Integrin Cytoplasmic Domain-associated Protein 1α (ICAP-1α) Interacts Directly with the Metastasis Suppressor nm23-H2, and Both Proteins Are Targeted to Newly Formed Cell Adhesion Sites upon Integrin Engagement*

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Cell adhesion-dependent signaling implicates cytoplasmic proteins interacting with the intracellular tails of integrins. Among those, the integrin cytoplasmic domain-associated protein 1α (ICAP-1α) has been shown to interact specifically with the β1 integrin cytoplasmic domain. Although it is likely that this protein plays an important role in controlling cell adhesion and migration, little is known about its actual function. To search for potential ICAP-1α-binding proteins, we used a yeast two-hybrid screen and identified the human metastatic suppressor protein nm23-H2 as a new partner of ICAP-1α. This direct interaction was confirmed in vitro, using purified recombinant ICAP-1α and nm23-H2, and by co-immunoprecipitation from CHO cell lysates over-expressing ICAP-1α. The physiological relevance of this interaction is provided by confocal fluorescence microscopy, which shows that ICAP-1α and nm23-H2 are colocalized in lamellipodia during the early stages of cell spreading. These adhesion sites are enriched in occupied β1 integrins and precede the formation of focal adhesions devoid of ICAP-1α and nm23-H2, indicating the dynamic segregation of components of matrix adhesions. This peripheral staining of ICAP-1α and nm23-H2 is only observed in cells spreading on fibronectin and collagen and is absent in cells spreading on poly-L-lysine, vitronectin, or laminin. This is consistent with the fact that targeting of both ICAP-1α and nm23-H2 to the cell periphery is dependent on β1 integrin engagement rather than being a consequence of cell adhesion. This finding represents the first evidence that the tumor suppressor nm23-H2 could act on β1 integrin-mediated cell adhesion by interacting with one of the integrin partners, ICAP-1α.

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∥ The abbreviations used are: ICAP, integrin cytoplasmic domain-associated protein; BSA, bovine serum albumin; CHO, Chinese hamster ovary; NDPK, nucleoside diphosphate kinase; PBS, phosphate-buffered saline; ICAM-1, intercellular adhesion molecule 1; TRITC, tetramethylrhodamine isothiocyanate; ELISA, enzyme-linked immunosorbent assay.

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belonging to a family of highly conserved proteins in eu-
karyotes. Nm23 family proteins possess a nucleoside dipho-
phate kinase activity (13–15). Eight different genes of this 
family have now been identified in humans and were named

\[ \text{nm23-H1, nm23-H2, to nm23-H8} \] (16). Apart from their role in 
nucleotides metabolism, nm23 isoforms are reportedly involved in 
a variety of cellular functions (17). Nm23-H2 has been shown to 
bind to the nuclease hypersensitive element of the \( \text{c-myc} \) and 
PDGF-A (platelet-derived growth factor A) promoter (12, 18).

Interestingly, expression of the \( \text{nm23} \) genes is linked to sup-
pression of tumor metastasis, differentiation, apoptosis, prolif-
eration, and DNA mutation (19–21). Introduction of \( \text{nm23-H1} \) or 
-H2 reduces the metastatic potential and \( \text{in vitro} \) cell motil-
ity of tumor cells (22, 23). Kantor et al. (24) report that murine 
melanoma cell lines and human breast carcinoma cells stably 
transfected with \( \text{nm23-H1} \) lose their ability to migrate in re-
sponse to different factors. Zhu et al. (25) report that \( \text{nm23-H1} \) 
interacts with the Ras-related GTPase member Rad and re-
versibly converts GDP-Rad to GTP-Rad, thus acting as an 
exchange factor and a GTPase-activating protein for Ras. More 
recently, an association between \( \text{nm23-H1} \) and Tiam1, a pro-
duct of an invasion and metastasis-inducing gene, has been 
shown. This interaction could lead to the down-regulation of 
Rac1 activity (26). The mechanism of tumor suppression by 
\( \text{nm23} \) is still poorly understood, although some speculations 
about the role of the enzyme have been presented (19).

