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Protein–protein interactions: a mechanism regulating the anti-metastatic properties of Nm23-H1

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Abstract

Nm23-H1, also known as NDPK-A, was the first of a class of metastasis suppressor genes to be identified. Overexpression of Nm23-H1 in metastatic cell lines (melanoma, breast carcinoma, prostate, colon, hepatocellular, and oral squamous cell carcinoma) reduced cell motility in in vitro assays and metastatic potential in xenograft models, without a significant effect on primary tumor size. The mechanism of Nm23-H1 suppression of metastasis, however, is incompletely understood. Nm23-H1 has been reported to bind proteins, including those in small G-protein complexes, transcriptional complexes, the Map kinase, the TGF- β signaling pathways and the cytoskeleton. Evidence supporting these associations is presented together with evidence of resultant biochemical and phenotypic consequences of association. Cumulatively, the data suggest that part of the anti-metastatic function of Nm23-H1 lies in pathways that it interrupts via binding and inactivation of proteins.

Keywords

Nm23-H1; Protein interactions; Metastasis

Introduction

Nm23-H1, also known as NDPK-A and NME1, is a highly conserved 18 kDa protein. To date, ten different Nm23 genes (Nm23-H1 to Nm23-H10) have been identified in humans, with H1 and H2 being the most studied. One of the properties of Nm23-H1 that has led to considerable interest is its ability to downregulate metastasis formation independently of primary tumor size, acting as the first described metastasis suppressor gene (MSG). Steeg et al. first described Nm23-H1 as a potential regulator of metastasis formation via a comparative hybridization assay of metastatic and non-metastatic mouse melanoma cell lines (Steeg et al. 1988a). Since then, the over expression of Nm23-H1 in metastatic cell

lines (melanoma, breast carcinoma, prostate, colon, hepatocellular, and oral squamous cell carcinoma) has been repeatedly shown to reduce cell motility in in vitro assays and their metastatic potential in xenograft models (Liu et al. 2002; Steeg et al. 1988b; Leone et al. 1993; Miyazaki et al. 1999; Leone et al. 1991; Li et al. 2006; Steeg et al. 2008). These results were validated using an Nm23-M1 knockout mouse model (Arnaud-Dabernat et al. 2003); the Nm23-H1 protein shares a 94% and 89% homology with its mouse (M1) and rat (R1) counterpart, respectively. The induction of hepatocellular carcinoma in these mice led to a two-fold increase of lung metastases in the knockout mice compared to wild-type controls (Boissan et al. 2005). The metastasis suppressor field has now expanded to over 20 genes which regulate key signaling processes in metastasis (rev. in (Steeg 2003; Smith and Theodorescu 2009)). In contrast, high Nm23-H1 levels have been associated with a significant reduction in survival for hematological malignancies, neuroblastoma, and osteosarcoma patients (Oda et al. 2000; Niitsu et al. 2001). These different effects point to a specific role for Nm23-H1 dependent on the cancer type and may be regulated by multiple mechanisms and interacting proteins.

Multiple functions for Nm23 proteins have been proposed, including its nucleotide diphosphate kinase (NDPK) activity (Biggs et al. 1990b; Wallet et al. 1990), histidine protein kinase activity (Cuello et al. 2003; Wagner and Vu 2000), and a more recently characterized 3′–5′ exonuclease activity (Zhang et al. 2011). In addition, multiple proteins involved in disparate cellular functions have been reported as potential binding partners with Nm23-H1 with varying levels of rigor (Table 1). These interactions may help to explain the ability of Nm23-H1 to function as an MSG (Steeg et al. 1988b). In this review, we will describe proteins that have been identified as interacting with Nm23-H1, with special emphasis on the molecular pathways potentially regulated by these interactions. In general, evidence has been reported for proteins that bind Nm23 and disrupt its metastasis inhibitory activity, while other proteins appear to potentiate functional Nm23 pathways. In turn, Nm23 has been reported to affect the enzymatic activities of several binding proteins. These interactions could yield new information about the metastatic process as well as new potential therapeutic targets that have, to date, gone undiscovered.

Nm23 interacting proteins

Small G-protein function

One of the major in vitro phenotypes of Nm23-H1 over-expression has been the protein's ability to downregulate motility of cancer cells to a variety of chemoattractants (Horak et al. 2007). The Rho family GTPases, which are comprised of Rho, Rac, and Cdc42 subfamilies, have been extensively studied for their role in regulating cell migration in several different cell types and in response to multiple chemoattractants (Hall 1998). Rho family GTPases are key mediators of cell transduction pathways that regulate changes to the actin cytoskeleton and are activated by guanine nucleotide exchange factors (GEFs) and negatively regulated by GTPase-activating proteins (GAPs) and GDP dissociation inhibitors. Several GEFs for the Rho family GTPases have been reported to bind Nm23 proteins.

