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ORIGINAL PAPER

H-prune-nm23-H1 protein complex and correlation to pathways in cancer metastasis

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Abstract Cancer is a multi-step process, one of the latest events correspond to metastasis formation and dissemination, to date the major cause of deaths. The h-prune-nm23-H1 protein complex and its activation of PDE-cAMP activity have been shown to correlate with breast cancer progression and metastasis formation. Here, we describe the protein complex formation and its involvement in cell migration. By gene expression studies and protein-protein pull-down analyses coupled to mass spectrometry we have identified new genes and pathways along which the h-prune-nm23-H1 complex exerts its function. We review here h-prune binding to the glycogen synthase kinase (GSK-3β) and identified a new h-prune protein partner, Gelsolin, an ATP severing protein acting in focal adhesions, in a MDA-435 breast cancer cellular model. The results presented here underline the importance of this protein complex leading to new translational studies involved into the inhibition of cell migration, thus enhancing the potential of using this knowledge to direct inhibition of metastases formation in humans.

Keywords PDE · cAMP · NDPK · Breast · h-prune · nm23· GSK-3β · Gelsolin

Introduction

Metastasis spread is the major cause of death from breast cancer (Newcombe and Lantz, 1993). This phenomena is due to the ability of a cell to invade the surrounding tissues. Considerable progress has been made towards the identification of the genetic events occurring during the progression of breast cancer. These include mutations in the BRCA1 and BRCA2 genes, as well as mutation in p53, Her2/neu and some other regulatory proteins of the cell cycle, such as cyclin-D1 and p27 Kip1. Notably few main pathways are recapitulating cancer in human then being modelled in mice (Rangarajan and Weinberg, 2003). Indeed, metastasis spread is driven by a highly complex molecular network. Metastatic cells change their adhesive properties, loose contact with the other cells in the primary tumor, and make new contacts with the extracellular matrix of the host tissues encountered as they invade (Sleeman, 2000). To migrate away from the primary tumor, tumor cells also need to gain motility functions. These properties are shared by metastatic cells that disseminate through the lymphatic and vascular systems. An emerging field of research to date correspond to the study of metastasis suppressor genes, which are characterized on the basis of their inhibition function measured as the capacity to inhibit cell motility *in vitro* and *in vivo*. At this time, several metastasis-suppressor genes NM23 (NME1), KAI1, KiSS1, BrMS1 and MKK4 (MAP2K4) are discovered (Yoshida et al., 2000; Shevde and Welch, 2003). They all meet the criteria to inhibit the motility properties of the cancer cells to suppress the formation of spontaneous and macroscopic metastases without affecting the growth rate of the primary tumor (Yoshida et al., 2000). NM23-H1 the first discovered suppressor of metastasis gene, has been shown to have a role in breast cancer (Hartsough et al., 2001; Hennessy et al., 1991; Royds et al., 1993; Steeg et al., 1993). NM23-H1 encodes for a nucleoside diphosphate kinase (NDPK-A) that catalyzes the γ-phosphorylation of a nucleoside diphosphate from a nucleoside triphosphate (usually ADP phosphorylation to ATP). Of note this enzymatic activity is not responsible of the anti-motility function in

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cancer. Other activities have being characterized for nm23-H1 thus inducing an anchorage-independent colonization, and a histidine protein kinase activity (Kantor et al., 1993; Leone et al., 1993; Howlett et al., 1994; Hartsough and Steeg, 2000; Hartsough et al., 2002). Finally a role on induction and anticipation to neuronal differentiation in rat PC12 cells was also found (Lombardi et al., 2000).

In breast and melanomas cancer, high expression of NM23-H1 is associated with a decreased metastatic potential (Florenes et al., 1992), while in non-Hodgkin lymphomas and neuroblastomas; high NM23-H1 expression is associated with an adverse outcome (Florenes et al., 1992; Leone et al., 1993; Almgren et al., 2004). Additionally, the levels of NM23 mRNA in metastatic malignant melanomas show an inverse correlation to disease progression (Hartsough and Steeg, 2000; Niitsu et al., 2001). The nm23-H1 protein binds to the human prune (h-prune) (Reymond et al., 1999) and we have previously shown that the overexpression by gene copy numbers amplification of h-prune induces cell proliferation. Increase level of h-prune expression, as compared to moderate or low levels of nm23-H1 expression, leads to aggressive and metastatic sarcomas and breast carcinomas, thus indicating an inhibitory role for h-prune against the metastasis suppression function of nm23-H1 *in vivo* (Forus et al., 2001). H-prune involvement in the metastasis spread in breast cancer patients was then confirmed in our analyses of a large set of primary breast carcinomas (an analysis performed on a tumor collection, for which more than 15 years of clinical follow-up is available). The pro-motility effects observed *in vitro* were then associated significantly to lymph node status (N2-N3) and with metastasis formation (M1) *in vivo*, thus indicating that h-prune is independent to the estrogen receptor (ER) and progesterone receptor (PR) status and results in a new marker of advanced stage disease status in breast carcinoma (Zollo et al., 2005).

