrosine phosphorylation
of paxillin was not substantially affected. These seemingly contradictory observations
raise the possibility that the regulation of Arf1 GTPase by ASAP1 plays an important role
in the stabilization of paxillin in newly formed adhesions.
Experimental Procedures

DNA constructs

The mouse ASAP1 mammalian expression construct pFlagASAP1 was a gift from Paul A. Randazzo (NIH). This construct was generated by subcloning an N-terminal Flag tag and the mouse ASAP1 cDNA into pcDNA3 (Invitrogen) (18). The mouse CD2AP sequence was generously provided by Andrey S. Shaw (Washington Univ. in St. Louis). To generate Flag-tagged full length, SH3ABC and ΔSH3 variants of CD2AP, standard PCR was carried out. The PCR products were digested with BamHI and NotI and subcloned into pFlag2AB (23). The glutathione S-transferase (GST)-CD2AP SH3 constructs were generated by subcloning the corresponding BamHI/NotI fragments into pGEX4T-1 (Amersham Pharmacia). The *Listeria* ActA mitochondria-targeting sequence (mito) (22) was provided by Frank B. Gertler (MIT). Amino acids 361-610 of ActA were amplified with PCR and subcloned into the XbaI and BclI sites of pEGFP-C1 (BD Biosciences) to create GFP-mito. To make the GFP-SH3ABC-mito construct, the three N-terminal SH3 domains of CD2AP were amplified and inserted into the XhoI and BamHI sites of GFP-mito. The HA-tagged Arf1 variants (wild type, Q71L and T31N) and the GST-GGA expression vector were provided by James E. Casanova (University of Virginia).
Identification of ASAP1 binding partners by affinity chromatography

HEK293 cells were grown to 80% confluency and transfected with Flag-tagged ASAP1. 24 hours after transfection, cells were lysed with lysis buffer (20 mM HEPES-KOH, pH 7.8, 50 mM KCl, 150 mM NaCl, 1 mM EDTA, and 1% NP40, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 U/ml of aprotinin, 1 mM sodium vanadate). Affinity purification, sequencing and MS analysis of ASAP1 binding proteins were performed as previously described (24).

In vitro binding assays and GST-GGA pulldown assays

GST fusion proteins were expressed in BL21 E. coli and purified using glutathione-Sepharose (Amersham Pharmacia). Equal amount of GST fusion proteins or GST alone (5 μg) were incubated with 500 μl of 1 mg/ml cell lysates in modified radioimmunoprecipitation assay buffer (RIPA) (50 mM HEPES, pH7.5, 150 mM NaCl, 2 mM EDTA, 5% glycerol, 0.5% Triton X-100, 10 μg/ml leupeptin, 0.5 mM phenylmethylsulfonyl fluoride, 0.05 U/ml of aprotinin, 1 mM sodium vanadate) at 4°C for 1.5 hours. The beads were washed twice with modified RIPA buffer and once with Tris-Buffered Saline (TBS). Associated proteins were subjected to SDS-PAGE and western blotting using an anti-ASAP1 polyclonal antibody. To measure intracellular levels of Arf1-GTP, GST-GGA pulldown assays were performed as previously described (25).
Antibodies and co-immunoprecipitation assay

The anti-ASAP1 rabbit polyclonal antibody was a generous gift from Paul A. Randazzo (NIH). The anti-ASAP1 mouse monoclonal antibody (mAb) was purchased from BD Biosciences. Anti-CD2AP rabbit polyclonal antibody was purchased from Santa Cruz. Anti-Flag mAb M5 was purchased from Sigma. Anti-paxillin mAb, anti-FAK mAb and anti-GFP mAb were purchased from BD Biosciences. Anti-Paxillin phospho-Tyr31 and phospho-Tyr118 were purchased from BioSource Inc. Erk1 mAb was a gift from M. J. Weber (University of Virginia). Anti-HA and anti-Arf1 polyclonal antibodies were provided by James E. Casanova (University of Virginia). VDAC polyclonal antibody was a product from Oncogene Research Products.

To immunoprecipitate endogenous ASAP1, 5 µg of anti-ASAP1 mAb was incubated with 500 µl of Phosphate-Buffered Saline (PBS) containing 50 µl of anti-mouse IgG-coupled agarose beads (Sigma) at 4°C for 1 hour. The beads were washed 3 times with cold PBS and incubated with 750 µg of clarified cell lysates at 4°C for 2 hours. Immune complexes were collected by centrifugation, washed three times with 1.0 ml of lysis buffer, separated by SDS-PAGE and western blotted with anti-CD2AP antibody. Antibody binding was detected using horseradish peroxidase-conjugated sheep anti-rabbit IgG followed by Enhanced ChemiLuminescence (ECL, Amersham Pharmacia). To immunoprecipitate endogenous CD2AP, a similar procedure was followed except that polyclonal anti-CD2AP antibody was coupled onto 50 µl of Protein A-conjugated sepharose beads (Sigma). To immunoprecipitate Flag-tagged CD2AP variants, 20 µl of
M2-agarose (Sigma) was incubated with 500 µg of clarified cell lysates in lysis buffer at 4°C for 2 hours and the immune complexes were subsequently subjected to the washing steps as described above.