In this study, we report that ICAP-1α and \( \text{nm23-H2} \) interact directly, 
co-localize and concentrate in peripheral ruffles, and are re-
cruited to \( \beta_1 \) integrin-rich cell adhesion sites in cells spreading 
on fibronectin and collagen. This particular cell localization supports 
the view that this association is relevant to a physi-
ological process during the early stages of cell adhesion. It is 
the first report linking the tumor suppressor protein \( \text{nm23-H2} \) 
to the cell adhesion and migration machinery.

**EXPERIMENTAL PROCEDURES**

**Reagents—** Human fibroblasts (Hs-68) were kindly provided by Dr. C. 
Gauthier-Rouvire (Montpellier, France). Fibronectin was extracted from 
human amnion (Maxisorp, Nunc, Roskilde, Denmark) and 
\( \text{pACT2-ea-actinin-1} \) vectors were kindly provided by Dr. A. Duper-
ay (Grenoble, France). Monoclonal antibodies against \( \text{nm23-H1} \) or 
\( \text{ nm23-H2} \) were purchased from Cologer, Paris, France. 
Rhodamin-phallolidin was from Sigma-Aldrich. Rabbit anti-ICAP-1α 
as serum was raised in our laboratory by immunizing rabbits with 
\( \text{pAS2-1/ICAP-1α} \) protein as antigen. Briefly, a recombinant 
P. pastoris strain (Maxisorp) was cotransformed with either 
ICAP-1α proteins (40 mg/ml) or NDPK proteins (nm23-H2 or 
NDPK from \( \text{Dictyostelium discoideum} \), provided by Dr. I. Lasu), 
were isolated from positive yeast clones by a glass beads/phenol-chlo-
reform extraction protocol provided by the manufacturer (CLONTECH).

Escherichia coli 1066 bacteria were then electroporated with 
\( \text{pAS2-1 vector (CLONTECH distributed by Ozyme, Montigny le 
Bretonneux, France).} \) The sequence of the bait construct was verified by 
DNA sequencing, and the construct was introduced into Y190 yeast cells 
using a lithium acetate transformation protocol. The resulting 
\( \text{pAS2-1/ICAP-1α} \) construct was used as bait to screen 200,000 
human placenta MATCHMAKER cDNA library (6 × 109 cells) 
according to the manufacturer’s protocol. Briefly, Y190 ( \( \text{pAS2-1/ICAP1α} \) 
transformed by the library plasmids were selected by plating on SD 
medium lacking tryptophan and leucine (SD-WL). Interaction 
of proteins encoded by \( \text{pAS2-1/ICAP-1α} \) and by the \( \text{pACT2} \) library vectors was 
tested by growing the cells in the presence of 25 mg 3′-amino-
L-thyrosine (SD – \( \text{WL} + \text{3AT} \)). Histidinopositive colonies were 
further tested for LacZ activation. The growth of blue colonies in the 
histidine-deficient medium indicated a positive interaction. 42 positive 
 yeast colonies, as indicated by activation of both reporter genes (histi-
dine and lacZ) were independently identified and isolated. Plasmids 
were isolated from positive yeast clones by a glass beads/phenol-chlo-
reform extraction protocol provided by the manufacturer (CLONTECH).