H-prune belongs to the DHH superfamily of phosphoesterases that includes the RecJ nuclease identified since in bacteria and the Pyrophosphatases and Exopolyphosphatases (PPX1) from yeast and bacteria respectively (Aravind and Koonin, 1998; D'Angelo et al., 2004). Given the prediction of a phosphoesterase activity for h-prune and its association with the NDPK family of proteins, we have been investigating its biochemical properties and its potential role in metastasis. We have shown that h-prune retains a phosphodiesterase (PDE) activity, with a preferential affinity for cAMP over cGMP as substrate (D'Angelo et al., 2004). The cyclic nucleotide PDEs belong to a superfamily of proteins that catalyze the hydrolysis of 3′, 5′-cyclic nucleotides (cAMP; cGMP) to their corresponding nucleoside 5′-monophosphates (Beavo and Brunton, 2002; Beavo et al., 1994). In the aggressive MDA-MB435-C100 breast cancer cell line the overexpression of nm23-H1 has been shown to significantly reduce its metastatic phenotype both *in vitro* and *in vivo* (Hartsough and Steeg, 2000; Mao et al., 2001; Tseng et al., 2001). At the same time, h-prune overexpression is able to change the "low" motility phenotype into the "high" motility phenotype mainly due to its increase in cAMP-PDE activity. This latest function was found inhibited by dipyridamole (an anti-platelet aggregation drug) and one of the most eight selective PDE inhibitors tested in our previous studies. Indeed dipyridamole was then used *in vitro* showing an inhibition of cell motility in the breast cancer model overexpressing h-prune protein (D'Angelo et al., 2004). Comparing those results to the one reported in clinical trials demonstrating its positive effect on the use in chemo-adjuvant therapy together with α-interferon and 5-fluoro-uracil (reviewed by Hejna et al., 1999), we decided to further study this drug, thus enhancing the potential benefit of the use of its use in breast cancer metastasis treatment (Zollo et al., in preparation).

We have additionally demonstrated that both the h-prune cAMP PDE activity and the formation of h-prune-nm23-H1 complex are both correlated to an increase in cell motility properties in the MDA breast cancer cell line (D'Angelo et al., 2004). Furthermore, we found that nm23-H1 phosphorylation by casein kinase I (CKI) at aminoacids S122 and S125 is promoting this h-prune/ nm23.H1 complex formation. Thus demonstrating that the inhibition of nm23-H1 phosphorylation by IC261, a specific CKI δ/ε inhibitor, impairing the h-prune/nm23 interaction, results in a final decrease in cell motility properties of those breast cancer cells (Garzia et al. in preparation). Because of those previously reported findings we thought to identify other h-prune-nm23 protein partners, the network of genes involved in to the increase of cell motility induced by h-prune overexpression, thus performing a whole gene expression profiling analyses in MDA breast clones, overexpressing nm23-H1 alone (MDA-H1-177) and together with h-prune overexpressed clone (MDA-H1-177-prune #8). These analyses show several genes involved in cell motility properties being upregulated and reviewed in D'Angelo and Zollo (2004).

Infact in the breast clone overexpressing h-prune and nm23-H1 (MDA-H1-177-prune #8), the phosphatidylinositol 4-kinase type II (PI 4-K) gene, a key enzyme involved in the modelling of the actin cytoskeleton was found up-regulated. PI 4-kinases converts PI into PI-4-phosphate (PI-4-P), a critical molecule in phosphatidylinositide intermediate signalling pathways. At this time it is known that both PI-4-P and PI-4,5-P2 can interact with actin-binding proteins to regulate actin polymerization. A second important gene found overexpressed was L-plastin, which it was found already to be involved in prostate carcinoma cell invasion (Zheng et al., 1999). Then other genes were found down regulated in the MDA-H1-177-prune #8 clone. These genes are involved in extracellular matrix contacts and cellular adhesion, examples are plakophilin, LIM, (plakin) in

(the cytoskeleton re-organization) and EXT1 (known to act as an oncosuppressor gene). These analyses suggest the involvement of the protein-protein complex formation with cellular motility acquired function in breast cancer. While these analyses were performed at mRNA level the data produced by these studies are relatively important; the genes found up-regulated or down-regulated in those breast cancer models are underlining the direct or indirect gene partners of h-prune-nm23-H1 complex, as being those considered new targets for future applications in translational research studies.