**Cell culture, transfection and immunofluorescence microscopy**

HEK293 and REF52 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen), 10 µg/ml penicillin and 0.25 µg/ml streptomycin (Invitrogen). For transient transfection experiments, cells were grown to ~70% confluency in 100 mm dishes and transfected with 4 µg DNA using PolyFect™ (Qiagen). Twenty-four hours after transfection, REF52 cells were trypsinized and replated on FN in DMEM, fixed with 4% paraformaldehyde and immunostained as described previously (26).

**Mitochondria staining, fractionation and cell spreading assay**

To visualize the subcellular localization of mitochondria, pre-warmed DMEM/10% fetal bovine serum containing 125 nM of MitoTracker Red CMXRsos (Molecular Probes) was added onto REF52 cells previously plated on FN. After 40 minutes incubation at 37°C, the medium was removed and the cells were washed and fixed with 4% paraformaldehyde as described above. The mitochondria and the GFP fusion proteins carrying mito-targeting sequence were detected with fluorescence microscopy. The mitochondria fractions from whole cell lysates were prepared using the procedure of
Eskes et al. (27) with a modified mitochondria homogenization buffer (0.2 M mannitol, 10 mM HEPES-NaOH, pH 7.4, 50 mM sucrose, 1 mM EDTA supplemented with proteases inhibitors). For cell spreading assays, REF52 cells were trypsinized and re-plated on FN for the indicated times 24 hours after transfection. The quantitation of cell spreading was performed as described previously (17,28).

**Cell migration assays**

Cell migration was assessed using modified transwell chambers (8 µm pores, Becton Dickinson) as previously described (29). To assess FN stimulated haptotaxis, the underside of the membrane was coated with 1.5 µg/ml FN suspended in PBS in the lower chamber and PBS was placed in the upper chamber. After incubating the chambers overnight at 4°C, the FN solution was removed and DMEM was added to each chamber. For EGF-mediated chemotaxis, DMEM supplemented with 10 ng/ml EGF was added to the lower chamber and unsupplemented DMEM was added to the upper chamber. REF52 cells (1 X 10^5) were suspended in the upper chamber and allowed to migrate for 6 hours at 37°C. Nonmigrating cells were removed with a cotton swab. Cells migrating through the membrane were washed twice with PBS, fixed in 4% paraformaldehyde at room temperature for 20 min, and counted under a Zeiss Axiovert 135TV inverted microscope. Nontransfected cells were stained with crystal violet prior to counting to aid in visualization.
**Preparation of siRNAs and transfection**

Target specific siRNA duplexes with symmetric 3’ dTdT overhangs were synthesized by Dharmacon. Two siRNA sequences were selected from position 902-920 (5’- CAG CUA ACU GCA CUC CGA G -3’) and position 1642-1660 (5’- UGA UAU UAU GGA AGC AAA U -3’) of mouse ASAP1b (GenBank accession number AF075462). Both siRNA duplexes reduced ASAP1 expression to a similar extent and induced similar cellular phenotypes. Transient transfection of REF52 cells with siRNAs was performed using a calcium phosphate protocol (30).

**Statistical analysis**

Each experimental group was analyzed using single-factor analysis of variance. P values were obtained by performing two-tailed student’s t test using Microsoft Excel software. Statistical significance was defined as P < 0.05.
Results

Identification of Arf-GAP ASAP1 Binding Partners

To better understand the role of ASAP1 in cellular processes regulating dynamic actin organization, we exploited affinity chromatography and mass spectrometry (MS) to identify proteins that interact with full length ASAP1 (Fig. 1). Using Flag-tagged ASAP1 coupled to agarose beads, several proteins were identified as ASAP1 binding partners. MS analysis revealed sequence matches with known binding partners for ASAP1 such as FAK, as well as a novel binding protein, an 80 kDa adaptor protein CD2AP/CMS, hereafter referred to as CD2AP (Fig. 1B). Peptides identified by MS represent 42% coverage of full length CD2AP. To further validate this interaction, Flag-ASAP1 was immunoprecipitated from extracts of HEK293 cells and ASAP1 was recovered by elution with two sequential applications of Flag peptide followed by elution with 1% SDS. The individual fractions were analyzed with SDS-PAGE and Western blotting along with lysate control (Fig. 1C). As shown in Fig. 1C, the profile of CD2AP elution paralleled that of Flag-tagged ASAP1, indicating CD2AP stably associates with ASAP1.

CD2AP associates with ASAP1 in vivo

To demonstrate that CD2AP and ASAP1 form stable complexes within the cell, we performed co-immunoprecipitation experiments. Endogenous ASAP1 was immunoprecipitated from HEK293 cell lysates using an ASAP1-specific antibody.
Endogenous CD2AP was readily detected in ASAP1 immune complexes as revealed by blotting with a CD2AP-specific antibody (Fig. 2A, lane 2). The association of ASAP1 with both FAK (17) and CD2AP raises the possibility that localization of ASAP1 within the cell is spatially controlled by its binding partners. As shown in Fig. 2B, when endogenous ASAP1 was immunoprecipitated from HEK293 cell lysates, both FAK and CD2AP were found in the immune complexes. However, in a reciprocal experiment, only ASAP1, not FAK was detected in CD2AP immunoprecipitation complexes (Fig. 2C). These results indicate that intracellular ASAP1 is present in at least two pools, one associated with FAK, the other with CD2AP.