**Purification of Proteins—** Recombinant His-tagged ICAP-1α protein 
was purified from BL21(DE3) E. coli strain transformed with the 
prokaryotic expression vector pET19b/ICAP-1α (pET19b plasmid 
purchased from Novagen). The expression of ICAP-1α was induced with 
1 mg isopropyl-1-thio-\( \beta \)-D-galactopyranoside at 30 °C for 3–5 h. Bacteria 
resuspended in PBS were sonicated and centrifuged (20,000 × g, 30 
min, 4 °C). Soluble His-tagged ICAP-1α protein was purified by 
affinity for nickel-nitrilotriacetic acid resin (Ni-NTA, Qiagen), washed with 40 
ml of wash buffer (60 mM imidazole, 500 mM NaCl, 20 mM Tris-HCl, pH 7.9), 
and eluted with elution buffer (1 M imidazole, 500 mM NaCl, 20 mM 
Tris-HCl, pH 7.9). After dialysis against PBS to eliminate imidazole, 
the protein purity was checked on SDS-PAGE. The same protocol 
was used for ICAP-1α fragments. Recombinant \( \text{nm23-H2} \) protein 
and NDPK from \( \text{Dictyostelium discoideum} \), provided by Dr. I. Lasu, 
were prepared as described previously (28).

**Solid Phase-based Binding Assays—** The interaction between recomb-
ninant ICAP-1α and recombinant \( \text{nm23-H2} \) was analyzed using a solid 
phase binding assay. Mouse monoclonal antibody 4D1D6 (Maxisorp) 
was coupled to 9B10 ascites (CLONTECH). The ICAP-1α-bound resin was washed with PBS, 300 
mM NaCl, 5 mM imidazole, blocked with PBS, 3% BSA, and used for 
pull-down experiments. Interaction assays were performed for 30 
min at room temperature using recombinant \( \text{nm23-H2} \) or HeLa cell 
lysates as the source of cellular \( \text{nm23-H2} \). Purified recombinant 
\( \text{nm23-H2} \) (5 mg) was diluted in PBS, 3% BSA, 300 mM NaCl, 5 mM imidazole, and 
HeLa cells were lysed in 1% Nonidet P-40, 10% glycerol, 20 mM Tris, 
\( \text{pH 8.5} \), 157 mM NaCl containing protease inhibitors. Bound proteins were 
 washed with PBS, 300 mM NaCl, 5 mM imidazole, eluted by boiling in 
Laemmli sample buffer, and analyzed by Western blotting using an 
affinity-purified anti-nm23-20 monoclonal antibodies specific to \( \text{nm23-H2} \).

**Comunonprecipitation Experiments—** CHO cells were transiently 
transfected with pcDNA3.1/ICAP-1α or pcDNA3.1 vector using Exgen 
(Euromedex, Souffelweyersheim, France). Twenty-four hours after the 
transfection, 10% Nonidet P-40, 30 mM Tris (pH 8) containing 
buffer containing protease and phosphatase inhibitors for 45 min. 
The cell lysates (500 mg of proteins) were incubated with 20 μl of 9B10 ascites 
containing anti-ICAP-1α monoclonal antibody for 2 h. Subsequently, 
the samples were mixed with 60 μl of immobilized protein G (Sigma-
Aldrich). After incubation for 1 h, the beads were washed four times 
with the lysis buffer, and the bound proteins were released from the
beads by boiling in 20 μl of SDS-PAGE Laemml sample buffer for 5 min. The samples were analyzed by Western blotting with either rabbit polyclonal anti-ICAP-1α antibodies (to check the immunoprecipitation of ICAP-1α) or affinity-purified rabbit polyclonal anti-nm23-H2 antibodies (to evaluate the interaction between ICAP-1α and endogenous nm23-H2). Immunological detection was achieved with hors eradish peroxidase-conjugated secondary antibody, and the staining was carried out with ECL according to the manufacturer’s instructions (Amer sham Biosciences).