Tiam1—Tiam1 is a GEF for the Rac1 GTPase (Habets et al. 1994). Tiam1 was first identified in T-lymphoma cells as an invasion and metastasis inducing gene and has been shown to stimulate the activity of c-Jun kinase (JNK) via the production of GTP-loaded Rac1 (Minard et al. 2004). Otsuki et al. showed that while Nm23-H1 has no direct interaction with Rho family GTPases, transiently transfected Nm23-H1 and Tiam1 co-immunoprecipitated from 293T cells. Endogenous co-immunoprecipitation was demonstrated using anti-Tiam1 antibody on a lysate of mouse whole brain. Co-expression of Nm23-H1 and Tiam1 in 293T cells was used as a readout of Rac function and the addition of Nm23-H1 variably decreased Rac-GTP levels, although it cannot be proven that this resulted directly from Tiam1 or another pathway. A transphosphorylation of Rac by Nm23-H1 was also proposed, although subsequent evidence in multiple other biochemical assays indicates that this is likely due to Nm23-H1 NDPK activity.

Lbc—The Lbc proto-oncogene also encodes a Rho GEF. It was reported to bind Nm23-H2, but not -H1 in co-immunoprecipitation assays in transiently transfected COS cell lysates; Nm23-H2 was reported to suppress RhoA activity in vitro (Iwashita et al. 2004).

Dbl-1—Another GEF that has been characterized as being modulated by the effects of Nm23-H1 is Dbl-1, which was originally identified from a diffuse B-cell lymphoma DNA library (Ron et al. 1988). Subsequent screening has identified 45 additional members of the Dbl-1 GEF family, making it one of the largest known families of GEFs in mammalian cells (Aittaleb et al. 2010). Cdc42, as previously mentioned, is a Rho family member that can be localized to the plasma membrane in response to signals such as integrin and focal adhesion kinase (FAK) activation. Once there, Cdc42 is activated by the exchange of GDP to GTP by Dbl-1, which leads to downstream activation of several cellular pathways involved in DNA synthesis, transcription activation, translation regulation, and cytoskeletal reorganization.

Murakami et al. (2008a, b) utilized a yeast two-hybrid screen to identify potential binding partners of Nm23-H1. They identified Dbl-1 as a potential interactor and confirmed this interaction with co-immunoprecipitation experiments using transiently transfected 293T cells with GST-tagged Nm23-H1. Interestingly, the authors found that the Nm23-H1 complex with Dbl-1 could regulate the activation of Cdc42 by blocking the GDP/GTP exchange activity of Dbl-1. This regulation thereby reduced the ability of cells to migrate, which could be rescued by increased expression of Dbl-1 to overcome the inhibitory effects of Nm23-H1. In their proposed model system, Nm23-H1 would therefore serve as a mechanism to disrupt the activation of Cdc42 in response to chemoattractants by cancer cells by modulating the GDP/GTP exchange by Dbl-1.

The independent identification of three GEFs as Nm23-interacting proteins suggests that this signaling pathway may be biologically relevant. One hypothesis is that Nm23, through its NDPK activity, alters the GEF activity of these proteins; alternatively binding to Nm23 may block their normal function. Whether these proteins are substrates of a histidine protein kinase activity has not been determined.

Two small G-proteins of the Ras family were also reported to directly bind Nm23 proteins. Both reported outcomes that were unexpected in the metastasis suppressor literature.

ARF6—Palacios et al. (2002) demonstrated that constitutively activated ARF6 bound to Nm23-H1 in transiently transfected MCF-7 breast cancer cells and recruited Nm23-H1 to cell–cell junctions. ARF6 is a member of the ARF family of Ras-related GTPases and has been shown to control endocytic traffic and actin remodeling in a variety of cell types (D’Souza-Schorey and Chavrier 2006). In epithelial cells, ARF6 promoted E-cadherin endocytosis during adherens junction disassembly and cell migration (D’Souza-Schorey 2005). Moreover, increased ARF6-GTP in cells was associated with the loss of epithelial phenotype and acquisition of a migratory phenotype (Palacios and D’Souza-Schorey 2003). In addition, Palacios et al. (2002) showed that ARF6-GTP, through the interaction with and recruitment of Nm23-H1, facilitated the disassembly of cell adherens junction by a dynamin-dependent endocytosis in MCDK II cells. Nm23-H1 promoted this process through its NDPK activity, providing a source of GTP for vesicle internalization and through the downregulation of Rac1 activity by inhibiting Rac1 association with the nucleotide exchange factor Tiam-1. These data suggest a pro-migratory role for Nm23-H1, which may be relevant in explaining the association of high Nm23-H1 expression with adverse outcomes in non-Hodgkin lymphomas and neuroblastoma (Hartsough and Steeg 2000).