The N-terminal SH3 Domains of CD2AP stably associates with ASAP1 both in vivo and in vitro

Both ASAP1 and CD2AP are multi-domain proteins containing SH3 domains and proline-rich motifs (Fig. 1A). To map the ASAP1-binding site on CD2AP, three Flag-tagged CD2AP variants encompassing the full length, the N-terminal region or the C-terminal moiety of CD2AP were expressed in HEK293 cells, immunoprecipitated with anti-Flag antibody and assayed for the presence of ASAP1. As shown in Fig. 3A, endogenous ASAP1 bound to the full length and the N-terminal variant of CD2AP in approximately equal amounts (lanes 3 and 6) whereas the C-terminal variant of CD2AP failed to associate with ASAP1 (lane 9). These data show that CD2AP interacts with ASAP1 through its N-terminal SH3 domains.
The N-terminal region of CD2AP contains three SH3 domains (Fig. 1A, designated as SH3A, B and C, respectively). To examine which SH3 domain contributes to CD2AP-ASAP1 interaction, we generated four GST fusion proteins encompassing either individual SH3 domain of CD2AP or the entire N-terminal region with all three SH3 domains. These GST fusions were expressed and purified from BL21 *E. coli* then utilized to pulldown endogenous ASAP1 from HEK293 cell lysates. As shown in Fig. 3B, both SH3A and SH3B readily associated with ASAP1 *in vitro* whereas SH3C failed to do so. These data indicate that only the first two SH3 domains of CD2AP contribute directly to CD2AP-ASAP1 interaction.

**Mis-localization or reduced expression of ASAP1 inhibits cell spreading on FN**

The role of Arf-GAPs such as ASAP1 in cytoskeletal remodeling most likely reflects its localization to either focal adhesions, membrane ruffles, or both. Because the subcellular localization of ASAP1 may be spatially controlled during cell spreading and migration, we speculated that the depletion of endogenous ASAP1 from its correct destination would compromise its function and lead to corresponding phenotypes.

The C-terminus of *Listeria monocytogenes* ActA is targeted to mitochondrial membranes when exogenously expressed in eukaryotic cells (22). As shown in Fig. 4A, expression of a fusion protein containing GFP fused to amino acids 361-610 of ActA efficiently targeted GFP to mitochondria as revealed by co-localization with mitochondria markers Mito-Tracker (Fig. 4A) and cytochrome C (data not shown). We
exploited the strong association between CD2AP and ASAP1, making a GFP-expression construct in which GFP was fused to the N-terminal SH3 domains of CD2AP which in turn was fused to the mitochondria-targeting sequence of ActA (hereafter referred to as mito). We hypothesized that this fusion protein (hereafter referred to as SH3ABC-mito) would serve as a molecular sink to trap ASAP1 onto mitochondria membranes. When expressed in REF52 cells, SH3ABC-mito efficiently sequestered endogenous ASAP1 onto mitochondria as evidenced by the co-staining of mitochondria and ASAP1 (Fig. 4B, panels c and f). In parallel cell fractionation experiments, the mitochondrial fraction from SH3ABC-mito expressing cells was significantly enriched for ASAP1 compared to GFP-mito expressing cells (Fig. 4C, compare lanes 4 and 6).

To assess the consequences of mis-localization of ASAP1 on cell spreading, cells expressing SH3ABC-mito were plated on FN and the rate of cell spreading was measured (Fig. 4D). SH3ABC-mito-transfected cells exhibited a significant retardation of cell spreading compared to GFP-transfected control cells after plating on FN for one hour. Four hours after initial plating, approximately 50% of SH3ABC-mito-transfected cells still displayed rounded phenotype, indicating that mis-localization of ASAP1 prevents efficient cell spreading on FN.

The importance of ASAP1 in the cellular response to FN stimulation was further addressed using small interfering RNAs (siRNAs) targeted to ASAP1 to reduce intracellular levels of ASAP1. Two different siRNAs specific for ASAP1 were transfected into REF52 cells. Each oligonucleotide was efficient at reducing ASAP1
expression. As shown in Fig. 5 (panels A and B), a pool of such siRNAs reduced ASAP1 levels in REF52 cells by 75%. To assess the effects of reducing ASAP1 levels on cell spreading, cells treated with siRNAs for ASAP1 or control siRNA (for luciferase, a gene not expressed in eukaryotic cells) were plated on FN and the rate of cell spreading was determined. As shown in Fig. 5C, attenuation of ASAP1 levels resulted in a significant delay of cell spreading on FN. These data support the observations shown in Fig. 4D, indicating that ASAP1 function is necessary for efficient signaling in response to FN stimulation.