Immunofluorescence Staining of Cells—Hs68 cells were cultured as a monolayer in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and harvested with trypsin/EDTA. The cells were plated on coverslips that were precoated with 25 μg/ml human plasma fibronectin and incubated for different lengths of time (as specified for each experiment) in a 37 °C incubator under a 5% CO2, 95% air atmosphere to obtain cells at different stages of spreading. Within the first hour of plating, extensive membrane ruffling was observed in many of the cells that were spreading on fibronectin. Under these experimental conditions, most of the cells were fully spread within 4 h. The cells were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.2% Triton X-100 in PBS. Nonspecific sites were blocked in 10% goat serum for 1 h at room temperature. Cells were stained for 1 h with either monoclonal or polyclonal antibodies in a moist chamber. Anti-nm23-H2 monoclonal antibody (Seigakaku) and polyclonal antibodies were used at a final concentration of 50 μg/ml. Anti-ICAP-1α 4D16 monoclonal antibody (from hybridoma supernatant) was used at a ratio of 1:3 and anti-ICAP-1α polyclonal antibodies were used at 1:500. The 4B7R monoclonal antibody specific for human β1 integrin was used at 5 μg/ml anti-Rac1 was used at 2.5 μg/ml, and anti-tubulin at 1:100. After rinsing, coverslips were incubated with appropriate Alexa-conjugated secondary antibodies (Molecular Probes, distributed by Interchim) for 30 min. For actin staining, coverslips were incubated with TRITC-phalloidin. The cells were mounted in Mowiol solution and viewed using a confocal laser scanning microscope (Zeiss LSM 410).

RESULTS
Identification of nm23-H2 as a Binding Partner of ICAP-1α—To identify the proteins directly involved in ICAP-1α-mediated transduction signals, we used the full-length ICAP-1α cDNA fused to the GAL4 DNA-binding domain as bait in a yeast two-hybrid system to screen a human placenta cDNA library. Forty-two positive clones were obtained and sequenced. BLAST searches in cDNA data bases revealed that four inserts overlap with the cytoplasmic domain of β1 integrin, confirming the reported ICAP-1α/β1 integrin interaction already described (9). Four additional inserts coded for the human protein named nm23-H2. Introduction of only pAS-2/ICAP-1α or pACT2/nm23-H2 construction did not result in activation of both reporter genes, indicating that neither ICAP-1α nor nm23-H2 can activate the reporter genes in the absence of the other binding partner (Fig. 1). In additional control experiments, another “bait,” the cytoplasmic domain of ICAM-1, and another “prey,” α-actinin-1, were tested for interaction with nm23-H2 and ICAP-1α, respectively. In all of these cases, no detectable β-galactosidase activity was observed, characterizing the specificity of our screen.

Nm23-H2 Binds to ICAP-1α In Vitro and ex Vivo—To confirm the direct interaction of nm23-H2 with ICAP-1α, we carried out an ELISA-based solid phase binding assay. These experiments revealed a saturable binding of ICAP-1α to nm23-H2 and vice versa (Fig. 2A). In contrast, NDPK from D. discoideum did not bind to ICAP-1α. In an independent approach to corroborate these results, we incubated recombinant His-tagged ICAP-1α bound to Ni(II) chelating resin with a solution of purified recombinant nm23-H2 protein or with HeLa cell lysates containing endogenous nm23-H2 (Fig. 2B). In both cases, nm23-H2 bound to ICAP-1α protein as revealed by Western blot analysis. The results obtained with these pull-down assays indicate that recombinant ICAP-1α interacts with recombinant nm23-H2 protein detected as a monomer and an SDS-resistant dimer. When endogenous nm23-H2 from HeLa cells was used instead, only the dimeric form of nm23-H2 was retained by ICAP-1α. We presume that this dimer is due to oxidative conditions in our experimental procedure. The interaction with nm23-H2 was also tested with recombinant protein containing the N-terminal (1–99) or C-terminal (100–200) half of ICAP-1α protein by pull-down assay (Fig. 2B) and solid phase assay (Fig. 2C). Only the C-terminal polypeptide was able to interact strongly with recombinant or cellular nm23-H2, supporting the idea that the nm23-H2 binding site is localized at the ICAP-1α C-terminal half. To determine whether the interaction between ICAP-1α and nm23-H2 also occurred ex vivo, we expressed ICAP-1α by transient transfection in CHO cells. Soluble extracts were prepared as described under “Experimental Procedures.” Only in ICAP-1α-transfected cells, immunoprecipitation of ICAP-1α using the anti-ICAP-1α 9B10 monoclonal antibody resulted in a co-immunoprecipitation of endogenous nm23-H2 as detected by Western blot analysis using an affinity-purified polyclonal antibody (Fig. 2D). Thus, consistent with ICAP-1α/nm23-H2 interaction detected in yeast cells and in vitro, ICAP-1α and nm23-H2 form a complex in mammalian cells.