Addressing this issue by yeast two hybrids experiments, Kobayashi et al. (2006) together with our group, found GSK-3β binding to h-prune protein. The serine/threonine kinase glycogen synthase kinase-3 (GSK-3) was first described in a metabolic pathway for glycogen synthase regulation that is sensitive to insulin-mediated inhibition (Plyte et al., 1992). GSK-3 has subsequently been shown to regulate several physiological responses, including protein synthesis, gene expression, subcellular localization of proteins, and protein degradation in mammalian cells by phosphorylating many protein substrates (Cohen and Frame, 2001; Doble and Woodgett, 2003; Grimes and Jope, 2001). There are two members of GSK-3 in mammals (GSK-3α and GSK3-β, since reported by Woodgett, 1990). GSK-3 is highly conserved through evolution and plays a fundamental role in cellular response. GSK-3β was also found to binds to Axin, Axil, and AKAP220 (Ikeda et al., 1998; Tanji et al., 2002). Axin binds to not only GSK-3 but also β-catenin, APC, and Dvl, all of which are important components in the Wnt signalling pathway (Kikuchi, 1999; Wodarz and Nusse, 1998). In the Axin complex, GSK-3 phosphorylates β-catenin, APC, and Axin efficiently (Ikeda et al., 1998; Kishida et al., 1999), and thereby induces ubiquitination of β-catenin, leading to its degradation. Evidences have been accumulated that GSK-3β regulates cellular architecture in neuronal cells (Frame and Cohen, 2001; Jope and Johnson, 2004). Two microtubule associating proteins (MAPs), Tau and MAP1B, are phosphorylated by GSK-3β, which regulates their binding to microtubules, thereby modulating microtubule dynamics. An inactive pool of GSK-3β has been found to be localized at the leading edge of the cells alongside F-actin, and semaphorin 3A and lysophosphatidic acid activate GSK-3, causing growth cone collapse and neurite retraction (Eickholt et al., 2002). GSK-3 mediates Par6-PKCζ-dependent promotion of neural polarization, and cell protrusion in hyppocampal neuron and astrocytes (Etienne-Manneville and Hall, 2003; Jiang et al., 2006; Yoshimura et al., 2005). These findings are underlining new research hypothesis in h-prune/nm23-H1 protein complex function in brain development (see Carotenuto et al. in this issue). We think will be of importance those results for example involved on neural polarity and in cancer of central nervous system origin (Yoshimura et al., 2005). We demonstrate here h-prune binding to GSK-3β in breast cancer MDA435 cells by mean of coimmunoprecipitations experiments does hampering the value of the previous finding in colon rectal carcinoma cells. Within a systematic proteomic pull-down experiments we identified here gelsolin, as new interactor of h-prune/nm23-H1 in the breast MDA 435 cancer model. Gelsolin is an actin binding protein whose activity is dependent from calcium and phospholipids (phosphatidylinositol 4,5-bisphosphate (PIP2). This molecule is differentially regulating actin cytoskeleton thus depending on the binding of monomer of actin filament. Gelsolin sever actin filaments and is activated by Ca++, while phosphoinositides block its capping (Yin et al., 1988; Wegner et al., 1994; Kwiatkowski, 1999). Gelsolin, while binding to actin monomeric form, leads to a nucleating effect on filament polymerization; otherwise its binding to the barbed ends of the actin filament cause its severing, and the regulation of actin filaments length. The large diversity of actin binding proteins in the mammalian cells enable the cell to control different physiological processes including cell shape, cell motility, several signalling, pathways leading to apoptosis and differentiation. Time dependent modifications of actin cytoskeleton can critically regulate tumor-associated processes such as invasion and metastasis. Defining which pathway and protein networks are involved in h-prune-nm23 protein complex and its function during cancer progression will be of importance for additional translational studies and here we discuss our previous findings and identify a new h-prune protein partner.