**Mis-localization or reduced expression of ASAP1 inhibits cell migration**

Given the importance of focal adhesion turnover on cell motility and our previous report that ASAP1 overexpression disturbs focal adhesion architecture (17), the effects of ASAP1 depletion on cell motility were tested using a transwell migration assay. FN and EGF were used as haptotaxis- and chemotaxis-promoting agents, respectively. As shown in Fig. 6A, in cells treated with siRNAs for luciferase, both FN and EGF stimulated cell migration by 2 to 3 fold. On the other hand, siRNA-mediated reduction of ASAP1 resulted in a 50% decrease in the basal level of chemokinesis (black column). In addition, attenuation of ASAP1 expression significantly inhibited the haptotactic response to FN (white column) and the chemotactic response to EGF (gray column). Of note, the ASAP1-depleted cells appeared to retain the ability to respond to EGF as evidenced by an approximately 2 fold increase over basal chemokinesis. A similar
phenotype was obtained with ASAP1-mislocalized cells (Fig. 6B), indicating that ASAP1 function is indispensable for FN-stimulated haptotaxis.

**Abrogation of ASAP1 increases GTP loading on Arf1 and alters paxillin localization to adhesions**

ASAP1 exhibits strong GAP activity towards Arf1 in vitro (18). To investigate the mechanism by which ASAP1 contributes to the FN-dependent cell spreading and migration, we took advantage of the GTP-dependent interaction of Arf proteins with GGAs (Golgi-localized, γ ear-containing, Arf-binding proteins), a family of Arf-dependent adaptors (31). As shown in Fig. 7A, GST-GGA fusion proteins selectively precipitated the constitutively active, GTP-bound mutant Arf1Q71L, whereas the GDP-bound dominant negative mutant Arf1T31N was undetectable in the GST-GGA beads.

The GST-GGA pulldown assay was utilized to assess the change in the GTP-loading on endogenous Arf1 in ASAP1-depleted cells (Fig. 7B). As shown in Fig. 7B and 7C, the suppression of ASAP1 levels led to a 40% increase of GTP loading on Arf1. Mis-localization of endogenous ASAP1 also increased Arf1-GTP to a similar extent (Fig. 7D and E). These data are consistent with ASAP1 functioning as a GAP for Arf1 and the effects on cell adhesion being mediated, at least in part, by alteration in the level of GTP-Arf1.
Arf1 GTPase has been implicated in the translocation of paxillin to cell adhesions in
Swiss 3T3 fibroblasts (9). To investigate if the regulation of Arf1 by ASAP1 plays a
role in paxillin localization to adhesions in vivo, we examined paxillin localization in
both ASAP1-mislocalized cells and cells treated with ASAP1 siRNAs. As shown in Fig.
8A, paxillin was poorly organized in adhesions (e.g., showing an increase in diffuse
cytoplasmic staining) when ASAP1 was mis-targeted to mitochondria. The
redistribution of paxillin was quantitated by assessing paxillin localization in transfected
cells. As shown in Fig. 8B, about 40% of ASAP1-mislocalized cells exhibited a “minus
cell” phenotype (e.g., poorly organized paxillin), compared to only 15% of the cells
expressing GFP-mito. In parallel experiments we assessed the distribution of paxillin in
cells treated with ASAP1 siRNAs and control luciferase siRNAs. As shown in Fig. 9,
the reduction in ASAP1 expression resulted in an approximately 35% increase in the
number of cells exhibiting poorly organized paxillin. In contrast, treatment of cells with
ASAP1 siRNAs had no effect on the localization of vinculin to cellular adhesions (data
not shown). We and others have previously reported that overexpression of ASAP1
inhibits cell spreading and causes loss of paxillin from adhesions (17,19). These
observations taken together with the observations described above raise the possibility
that the recruitment of certain adhesion components such as paxillin requires the dynamic
turnover of Arf1 small GTPase rather than its GTP-bound active form.

Paxillin undergoes tyrosine phosphorylation at two major sites, Tyr31 and Tyr118,
aupon cell adhesion and activation of integrin signaling (32-34). These tyrosine
phosphorylation events are mediated by Src family kinases and generate two
SH2-docking sites for members of the Crk family adaptor proteins which in turn initiate
downstream signaling to coordinate integrin-mediated cell motility (35). As shown
above, abrogation of ASAP1 function disturbs normal paxillin localization to adhesions.
Because phosphorylation of paxillin Tyr31 and Tyr118 is driven by adhesion formation,
we speculated that attenuation of ASAP1 would negatively impact integrin-dependent
tyrosine phosphorylation of paxillin in a similar manner. Surprisingly, in cells treated
with ASAP1 specific siRNAs, the increase in paxillin tyrosine phosphorylation in
response to FN stimulation paralleled that in control cells as measured by
immunoblotting with as a phospho-Tyr31 specific antibody (Fig. 10A). In addition,
under conditions in which paxillin recruitment to adhesions was compromised and was
thus cytosolic (Fig. 10B, panels c and d), phosphorylation on paxillin Tyr118 was still
detectable (Fig. 10B, panels g and h). These observations raise the possibility that the
regulation of Arf1 GTPase by ASAP1 plays a role in the stabilization of paxillin in
adhesions rather than the translocation of paxillin to adhesions. Thus, compromising
ASAP1 function and Arf1 turnover in ASAP1-depleted cells may result in the enhanced
release of tyrosine-phosphorylated paxillin from adhesions.
Discussion

In this report we show that CD2AP, a ubiquitously expressed adaptor protein, stably associates with the Arf GTPase-activating protein ASAP1 through its N-terminal SH3 domains. Mis-localization of endogenous ASAP1 to mitochondria with a CD2AP SH3-mito fusion protein inhibits FN-mediated cell spreading and cell migration. Using a totally different approach, we show that suppressing ASAP1 expression with siRNAs retards cell spreading and inhibits cell migration. Abrogation of ASAP1 function using either mis-localization or siRNA strategies causes increased intracellular Arf1-GTP and loss of paxillin from cell adhesions. Given the previous studies (16,17,21) that overexpression of ASAP1 and related Arf-GAPs influences similar aspects of cellular function, we concluded that ASAP1 contributes to the process of adhesion assembly by regulating dynamic GTP/GDP cycling of Arf1 GTPase.