ICAP-1α and nm23-H2 Co-localize in Peripheral Ruffles and Are Recruited to β1 Integrin-rich Cell Adhesion Sites in Cell Spreading on Fibronectin—To ascribe a physiological role to the association between ICAP-1α and nm23-H2, we examined their co-localization in vivo. To analyze the subcellular localization of ICAP-1α, we generated a monoclonal ICAP-1α antibody that recognizes both recombinant and endogenous human ICAP-1α in immunofluorescence studies. By immunoblotting with His-tagged fusion proteins containing different domains of the ICAP-1α protein, we showed that this antibody recognizes an epitope located within the N-terminal 100 amino acid residues (not shown). This antibody was specific because it reacted neither with a His-tagged fusion protein containing the C-terminal region of ICAP-1α nor with other irrelevant His-tagged fusion proteins (data not shown).

2 TALON resin should not be exposed to high concentrations of a strong reducing agents such as dithiobisreitol, dithioerythritol, or β-mercaptoethanol. These reagents reduce the cobalt ions and thereby prevent them from binding His-tagged proteins.
ICAP-1α Interacts with nm23-H2

FIG. 2. Nm23-H2 interacts with ICAP-1α through ICAP-1α C terminus domain. A, left panel, interaction between recombinant nm23-H2 and recombinant ICAP-1α was measured by ELISA. Briefly, a 96-well tray (MaxiSorp, Nunc) was coated with either ICAP-1α protein (left graph) or NDPK proteins (nm23-H2 or NDPK from D. discoideum; right graph) and then blocked with a PBS, 3% BSA solution for 1 h at room temperature. Increasing concentrations of Nm23-H2 (left graph) or ICAP-1α (right graph) were incubated in PBS for 1 h at 37°C. After three washes in PBS/0.1% Tween 20, detection of nm23-H2 or ICAP-1α was performed using the affinity-purified polyclonal antibody directed against nm23-H2 or monoclonal antibody 9B10 directed against ICAP1-α. Both graphs show the ability of recombinant ICAP-1α to bind to human nm23-H2 (solid lines) and not to D. discoideum NDPK (dotted line). Nonspecific binding on BSA has been subtracted from the results. Data shown are the means of triplicate determinations, and error bars represent standard deviations. The figure illustrates one representative experiment of four performed with similar results. Right panel, Coomassie staining of the purified proteins used in these experiments. B, pull-down experiments were performed as described under “Experimental Procedures.” Left panel, recombinant nm23-H2 was incubated with empty resin (−) or resin bound to the indicated recombinant His-tagged ICAP polypeptides (full-length (FL) ICAP-1α: 1–99 N terminus; 100–200 C terminus). Retained nm23-H2 was then detected by Western blotting (WB) with affinity-purified polyclonal anti-nm23-H2. Right panel, alternatively, HeLa cell lysates were used as a source of endogenous nm23-H2. The amount of recombinant His-tagged ICAP1-α proteins retained by resin was checked by Western blotting the same membranes with the polyclonal anti-ICAP1-α antibodies. C, solid phase assay was performed using recombinant ICAP1-α(1–99) (dotted line) or ICAP1-α(100–200) (solid line) fragments as coated proteins to show their ability to interact with nm23-H2. The procedure used is described above in A. D, CHO cells were transfected with pcDNA3.1 or pcDNA3.1/ICAP-1α vector as indicated. Upper panel, interaction between ICAP-1α and nm23-H2 was determined by nm23-H2 immunoblot on ICAP-1α immunoprecipitates (IP) performed with anti-ICAP-1α 9B10 monoclonal antibody. Lower panel, immune complexes were probed with a polyclonal anti-ICAP-1α to show the amount of ICAP-1α immunoprecipitated with the monoclonal antibody 9B10.