Material and methods

Cell culture, transfection and immunoprecipitation

MDA-MB435 c100 and the h-prune MDA-435 stable clone #4 cells were maintained in Dulbecco's modified Eagle's medium with 10% foetal bovine serum; the stable clone growth was supplemented with 2 μg/ml of puromycin, at 37°C in 5% CO2. Expression plasmid for h-prune full-length was already described in (D'angelo and Zollo, 2004), construct for GSK-3β (pCGN/ GSK-3β) was kindly provided by Prof. A. Kikuchi. MDA-MB435 c100 cells (1.5×10^6) were transfected with 8 μg of plasmid DNA using the Polyfect system (Qiagen) using standard co-immunoprecipitation experiments. The cells were harvested 48 h after transfection and the total cell lysates were obtained with the standard protocol by lyses the cells in 20 mM Tris-HCl, 2 mM MgAc, 0.3 mM $CaCl_2$, 1 mM DTT, 2 μg/ml pepstatin, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 mM NaF, 1 mM Na_3VO_4. The immunoprecipitation was performed using anti-HA or anti-FLAG monoclonal antibodies (Sigma and Roche, respectively) in ratio of 1 μg :400 μg of total cell extract. The

co-immunoprecipitation experiments were then analyzed by standard Western blotting techniques.

Affinity purification of FLAG-Prune complexes

Complexes were affinity purified from total extracts from breast carcinoma cells MDA-MB435 c100 (negative control wt cells), MDA-435 #4 stable clone (overexpressing h-prune). Cells were washed in PBS and lysed in buffer A (50 mM Tris/HCl pH 7.5, 150 mM NaCl 10% Glycerol, 0.5 mM PMSF, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, and proteinase inhibitors). Total extracts were collected after centrifugation of the lysates for 40 min at 13200 r.p.m. in an Eppendorf F452411 rotor at 4°C. Extracts were incubated with Mouse IgG agarose beads (Sigma) for 2 h at 4°C to reduce not-specific binding. Extracts were collected after centrifugation for 5 min to 3000 r.p.m and then incubated with M2 anti-FLAG agarose-conjugated antibody (Sigma) for 4 h at 4°C, followed by extensive washes with buffer B (50 mM Tris/HCl pH 7.5, 300 mM NaCl 10% Glycerol, 0.5 mM PMSF, 1% Triton X-100, 1 mM NaF, 1 mM Na_3VO_4, and proteinase inhibitors). Elution of immunoprecipitate was performed with 3X FLAG peptide in buffer A. The eluted extracts were precipitated with methanol/chloroform before loading on 10% polyacrylamide SDS-PAGE. The gel was stained with Coomassie colloidal blue (Pierce). Protein bands were excised from the gel and de-stained by repetitive washings with 50 mM NH_4HCO_3 pH 8.0 and acetonitrile. Samples were reduced and carboxy-amidomethylated with 10 mM DTT and 55 mM iodoacetamide in 50 mM NH_4HCO_3 buffer pH 8. Tryptic digestion of the alkylated samples was performed at 37°C overnight using 100 ng of trypsin.

MALDI MS analyses

MALDI mass spectra were recorded on an Applied Biosystem Voyager DE-PRO mass spectrometer equipped with a reflectron analyser and used in delayed extraction mode**.** 1ml of peptide sample was mixed with an equal volume of μ-cyano-4-hydroxycynnamic acid as matrix (in acetonitrile/50 mM citric acid (70:30, v/v)), applied to the metallic sample plate and air dried. Mass calibration was performed by using the standard mixture provided by manufacturer. Raw data, reported as monoisotopic masses, were then introduced into MASCOT peptide mass fingerprinting search program (Matrix Science, Boston, USA) and used for protein identification.

LCMSMS analyses

LCMSMS analyses were performed on a Q-TOF hybrid mass spectrometer equipped with a Z-spray source and coupled on-line with a capillary chromatography system CapLC (Waters, Manchester, UK). After loading, the peptide mixture (10 μl) was first concentrated and washed at 10 μl/min onto a reverse-phase pre-column using 0.2% formic acid as elution solution (eluent). The sample was then fractionated onto a C18 reverse-phase capillary column (75 μm × 20 mm) at a flow rate of 280 nl/min using a linear gradient of elution solution eluent B (0.2% formic acid in 95% acetonitrile) in A (0.2% formic acid in 5% acetonitrile) from 7 to 60% in 50 min. The mass spectrometer was set-up in a data-dependent MS/MS mode where a full scan spectrum (m/z acquisition range from 400 to 1600 Da/e) was followed by a tandem mass spectrum (m/z acquisition range from 100 to 2000 Da/e). Peptide ions were selected as the three most intense peaks of the previous scan. A suitable collision energy was applied depending on the mass and charge of the precursor ion. ProteinLynx software, provided by the manufacturers, was used to analyze raw MS and MS/MS spectra and to generate a peak list which was introduced in the Mascot MS/MS ion search software for protein identification.