Different domains of ASAP1 have been implicated in the targeting of ASAP1 to its correct intracellular localizations. ASAP1 is found in perinuclear regions, focal adhesions and membrane ruffles (17,18). We have reported in an earlier study that ASAP1 binds to FAK through its C-terminal SH3 domain (17), which accounts for its localization to cell adhesions. In addition, ASAP1 possesses a PIP$_2$-interacting PH domain (18) that could potentially target ASAP1 to plasma membrane. In this study we show that ASAP1 forms stable complexes with the adaptor protein CD2AP. As shown previously (36-39), CD2AP localizes to membrane ruffles in different cell types. Cin85, a close relative of CD2AP, is recruited to RTK (receptor tyrosine kinase)/Cbl complexes.
upon growth factor stimulation (40). Therefore, the interaction of ASAP1 with CD2AP provides a possible mechanism by which ASAP1 localization on the plasma membrane is further refined upon growth factor receptor engagement. The observed localization of ASAP1 to the plasma membrane challenges the dogma that ASAP1 functions solely as an Arf1 GAP in that Arf6, not Arf1, is found at the cell periphery. The failure to see an increase in GTP loading of Arf6 upon inhibition of ASAP1 expression suggests that at least in REF52 cells, ASAP1 is not an efficient GAP for Arf6. Thus, a small functional pool of Arf1 is likely present at peripheral membrane regions.

Increasing evidence indicates that GTP/GDP turnover is important for the function of the Arf family of GTPases. A conceptually traditional role of Arf-GAPs is to promote the GTP hydrolysis on Arfs and thus is predicted to counter the phenotypes caused by Arf-GEFs or catalytically active Arfs. However, as demonstrated in this report, abrogation of ASAP1 function with different approaches caused similar phenotypes to those observed with ASAP1 overexpression, suggesting an important role for the dynamic cycling of Arf1 GTPase in adhesion assembly rather than its active GTP-bound form. In the case of Arf6, the expression of a constitutively active mutant Arf6Q67L generally blocks the actin-dependent protrusive activity caused by Arf6 GEF (41,42), consistent with Arf6 cycling between active and inactive forms to function properly. In a recent study, Hashimoto et al. showed that both Arf6Q67L and Arf6T27N inhibited the invasiveness of breast cancer cells MDA-MB-231 (43), indicating that a similar requirement for dynamic GTP/GDP turnover exists for Arf6 function as well.
As demonstrated in this study, both FN-dependent haptotaxis and EGF-dependent chemotaxis were impaired under conditions that attenuate ASAP1 function. However, cells with significantly reduced levels of ASAP1 retained the ability to respond to EGF, albeit at reduced levels. This contrasts to the relatively complete inhibition of chemokinesis and FN-stimulated haptotaxis. These results indicate that ASAP1 depletion leads to an inhibition of common pathways required for haptotaxis and chemotaxis. However, EGF may activate other signaling pathways that partially compensate for the motility defects induced by the loss of ASAP1 function. It is possible that other compensatory Arf GAPs are exploited by EGF under ASAP1-depleted conditions.

The role of ASAP1 in chemotaxis appears complex. In an earlier study, Furman et al. showed that overexpression of ASAP1 enhances cell migration toward PDGF (44). In contrast, ASAP1 overexpression was shown to inhibit the formation of PDGF-induced membrane ruffles in a GAP-dependent manner (19). We have observed an inhibitory effect of ASAP1 overexpression on EGF-dependent chemotaxis (Liu and Parsons, unpublished data). However, it is unclear to what extent PDGF and EGF signal to common migration machinery.

Arf1 is reported to mediate paxillin recruitment from a perinuclear region to adhesions (9). Both overexpression of ASAP1 (17) and abrogation of ASAP1 function alter the localization of paxillin but not vinculin to cellular adhesions. Surprisingly, tyrosine phosphorylation of paxillin was not significantly reduced under conditions in
which paxillin localization was affected. Because it is generally assumed that tyrosine phosphorylation of paxillin takes place in response to FAK activation and complex formation with paxillin (45), these observations indicate that paxillin stability in adhesions may be compromised in the absence of Arf1 turnover. We suggest that Arf1 may be an important regulator of adhesion dynamics in response to integrin engagement and activation.
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Figure legends