Despite the observed association of ICAP-1α with the cytoplasmic domain of β1 integrin, we obtained no evidence for ICAP-1α accumulation at β1 integrin- or vinculin-rich focal adhesion sites in fully spread cells. ICAP-1α was found primarily in the cytosol, with some concentrations in the perinuclear or nuclear region. We therefore analyzed the subcellular localization of ICAP-1α in cells during the early stages of spreading. H216 cells newly plated on fibronectin-coated coverslips were stained with either polyclonal or monoclonal anti-ICAP-1α antibodies. ICAP-1α was observed to be highly concentrated at the edge or at peripheral ruffles of spreading cells (Fig. 3). A similar localization of nm23-H2 was observed with the monoclonal as well as the specific polyclonal antibodies. Noticeably, until 45 min of adhesion, ICAP-1α co-localized with nm23-H2 in many cell adhesion sites resembling ruffles or lamellipodia at the cell periphery, suggesting that ICAP-1α and nm23-H2 are involved in integrin-mediated cell spreading (Fig. 3). As cells spread further (1 h after seeding), both ICAP-1α and nm23-H2 staining at the cell edges decreased. Thus, high concentrations of ICAP-1α and nm23-H2 appear transiently at the cell periphery during the process of spreading. We noted that immunostaining with anti-ICAP-1α antibodies showed a labeling similar to that of stress fibers in fully spread cells.

Previous studies have shown that α5β1 integrins accumulate in the peripheral ruffles of cells spreading on fibronectin (29). We confirmed such a localization of β1 integrin at the edge of the spreading cells and showed in addition co-localization of β1 integrins with ICAP-1α in the peripheral ruffles by co-staining...
and image capture were done with a confocal microscope. The
Occupied/H9252 were never detected in focal adhesions (Fig. 5).

The cells with a monoclonal anti-β1 integrin antibody (Fig. 4). When Hs68 fibroblasts adhered to the extracellular matrix protein fibronectin, F-actin-containing membrane ruffling was stimulated as the initial response upon Rac1 activation as described by others (1). Indeed, additional co-staining in the early state of spreading (30 min of spreading) showed the peripheral co-localization of ICAP-1α with actin and Rac1, but not with tubulin or vimentin, allowing a better characterization of these areas (Fig. 4).

**ICAP-1α and nm23-H2 Are Recruited to Areas Enriched in Occupied β1 Integrins**—Like other integrins, β1 integrins can exist in different functional states with respect to ligand binding. These changes involve both affinity modulation, by which conformational changes in the integrin heterodimer govern affinity for individual extracellular matrix proteins, and avidity modulation, by which changes in lateral mobility and integrin clustering affect the binding of cells to multivalent matrices. Here we used the monoclonal antibody 12G10, which recognizes a ligand-induced binding site (30), to investigate the functional state of β1 integrins co-localized with ICAP-1α and nm23-H2. During initial cell spreading, the 12G10 monoclonal antibody recognized engaged integrins at the cell edge after 30 min of spreading (early spreading) and in focal adhesions after 4 h of spreading (late spreading). Fig. 5 shows that as cells spread further, 12G10 staining decreased at the cell edges, indicating that localization of occupied integrins at the cell edges precedes the formation of focal adhesions. In contrast, although ICAP-1α or nm23-H2 co-localized with engaged β1 integrins at the edges of the cells during initial spreading, they were never detected in focal adhesions (Fig. 5).