Results

H-prune interaction with GSK-3β

Recently yeast two hybrids system experiments, by Kobayashi et al., 2006, found GSK-3β binding to h-prune protein. Knock-down of GSK-3β and h-prune by small interfering RNA (siRNA) suppressed cell migration. Thus h-prune formed a complex with paxillin and vinculin at focal adhesions. Loss of activity of GSK-3β or knockdown of GSK-3β and h-prune inhibited the disassembly of paxillin, the tyrosine phosphorylation of focal adhesion kinase (FAK), and the activation of Rac. These results indicate that the GSK-3β and h-prune cooperatively regulate the disassembly of focal adhesions to regulate cell migration (Kobayashi et al., 2006). In order to verify if h-prune ability to increase cell motility of breast cancer cell motility could be related to the binding to GSK-3β we first verified if such interaction was retained in a breast cancer cell lines. We co-immunoprecipitate those proteins in a mammary tumor cell line MDA-MB435-c100. While the binding of h-prune with GSK-3β was found (see Fig. 1), we did not found nm23-H1 in the same protein co-immunoprecipitation complex. At this time, we cannot exclude a direct involvement of nm23-H1 in GSK-3β signalling, in particular because of those recent findings reporting the involvement of CKI and Frat1 recently discovered and both involved in GSK-3β/Wnt signalling (review by Knippschild et al., 2005).

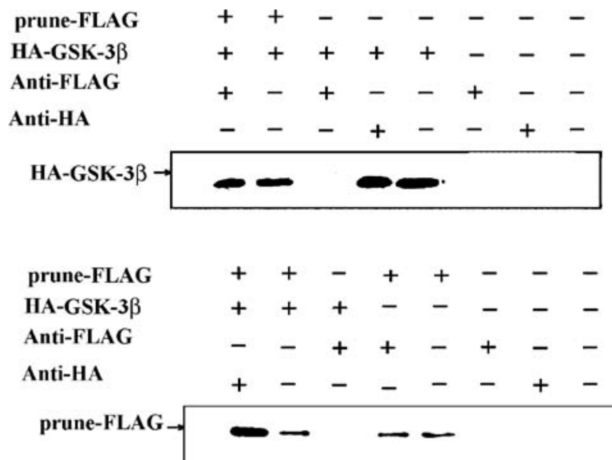

Fig. 1 GSK-3β and h-prune interaction in breast cancer cell line. H-prune and GSK-3β cDNA/protein were single transfected and cotransfected in MDA-MB435 breast cancer cell line. H-prune retains the FLAG tag otherwise GSK-3β has HA tag. Upper panel shows in lane 1 the cotransfection incubated with the anti-FlAG antibody to immunoprecipitate h-prune, the presence of the band of expected size after the Western blot analyses using an anti HA antibody reveals the presence of GSK-3β bound to h-prune. The lower panel shows in lane 1 (same experiment) but the immunoprecipitation was performed using an anti-HA antibody, the band in the Western blot revealed by the anti-FLAG antibody indicates the presence of h-prune in a complex with GSK-3β The demonstration of the functional consequence of this interaction is reported in the work presented by Kobayashi et al., 2006

A systematic approach to find new interactors of h-prune/nm23 complex in a breast cancer cell model

In view of these findings and in order to identify new protein-protein partners, we decide to perform pull-down experiments followed by mass-spectrometry analyses in the same breast carcinoma cellular model (see simplified model represented in Fig. 2, upper panel). We compared the proteins presented in pull-down experiments coupled to mass spectrometry between the breast clone overexpressing h-prune named MDA-prune #3 and the wild type breast cancer MDA-MB435c100 cell line. These experiments allow us to identify protein-protein interactions in a specific cellular compartment and in a time dependent manner. We took advantage of the FLAG tag at the carboxyl terminal of h-prune protein expressed stably in the MDA435c100 clone. This approach has the advantage to obtain protein/protein interactors that can directly or indirectly bind to h-prune protein, suggesting a potential network of interaction in which h-prune exert its role and induces cell motility. Within this approach we found gelsolin in the immunoprecipitation protein lysate products in the h-prune overexpressing clones, thus comparing to the wild type control cell line protein pull-down analyses (see Fig. 2, below panel). These findings are indicating a new role of h-prune in focal adhesions together with gelsolin, thus underlining its role on cell motility properties acquired by a breast cancer pro-metastatic cell.