**Fig. 1. Identification of CD2AP as an ASAP1 binding partner.** (A) Schematic representation of the structural domains of ASAP1, CD2AP and CD2AP variants used. The individual domains of ASAP1 are indicated: unique region, PH domain, GAP domain, Ankyrin repeats, proline-rich motif and SH3 domain. The three SH3 domains, proline-rich motif and coiled-coil domain of CD2AP are denoted for full-length CD2AP, N-terminal SH3ABC and C-terminal ΔSH3 variants. (B) Flag-tagged ASAP1 expressed in HEK293 cells was immunoprecipitated with M2-agarose beads and subsequently eluted with Flag peptide as described in Experimental Procedures. Lysate input (input) and bound proteins (IP) were analyzed with SDS-PAGE and visualized by silver staining. Bands present in the ASAP1 IP lane but not present in control lanes (24) were excised and subjected to MS analysis. Positions of CD2AP and Flag-ASAP1 are indicated. (C) Flag-tagged ASAP1 was immunoprecipitated as in (B) and subjected to two sequential elutions with Flag peptide (lanes 2 and 3) and a final elution with SDS (lane 4). The recovered proteins and lysate control (lane 1) were separated by SDS-PAGE and Western immunoblotted with antibodies specific for Flag, FAK and CD2AP, respectively.

**Fig. 2. Co-immunoprecipitation of CD2AP and ASAP1.** (A) Endogenous ASAP1 was immunoprecipitated from 500 µg of HEK293 whole cell lysate using an anti-ASAP1 antibody. The presence of endogenous CD2AP in the immune complexes was assessed using a rabbit anti-CD2AP antibody. The blot was stripped and the efficiency of
ASAP1 immunoprecipitation was determined by blotting with anti-ASAP1 antibody. In lane 1, 25 µg of whole cell lysates were analyzed for protein expression. (B) Endogenous ASAP1 was immunoprecipitated as in (A) and the presence of endogenous CD2AP and FAK in the immune complexes was determined using CD2AP-specific and FAK-specific antibodies, respectively. (C) Endogenous CD2AP was immunoprecipitated from extracts of HEK293 cells using an anti-CD2AP antibody. The presence of endogenous ASAP1 and FAK in the immune complexes was determined using ASAP1-specific and FAK-specific antibodies, respectively.

**Fig. 3. The N-terminal SH3 domains of CD2AP interact with ASAP1.** (A) Flag-tagged full-length CD2AP and N-terminal SH3ABC variant, C-terminal ∆SH3 variant (Fig. 1A) were transfected into HEK293 cells. The exogenously expressed CD2AP variants were immunoprecipitated from cell lysates using anti-Flag M2 agarose beads. Bound proteins were recovered with protein sample buffer and separated by SDS-PAGE. The presence of endogenous ASAP1 in the immune complexes was analyzed using ASAP1-specific antibody (IP, lanes 3, 6 and 9). Aliquots (25 µg) of cell lysate (L, lanes 1, 4 and 7) and flowthrough (FL, lanes 2, 5 and 8) were analyzed to assess the efficiency of the immunoprecipitation. (B) To map the targeting site of ASAP1, variants of CD2AP-SH3 domains (SH3A: lane 3; SH3B: lane 4; SH3C: lane 5; SH3ABC: lane 6) were fused to GST. The GST fusion proteins were incubated with 500 µg of HEK293 cell lysates as described in Experimental Procedures. The
associated proteins were subjected to SDS-PAGE and Western blotting using an anti-ASAP1 antibody.

**Fig. 4.** Mis-localization of endogenous ASAP1 to mitochondria inhibits cell spreading on FN.  (A) EGFP was fused to a mitochondria targeting sequence (amino acids 361-610 of *Listeria monocytogenes* ActA) as described in Experimental Procedures. Co-localization of Mito-Tracker staining with GFP indicated targeting of the fusion protein to mitochondria in REF52 cells.  (B) REF52 cells were transfected with GFP (panels a and d), GFP-mito (panels b and e) and GFP-SH3ABC-mito (panels c and f), respectively.  Images show the localization of GFP (panels a, b and c) and endogenous ASAP1 (panels d, e and f) detected by immunostaining with anti-ASAP1 antibody.  Note the apparent change in the morphology/organization of the mitochondria in response to expression of SH3ABC-mito.  It is unclear whether this is due to alterations in the shape of the poorly spread fibroblasts or is a consequence of targeting the SH3ABC-mito to the mitochondrial fraction.  (C) REF52 cells were transfected with GFP, GFP-mito and GFP-SH3ABC-mito, respectively.  24 hours after transfection, cell lysates were prepared in hypotonic homogenization buffer and mitochondrial fractions were isolated as described in Experimental Procedures.  For each transfection, 75% of the whole cell lysate (WCL) was used to prepare the mitochondrial fraction (Mit), 25% of the WCL was used as a loading control to analyze protein expression.  The proteins in WCL and Mit were separated with SDS-PAGE and Western blotted with antibodies
specific for ASAP1, a mitochondria marker VDAC (voltage-dependent anion channel) and GFP. To assess the efficacy of ASAP1 mis-localization to mitochondria, the amount of ASAP1 present in the mitochondrial fraction (ASAP1-Mit) and whole cell lysate (ASAP1-WCL) was determined using Alpha Innotech™ software. The ratios of ASAP1-Mit/ASAP1-WCL were corrected for the relative volumes of each fraction and the transfection efficiency and then expressed as Relative Enrichment (RE). The recovery of VDAC in the mitochondrial fraction of the individual experiments was 65% for cells transfected with GFP, 68% for GFP-mito and 70% for GFP-SH3ABC-mito, respectively. (D) REF52 cells were transfected with GFP, GFP-mito and GFP-SH3ABC-mito, respectively. 24 hours after transfection, cells were trypsinized and replated on FN for indicated amount of times. The extent of cell spreading was assessed as described previously (17, 28). The data represent mean ± S.D. for 3 independent experiments. The P values for the inhibition of cell spreading by SH3ABC-mito were less than 0.05.