**Targeting of Both ICAP-1α and nm23-H2 to the Cell Periphery Depends on the Integrins Engaged with the Extracellular Matrix Substrate**—Our observations presented above imply that the targeting of both ICAP-1α and nm23-H2 proteins is spatially and temporally linked to initial cell spreading. As ICAP-1α interacts specifically with β1 integrins, we hypothesized that ICAP-1α and nm23-H2 targeting to peripheral cell membranes during initial cell spreading should be observed on typical β1 integrin substrates and not on substrates specific for other integrins. In other terms, the composition of the extracellular matrix substrate should control the localization of both ICAP-1α and nm23-H2 proteins at the cell periphery. Indeed, we observed peripheral staining of both ICAP-1α and nm23-H2 in cells spreading on fibronectin and collagen, typical ligands of β1 integrins. However this localization was not observed when the cells were spread on poly-L-lysine, laminin 1, or vitronectin (Fig. 6). The involvement of α6β1 integrin in early spreading on laminin was ruled out because fluorescence-activated cell sorter analysis and immunofluorescence studies showed, on one hand, a very low level of α6 subunit in Hs68 cells, and on the other hand, the absence of α6 and β1 subunits in ruffles induced by laminin (data not shown). This observation indicates that the targeting of both ICAP-1α and nm23-H2 to the cell periphery is dependent on an engagement of β1 integrins interacting with fibronectin or collagen and is not just a consequence of cell adhesion.

**DISCUSSION**

Recent studies suggest that individual integrin α/β heterodimers can play unique roles in the regulation of cell migration, growth, survival, and differentiation (31–36). These regulatory functions of integrins involve specific interactions between the cytoplasmic domains of individual integrins and intracellular proteins involved in signal transduction or other
visualization is necessary for its function (38). Exis-
ting as in a hexameric form in solution and as this oligo-
mer-terminus and that this novel interaction can occur under phys-
ological conditions. The endogenous or recombinant nm23-H2 exists as in a hexameric form in solution and as this oligo-
merization is necessary for its function (38–40), the form interact-
ing with ICAP-1α should be hexameric.

Confocal fluorescence microscopy revealed unambiguously the subcellular co-localization of both proteins in lamellipodia and ruffles during the early stages of cell spreading. Moreover, the specificity and physiological relevance of the peripheral staining of ICAP-1α and nm23-H2 during the early stages of cell spreading is underlined by the fact that it was observed only when cells were plated on fibronectin and collagen, both of these matrices that engage β1 integrins. Indeed, this is consist-
ent with the known specificity of ICAP-1α for β1 integrins and strongly suggests that nm23-H2 targeting to specific occupied β1 integrins at the cell periphery is mediated by ICAP-1α. Co-localization of ICAP-1α and nm23-H2 at the cell edges precedes the formation of focal adhesions devoid of both proteins. Both ICAP-1α and nm23-H2 are recruited only into these nas-
cent substrate adhesion sites. This points out the molecular diversity of cell-matrix adhesions, indicating dynamic changes in the morphology, molecular composition and locations of cell matrix adhesions depending on spreading time. Therefore the recruitment of ICAP-1α and nm23-H2 is spatially and tempo-
raly linked to the formation of newly formed adhesion sites and may play a role in regulating focal adhesion assembly and/or downstream events initiated at integrin-dependent focal contacts, such as altered cytoskeletal organization or intracel-
lar signaling. Complementing this idea, we have recently shown that ICAP-1α quickly disassembles focal adhesions, probably because of a competition with talin for binding to the β1 integrin tail. At the leading edge of the migrating or spread-
ing cell, ICAP-1α could thus prevent focal adhesion assembly, contribute to lamellipodia extension, and promote integrin functions not requiring focal adhesion formation. This hypo-
thesis is strengthened by the observations of Reddy et al. (41), who show that conversely to ICAP-1α and nm23-H2, talin colocalizes with integrins in focal adhesions but is absent from cell periphery at 30 min of spreading.