A role for Nm23-H1 in cell–cell adhesion has recently been suggested by Boissan et al. (2010). The authors confirmed the localization and role of Nm23-H1 in cell–cell adhesion, they described an anti-motility effect of Nm23-H1, compared to the pro-motility described by Palacios et al. Boissan et al. showed that the depletion of Nm23-H1 in hepato-carcinoma cells (HepG2) resulted in delocalization of plasma membrane junction proteins (E-cadherin, β -catenin, γ -catenin) without altering their expression levels and increased cellular motility. Therefore, it appears that the function of Nm23-H1 in the cell–cell adhesion and its effects on motility are cell line-dependent.

Rad—Another protein that has been described as interacting with Nm23-H1 is Rad, a member of the Ras-related GTPases. Co-immunoprecipitation experiments carried out by Kahn et al. have shown an interaction between Nm23-H1 and Rad in a human muscle lysate. Nm23-H1 was reported to function as a Rad GAP, and evidence of specificity was provided by using other small G-proteins; further experiments concluded that Nm23-H1 was a functional GEF for Rad and other small G-proteins (Gem, Ras). In reverse experiments, addition of Rad to Nm23 accelerated its NDPK activity. Using K-1735 murine melanoma cells, Rad overexpression accelerated proliferation as measured by ^3H -thymidine incorporation, but Nm23-H1 overexpression abrogated this effect; this constitutes one of the few published reports of Nm23 affecting proliferation. A further examination showed that in breast cancer cell lines the forced overexpression of Rad increased proliferation in vitro as well as tumor formation (Tseng et al. 2001). The co-expression of Rad and Nm23-H1 overcame the effects of Rad on the growth of the cells in culture as well as primary tumor growth; unfortunately, the authors failed to characterize the presence of metastases in their assays.

Transcriptional regulation

Originally, Nm23-H2 was proposed to be a transcription factor for *c-myc* and other genes, a function that was highly debated. Rather, the examples cited below indicate that Nm23

proteins may be functional parts of several transcriptional complexes and thereby alter the expression of metastasis-related genes.

EBNA1-3C—Epstein–Barr virus (EBV) is an oncogenic virus involved in lymphoid tumorigenesis. Latent EBV antigens, Epstein–Barr nuclear antigen (EBNA)-2, EBNA-3C, and latent membrane protein-1 (LMP-1), are critical factors for cellular transformation. EBNA-3C, when introduced into a cell, induces the transcription of specific cellular genes. One of the best characterized interactions of Nm23-H1 is with EBNA-3C, demonstrated in protein pull-down assays, transiently transfected cell lines, and stably EBNA-3C-transfected cell lines with endogenous Nm23-H1 expression (Subramanian et al. 2001b). Subsequent work has confirmed this interaction in lymphoblastoid cells (Murakami et al. 2005). The Robertson lab developed the hypothesis that the Nm23-H1/EBNA-3C interaction regulates the latter's transcriptional activity, including the targets Cox-2 and α V integrin; these effects may be indirect, mediated through interactions with the Sp1, Necdin, and GATA transcription factors (Kaul et al. 2009; Choudhuri et al. 2006). EBNA-3C has been demonstrated to induce tumor cell motility in vitro and metastasis in vivo, and increased Nm23-H1 expression counters this phenotype (Murakami et al. 2005; Kaul et al. 2007; Subramanian et al. 2001a).

Estrogen receptors (ER)—Estrogen is a mitogen for estrogen receptor-positive (ER+) breast and other cancers. The binding of estrogen to its receptor induces the translocation of the complex to the nucleus and subsequent binding to estrogen response element (ERE) in specific genes that in turn can drive tumorigenesis and progression. After the receptor binding to the ERE, many co-regulatory proteins are recruited. According to a report by Curtis et al., Nm23-H1 co-immunoprecipitated ER α from lysates of MCF-7 breast carcinoma cells; Nm23-H2 was not pulled down. This interaction increased ER α -ERE complex formation, and modulated ER α -mediated transcription. Through the knockdown of Nm23-H1 in MCF-7 breast cancer cells, increased gene expression of cathepsin D and Bcl-2 was shown, which are ER targets involved in cell migration, apoptosis, and angiogenesis (Curtis et al. 2007). Conversely, Nm23-H2 was reported to co-immunoprecipitate ER β in transiently transfected HeLa cells, with modulatory effects on transcriptional activation (Rayner et al. 2008).

ROR α /RZR—The ROR (retinoid acid receptor-related orphan receptor α)/RZR β (retinoid Z receptor β) nuclear orphan receptor superfamily functions in development, differentiation, and apoptosis (Meyer et al. 2000; Sun et al. 2000). They act as ligand-activated transcription factors. Paravicini et al. (1996) reported an interaction between ROR α and Nm23-H1 and –H2, and RZR β and Nm23-H2 using in vitro GST-Nm23 fusion protein pull-down assays. A functional consequence of this interaction has not been reported.