Fig. 5. siRNAs-mediated reduction of ASAP1 expression inhibits cell spreading on FN. (A) siRNAs specific for Luciferase (Luc) or ASAP1 were transfected into REF52 cells as described in Experimental Procedures. Cells were lysed 72 hours after transfection and the expression level of ASAP1 was examined by immunoblotting with an anti-ASAP1 antibody. Equal loading of proteins was determined by immunoblotting with an Erk1 specific antibody. (B) The levels of ASAP1 were determined by
quantitation of multiple experiments using Alpha Innotech™ software. The data represent mean ± S.D. for 3 independent experiments (*, P value = 0.004). (C) REF52 cells were transfected with siRNAs specific for Luciferase (Luc) or ASAP1 as described in (A). 72 hours after transfection, cells were harvested and spreading assays were performed as described in Fig. 4D. The P values for the inhibition of cell spreading by reduced ASAP1 expression were less than 0.05.

Fig. 6. Abrogation of ASAP1 alters cell migration. (A) REF52 cells were transfected with siRNAs specific for Luciferase or ASAP1. 72 hours after transfection, cells were placed in the upper chambers of transwell dish and migration towards unsupplemented culture medium (mock), 1.5 µg/ml FN (FN) or 10 ng/ml EGF (EGF) was measured as described in Experimental Procedures. The data represent the mean relative migration ± S.D. for 3 independent experiments (*, P value < 0.05). (B) REF52 cells were transfected with GFP-tagged mito or SH3ABC-mito constructs. 24 hours after transfection, cells were placed in transwell chambers and migration of GFP-positive cells was determined as described in Experimental Procedures. The data represent mean ± S.D. for 3 independent experiments (*, P value < 0.05).

Fig. 7. Depletion of ASAP1 induces an increase of Arf1-GTP. (A) REF52 cells were transfected with HA-tagged Arf1 Q71L, T31N or WT variants. Cell lysates were incubated with GST-GGA resins and bound proteins were resolved by SDS-PAGE as
described in Experimental Procedures. The amount of GTP-bound Arf was measured by immunoblotting with an anti-HA antibody. (B) REF52 cells were transfected with siRNAs specific for Luciferase (Luc) or ASAP1. 72 hours after transfection, cells were lysed and GST-GGA pulldown assays were performed as in (A) except that GTP-bound endogenous Arf1 was measured using an antibody specific for Arf1. (C) Quantitation of GST-GGA pulldown assays in (B). The ratios of Arf1-GTP/total Arf1 were determined by quantitation of the Arf1 protein detected in the immunoblot. The data represent mean ± S.D. for 3 independent experiments (*, P value < 0.05). (D) REF52 cells were transfected with GFP, GFP-mito or GFP-SH3ABC-mito. 24 hours after transfection, cells were lysed and GST-GGA pulldown assays were carried out as in (B). (E) Quantitation of GST-GGA pulldown assays in (D) was carried out as described above. The data represent mean ± S.D. for 3 independent experiments (*, P value < 0.05).

Fig. 8. Mis-localization of endogenous ASAP1 to mitochondria inhibits paxillin localization to adhesions. (A) REF52 cells were transfected with GFP (panels a, d, g), GFP-mito (panels b, e, h) or GFP-SH3ABC-mito (panels c, f, i). 24 hours after transfection, cells were trypsinized, replated on FN-coated coverslips for 2 hours, and stained for ASAP1 or paxillin. Representative images show GFP (panels a, b, c), ASAP1 (panels d, e, f) and paxillin (panels g, h, i). (B) To quantitate the loss of paxillin from adhesions, the transfected cells were scored as “plus” if the cells exhibited well organized paxillin-containing adhesions or “minus” if paxillin staining was diffuse and
poorly organized. The data represent mean ± S.D. for 3 independent experiments (*, P value < 0.05). In each experiment approximately 100 cells were analyzed.

**Fig. 9. Reduced expression of ASAP1 inhibits paxillin localization to adhesions.** (A) REF52 cells were transfected with siRNAs specific for Luciferase or ASAP1. 72 hours after transfection, cells were plated on FN-coated coverslips as in Fig. 8A and co-stained for ASAP1 (panels a and b) and paxillin (panels c and d). (B) Quantitation of “plus” and “minus” cell phenotypes was carried out as described in Fig. 8B. The data represent mean ± S.D. for 3 independent experiments (*, P value < 0.05). Approximately 100 cells were assessed in each individual experiment.