In line with the concept of lamellipodia extension, this view could provide the functional significance of nm23-H2 associa-
tion with ICAP-1α. A previous report suggested that ICAP-1α interactions with the β1 integrin tail may support cell migration (37). Indeed, in these experiments, over-expression of ICAP-1α in COS-7 cells was associated with increased β1 integrin-dependent cell migration on fibronectin. Furthermore, mutations of the ICAP-1α binding sites localized on β1 integrin cytoplasmic tail abolished adhesion, invasion, and metastasis (42). On the other hand, numerous observations suggested that

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**Fig. 4.** Characterization of ICAP-1α-containing peripheral ruffles. Hs68 cells were plated on fibronectin, fixed with paraformalde-
hyde 30 min after plating, and co-stained with polyclonal anti-
ICAP-1α antibodies and rhodamin-phalloidin for actin staining or
monoclonal antibodies directed against tubulin, vimentin, integrin
(4B7R), or Rac1. ICAP-1α co-localizes with actin, integrin, and Rac1.
Visualization of a single section and image capture were done with a
confocal microscope. The bar represents 10 μm in all cases.

**Fig. 5.** ICAP-1α and nm23-H2 are localized at the edges of cell
in lamellipodia containing occupied integrins. Cells were plated
on coverslips coated with fibronectin, fixed after 30 min or 4 h, and
stained with polyclonal anti-ICAP-1α antibodies or nm23-H2 and mon-
oclonal 12G10 antibody directed against occupied β1 integrin. ICAP-1α
and nm23-H2 proteins co-localize with β1-occupied integrins only dur-
ing the early stage of spreading (30 min). Focal adhesions observed at
4 h of spreading contained neither ICAP-1α nor nm23-H2. Visualization
of a section and image capture were done with a confocal microscope.
The bar represents 10 μm in all cases.

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3 D. Bouvard, L. Vignoud, S. Dupé-Manet, N. Abed, C. Marie, R.
Fässler, and M. R. Block, submitted for publication.
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and stained with polyclonal anti-ICAP-1/H9251 counterbalance the interaction between ICAP-1/H9262 sent 10 image capture were done with a confocal microscope. The collagen and fibronectin, which was not evident when they were spread H2. We observed a peripheral staining in Hs68 fibroblasts spread on the interaction between nm23-H2 and ICAP-1/S122P and H118Y mutations were identified in melanoma of metastasis suppressor in tumor cell lines (44). For example, the nms nonspecific exchange of phosphoryl groups between nucleotides cated roles in the cell physiology than the mere catalysis of a exchange factor, Tiam1, involved in control of metastatic po-
lates Tiam1 and therefore inhibits Rac1 activation in vivo. Because nm23-H2 is able to form heterooligomers with other nm23 isoforms and because Rac is co-localized with ICAP-1a and controls lamellipodia extension (for review see Ridley (3)), one can speculate that the interaction of ICAP-1a with nm23-H2 may contribute to the overall regulation of Rac activity at the cell periphery. We can not rule out the possibility that the interaction between nm23-H2 and ICAP-1a might also counterbalance the interaction between ICAP-1a and β integrin, given the possibility of the dynamic of ruffles during cell spreading. Because the phosphorylation state of ICAP-1a could control cell adhesion, one can speculate that NDPK in the vicinity could somehow control phosphate donor availability.

In conclusion, the interaction between ICAP-1a and nm23-H2 may drastically change the understanding of the metastasis suppressor function of the nm23 protein family and will provide an alternative interpretation of the implication of these proteins in tumor invasion and metastasis. Focal adhesions form and disappear continuously during cell migration, and the cell spreading process as well as the interaction between ICAP-1a and nm23-H2 provide novel insight into the molecular basis of the dynamic nature of focal adhesion.

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Fig. 6. Effects of matrix composition on targeting of ICAP-1α and nm23-H2 to the cell edges. Cells were plated on coverslips coated with 25 μg/ml of collagen I (Co 1), collagen IV (Co 4), fibronectin (FN), vitronectin (VN), polylysine (PL), or laminin (LM) after 30 min, and stained with polyclonal anti-ICAP-1/H9251, counterbalance the interaction between ICAP-1/H9262.
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