Discussion

Gelsolin and GSK-3β have been found as new protein interactors of h-prune in breast cancer cell model, both proteins recently have been involved, with primary roles, in the signalling highly active in cytoskeleton remodelling, thus promoting cell motility. Several studies have shown a down-regulation of gelsolin in human tumors, examples are those reported data in lung, prostate, and ovarian cancer (Dosaka-Akita et al., 1998; Lee et al., 1999). One of the most important process involved in a tumor cell transformation phenotype corresponds to the miss-regulation of actin filaments as demonstrated by Clark et al. (2000) in a gene expression analysis using metastatic melanoma cells. They identified a set of genes selectively up-regulated in metastatic lines and several of these genes encode proteins that regulate the actin cytoskeleton. Cells derived from the gelsolin null-mice exhibit a variety of motility defects, indeed fibroblasts have a pronounced actin stress fibers, consistently with an inability to sever and remodel actin filaments. These cells are impaired to the ruffling process in response to growth factors, and exhibit defects in chemotaxis and in wound healing. Additionally, in central nervous system neurite retraction is defective, and neurons are more susceptible to glutamate-induced excito-toxicity (Witke et al., 1995; Azuma et al., 1998; Lu et al., 1997). Gelsolin down-regulation appears to be subjected to epigenetic modifications, mediated in part by histone acetylation/deacetylation mechanisms (Mielnicki et al., 1999). The frequency of gelsolin deficiency was found to be increased significantly in the progression from atypical ductal hyperplasia to ductal carcinoma in situ to invasive breast carcinoma, thus being gelsolin expression found as an independent marker of prognosis (Winston et al., 2001).

The question whether gelsolin alteration could be involved in tumor pathogenesis or is a simply side effect of the cancer progression, is to date still debated. It was additionally shown that in a gelsolin knock-out (KO) mammary epithelial cell (MCF-10a), an epithelial-mesenchymal transition (EMT) was encountered, a process occurring during development and metastasis by which epithelial cells change their morphology to acquire a fibroblast-like phenotype, thus showing a reduced cell adhesion and increased motility properties. Gelsolin was found to trigger the switch that controls E- and N-cadherin conversion through Snail transcription factor activation, leading to the development of human mammary tumors (Tanaka et al., 2006).

Otherwise, an additionally role of gelsolin in the acquisition of invasion properties in epithelial cell, is dependent on Ras/Rac signaling, since described by De Corte et al. (2002). Wild type ARF6 and PIP5K1a down-regulates the cell invasion function induced by gelsolin. This phenomena occurs despite both protein activities were found not required for collagen invasion (induced by gelsolin), as resulted from