**Fig. 10. Reduced expression of ASAP1 does not significantly alter tyrosine phosphorylation of paxillin in response to FN stimulation.** (A) REF52 cells were transfected with siRNAs specific for Luciferase or ASAP1. 72 hours after transfection, lysates were prepared from cells detached and held in suspension for 1 hour (S) or detached, held in suspension for 1 hour and replated on FN for indicated amount of time (15’, 30’, 60’). The lysates were analyzed with Western blotting using antibodies specific for Paxillin phospho-Tyr31, paxillin and ASAP1. (B) REF52 cells were transfected with indicated siRNAs as in (A). 72 hours after transfection, cells were trypsinized and re-plated on FN-coated coverslips as in Fig. 8A and co-stained for
paxillin (panels a and c) and ASAP1 (panels b and d), or paxillin (panels e and g) and paxillin phospho-Tyr118 (panels f and h).
Fig. 1

A

ASAP1
Unique → PH → GAP → ANK repeat → Pro → SH3

CD2AP
SH3A → SH3B → SH3C → Pro → CC

CD2APSH3ABC
SH3A → SH3B → SH3C

CD2AP∆SH3
Pro → CC

B

Input

IP

215
120
84
60
39.2

Flag-ASAP1

CD2AP

C

Elution

L  E1  E2  SDS

M5 Flag

FAK

CD2AP
A) Lysate IP

1. IP: anti-ASAP1
   IB: anti-CD2AP

2. IP: anti-ASAP1
   IB: anti-ASAP1

Fig. 2

B) IP with: αASAP1

1. IB with: αASAP1

2. IB with: αCD2AP

C) IP with: αCD2AP

1. IB with: αFAK

2. IB with: αCD2AP
Fig. 3

A

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<thead>
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<th>Flag-SH3ABC</th>
<th>Flag-ΔSH3</th>
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ASAP1

M5 Flag

B

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<th>Lysate</th>
<th>GST</th>
<th>A</th>
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ASAP1

GST fusions loaded
Fig. 4

A

B

C

D

ASAP1

GFP

GFP-mito

GFP-SH3ABC-mito

12.4% 11.6% 67.8%

WCL  Mit  Mit  Mit

MitWCL MitWCL MitWCL

GFP-mito

GFP

GFP-mito

GFP-SH3ABC-mito

Cell Spreading (%)

Time (minutes)

0 20 40 60 80 100

0 20 40 60 80 100

0 60 120 180 240

0 60 120 180 240

Fig. 4
Fig. 5

A

siRNAs

<table>
<thead>
<tr>
<th>Luc</th>
<th>ASAP1</th>
</tr>
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1

ASAP1

2

Erk1

B

Relative intensity (arbitrary units)

0 0.2 0.4 0.6 0.8 1 1.2

0 20 40 60 80 100

Luc | ASAP1 |

* 

C

Cell Spreading (%)

0 20 40 60 80 100

0 60 120 180 240

Time (minutes)

Luc

ASAP1
**Fig. 6**

(A) Relative Migration

- **Luciferase**
  - mock
  - FN
  - EGF

(B) Relative Migration

- **mito**
  - mock
  - FN
  - EGF

- **SH3ABC-mito**
  - mock
  - FN
  - EGF
Fig. 7

A

<table>
<thead>
<tr>
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<tr>
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Arf1-HA

B

**siRNAs**

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<td>Total Arf1</td>
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<td>ASAP1</td>
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<td>Erk1</td>
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C

Graph showing the relative intensity of Arf1-GTP/total Arf1 for Luc and ASAP1.

D

**GFP-tagged**

<table>
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<tr>
<th>GFP</th>
<th>mito</th>
<th>SH3ABC-mito</th>
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<tbody>
<tr>
<td>Arf1-GTP</td>
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<tr>
<td>Total Arf1</td>
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<tr>
<td>GFP</td>
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E

Graph showing the relative intensity of Arf1-GTP/total Arf1 for GFP, GFP-mito, and GFP-SH3ABC-mito.
Fig. 8

A

<table>
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<tr>
<th>Transfected with:</th>
<th>GFP</th>
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<th>GFP-SH3ABC-mito</th>
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<tr>
<td>Paxillin</td>
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<td><img src="image" alt="A-h" /></td>
<td><img src="image" alt="A-i" /></td>
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</tbody>
</table>

B

![Bar chart](image)

- **y-axis:** Cell Percentage (%)
- **x-axis:** GFP, GFP-mito, GFP-SH3-mito

- **Legend:**
  - plus cell
  - minus cell

*Significant difference*
Fig. 9

A

<table>
<thead>
<tr>
<th>siRNAs</th>
<th>Luciferase</th>
<th>ASAP1</th>
</tr>
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<td>ASAP1</td>
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<td>b</td>
</tr>
<tr>
<td>paxillin</td>
<td>c</td>
<td>d</td>
</tr>
</tbody>
</table>

B

Cell Percentage (%)

- Luciferase
  - plus cell: 90%
  - minus cell: 10%

- ASAP1
  - plus cell: 60%
  - minus cell: 50%
Fig. 10

A

Luciferase

on FN

S 15' 30' 60'

ASAP1 siRNAs

on FN

S 15' 30' 60'

Paxillin P-Tyr31

Paxillin

ASAP1

B

Luciferase

ASAP1

Paxillin

a b

c d

e f g h

Pax P-Tyr118

Paxillin

Pax P-Tyr118
Mis-localization or reduced expression of Arf GTPase-activating protein ASAP1 inhibits cell spreading and migration by influencing Arf1 GTPase cycling
Yunhao Liu, Gil M. Yerushalmi, Pablo R. Grigera and J. Thomas Parsons

J. Biol. Chem. published online January 4, 2005

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