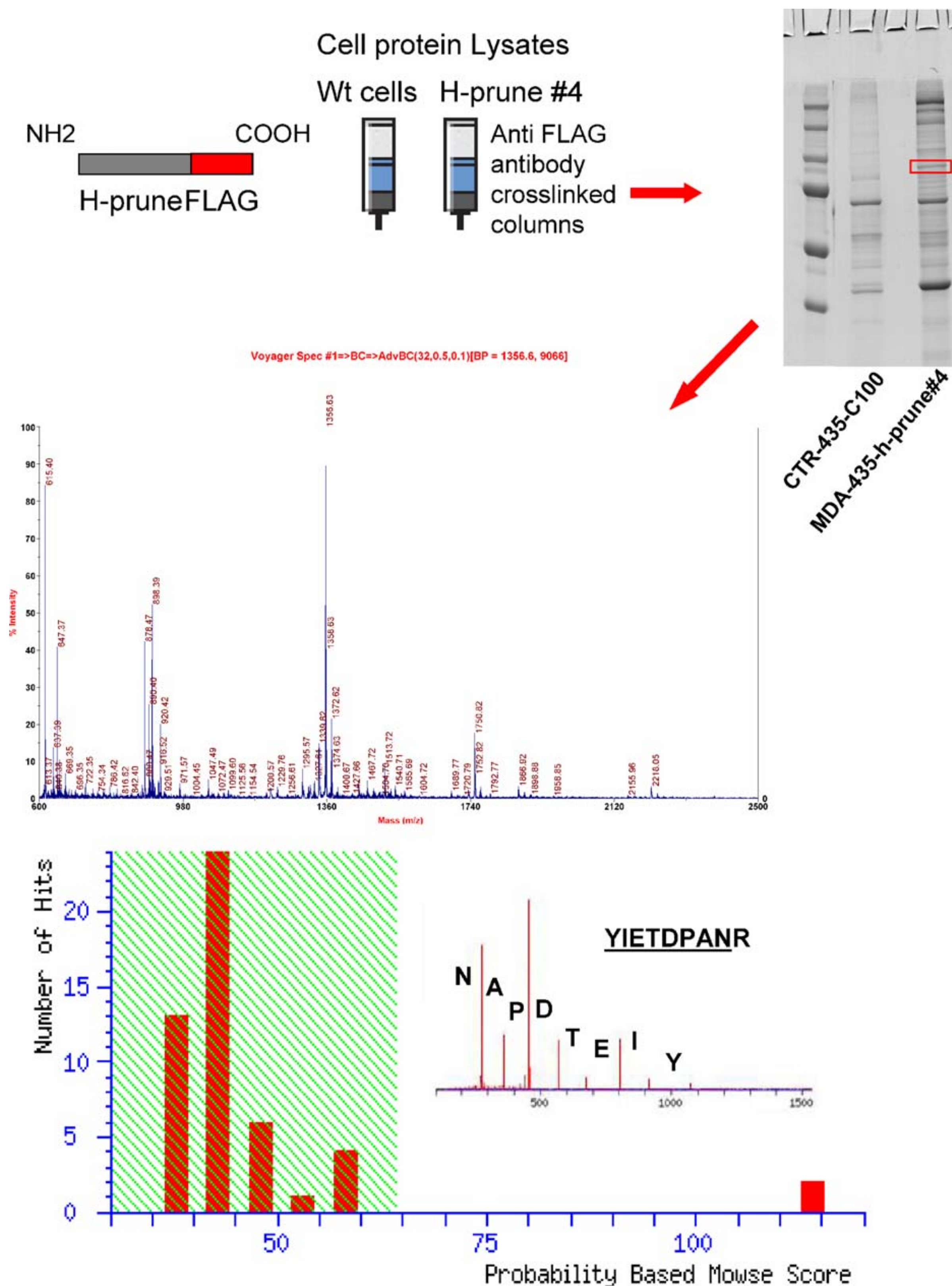

Fig. 2 Pull-down and mass-spectrometry analyses to identify a new putative interactor of h-prune. H-prune-containing protein complexes were isolated by immunoprecipitation with anti-FLAG antibodies and the individual components were fractionated by SDS-PAGE. The protein band indicated by the red square was excised from the gel, digested with trypsin and the resulting peptide mixture was analysed both by MALDI-MS and by nano LC-MS/MS. Mass spectral data were inserted into the Mascot software leading to the identification of the Gelsolin protein band (several peptides and amminoacid sequences were identified)

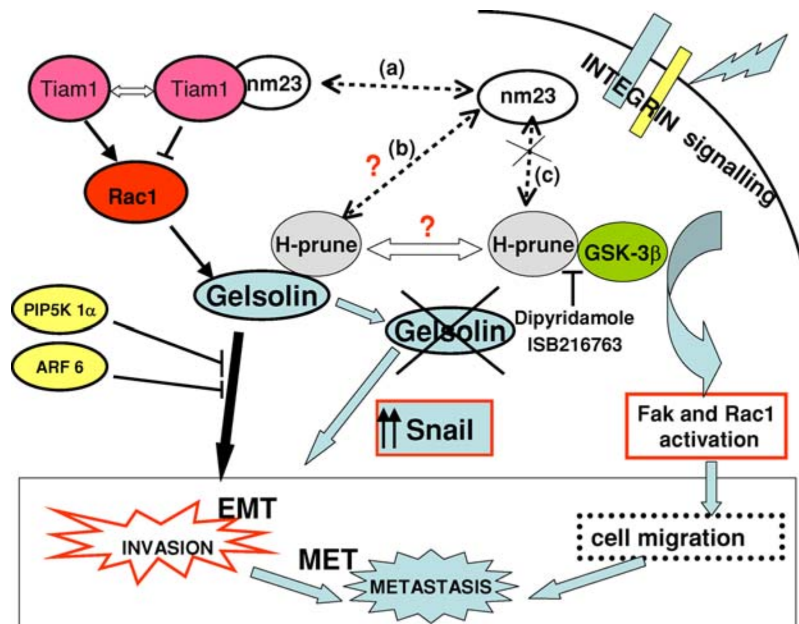

Fig. 3 The network of signalling pathways involved in h-prune-nm23-H1 and their different protein interactors. a–b) The nm23-H1 role will be integrated in the regulation of Rac1 (a small GTPase protein) induced cell migration by subtraction of Tiam1 (the Rac1 guanine-nucleotide exchange factor). This is becoming a key point in deciphering actin dynamics following integrin signalling. This was observed after the findings related to the gelsolin function as one of the major actin binding protein, thus operating through a signalling of Rac1. Is still an open question when and how during the tumor progression h-prune and nm23-H1 binds to gelsolin. If this will occur, another question will be raised if the binding could be responsible of a direct activation leading to invasion, or of a down-regulation of gelsolin activity, thus leading to Epithelial Mesenchymal Transition (EMT) c) nm23-H1 was not found in the protein complex formed by h-prune and GSK-3β in the focal adhesion. How it is regulated the formation of this complex and which enzymatic activity could be ascribed to h-prune is still an open question. The presence of two drugs (dipyridamole and the ISB216763) that potentially inhibited these complexes formation *in vitro* will be of importance in future translational research studies

a transfection of dominant negative protein isoforms in the gelsolin stable clones in MDCK cell line (De Corte et al., 2002).

In this latest cellular environment the role of nm23-H1 has been already investigated demonstrating the ADP-Rybosilation-Factor-6 (ARF6-GTP), a constitutively activate form of ARF6 protein, interacts with nm23-H1 and recruits it to the adherens junction (Palacios et al., 2002). It was since hypothesized that nm23 provides a source of GTP to ARF6 and that this latest protein mediate the recruitment of nm-23-H1 in to the cell-cell junctions, thus inducing a decrease in the cellular levels of Rac1-GTP. Consistent with these previous findings nm23-H1 down-regulates activation of Rac1 (Palacios et al., 2002). Although several studies have shown that Rac1-GTP promotes cell-cell adhesion at adherens junctions, and that dominant-negative Rac1 compromises junctional integrity; it is of note that activation of Rac1 has also been linked to the migratory phenotype (Santy and Casanova, 2001; Ridley et al., 1995; Fenteany et al., 2000). Because of h-prune function appertaining to the DHH family of proteins that includes exopolyphosphatases and nucleases (at this time not yet characterized), we propose here, that h-prune might be also posses an alternative enzymatic activity able to influence gelsolin functional behavior, influencing the cellular level of its cofactor PIP2 metabolism. This hypothesis needs further experiments in the next future. Additionally an interesting question needs to be address. Thus h-prune and gelsolin complex takes part to cancer progression and are both correlated to breast tumor behaviour and prognosis? H-prune is highly expressed in breast advanced cancer, otherwise gelsolin seems to be down-regulated, we think it is possible that this phenomena (loss of gelsolin) can occur by timing of this complex formation. It would be of interest if this can be related to the Epithelial Mesenchymal Transition (EMT) process occurring in the early and advanced malignancies. If h-prune enhances the loss of gelsolin activity during the tumor progression, this effect could be translated in to the additional function of h-prune protein and correlated to EMT occurring processes induced by loss of gelsolin in the breast cancer cells. Thus if gelsolin/h-prune complex is involved directly into gelsolin down-regulation, antagonizing this complex formation would be an advantage and might be considered in the translational research area an important tool to impair the tumor progression by inhibition of the protein complex formation. It is still an open question if gelsolin could be activated *in vivo* during tumor progression and takes part in invasion processes as already demonstrated *in vitro* (De Corte et al., 2002). We hypothesize here the presence of three main signalling actions related to the integrin pathways where we have encountered h-prune/nm23-H1

complex formation at this time (see model in Fig. 3). Rac1 seems to be necessary for gelsolin induced invasion and it is known that nm23-H1 could counteract this activation through the binding of Tiam1 (Palacios et al., 2002). The role of different regulators of gelsolin activity could be of interest in the valuation of new innovative therapeutic compounds. The role of h-prune/GSK-3β complex in the focal adhesion turnover suggests us to further investigate the presence of nm23-H1 in this complex might be timing dependent. This result can be verified in patients specimens to correlate their level of expression and presence to their prognosis. Because of the relevance of h-prune and GSK-3β activity on the motility phenotype induced by this protein complex, the combined use of inhibitors (dipyridamole and ISB216763) of both proteins seems to be an appealing approach for further translational studies. Inhibition of the binding between these two proteins, to antagonize the complex formation, could be additionally useful to discriminate between the two effects of the complex formation; a) the enzymatic activity and b) the recruitment of other signal transduction proteins. Despite the molecular biology advances made in this field of studies, more have to be discovered on understanding the mechanism insight of h-prune, GSK-3β and gelsolin in regulation on focal adhesions function and their involvement into the integrin pathways of action, then influencing motility and cancer metastasis. The function of nm23-H1 in this protein complexes and its potential peculiar timing of expression in a cellular compartment, at the present, is not known. This will be a valuable subject of further researches to ascribe definitively to these highly evolutionary conserved proteins, a role in cell migration, development and metastasis cancer formation in human.

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