Oct transcription factor—The OCA-S co-activator complex for the Oct transcriptional complex has been reported to contain Nm23-H1. Nm23-H1 was immunoprecipitated from T24 cell extracts together with NPAT, Oct-1, P60/Sti1, NF-YA and p38 (Zheng et al. 2003). The function of Nm23-H1 in this complex remains unknown and additional experiments establishing a role of Nm23-H1 are required.

SET—SET, also known as PHPII, TAF1 β and IB_{2b}PP2A, is a nucleosome assembly protein that mediates the Granzyme A-triggered caspase-independent cell death pathway. Evidence for the association of SET and Nm23-H1 was published by Fan et al. (2003), including co-elution of Nm23-H1 in a SET purification scheme, co-immunoprecipitation of the recombinant proteins, and co-immunoprecipitation of the proteins from a lysate of K562 cells. SET is reported to translocate to the nucleus with Nm23-H1 (Fan et al. 2003; Chowdhury et al. 2006). Once into the nucleus, SET is degraded and Nm23-H1 is released; a DNA-nicking activity of Nm23-H1 was then proposed to be activated, resulting in apoptosis or DNA instability (Li et al. 2010). The DNA nicking activity was described as an endonuclease, which has been reported to be a co-purifying contaminant (Goswami et al. 2006), and the function of Nm23-H1 in this complex remains unknown. The SET protein has also been linked to chromatin modification, transcriptional activation, DNA repair and mRNA stability, and so this interaction may pertain to transcriptional complexes.

Cytoskeletal pathways

Given the dynamic changes to the cytoskeleton involved in tumor cell motility, a key component of tumor metastasis, it is unsurprising that Nm23 proteins have been reported to interact with the cytoskeletal machinery. A variety of interactions have been reported, and it is unclear how many are discrete or alternatively parts of a large multi-protein functional complex. Interestingly, the cytoskeletal interactions of Nm23 have been observed in cells from a variety of organisms and tissues, and during development; the ubiquity of this association suggests that it may serve as essential function (Biggs et al. 1990a; Otero 1997).

Vimentin—Otero et al. (1997) reported that NDPK (without indicating the specific Nm23 isoform) co-purified with vimentin from frog heart, lung, and liver extracts, and that vimentin eluted from NDPK affinity columns. A subsequent study by Roymans et al. (2000) using rat NDPK proteins, showed a one-way co-immunoprecipitation of NDPK in the C6 rat glioma cell line, primarily with the R2 (homologue of Nm23-H2) form but also including R1 (homologue of Nm23-H1) protein; similar associations were observed by co-immunofluorescence. Vimentin is a member of the intermediate filament family of proteins and promotes changes in cell shape, adhesion, and motility during cancer invasion (Mendez et al. 2010). Otero (2000) reported an interesting experiment in which vimentin was incubated with either BSA or NDPK; intermediate filaments assembled in the presence of NDPK formed bundles and became more densely packed. If NDPK has such an effect in vivo, over-expression of Nm23/NDP kinase could reduce cytoskeleton plasticity and cell movement, contributing to an anti-metastatic activity.

Tubulin—Several studies reported a possible interaction of Nm23-H1 with microtubules, with varying conclusions. Microtubules represent a dynamic structure in cells which grow at one end (plus end) by the polymerization of tubulin dimers (powered by the hydrolysis of GTP), and shrink at the other end (minus end) by the release of tubulin dimers (depolymerization). Microtubules participate in a wide variety of cell activities, most of which are involved in motion. While a co-immunoprecipitation has been reported between the mouse homolog of Nm23-H1 and β -tubulin in differentiated mouse myogenic C2C12 cells, co-immunoprecipitation experiments performed in MCF7 and MDA-MB-231 breast

carcinoma cells have not shown binding (Lombardi et al. 1995; Pinon et al. 1999). It has been hypothesized that Nm23, by its NDPK activity, could provide a high concentration of nucleotides required for both microtubule polymerization and vesicular dependent movement along the microtubules driven by the motor proteins kinesin and dynein. Other reports suggested an indirect interaction between human Nm23 and micro-tubules (Islam and Burns 1985). Recently, reports have shown an interaction with microtubules and two other Nm23 family members, Nm23-H7 and H9 (Ikeda 2010; Sadek et al. 2003).

Integrin cytoplasmic domain-associated protein 1 α (Icap-1 α)—Cellular adhesion and signaling in response to extra-cellular matrix is mediated by integrin complexes organized into transient focal complexes and more stable focal adhesions, from which signals pass to the cytoskeletal apparatus. Icap-1 α binds to the cytoplasmic tail of β 1 integrin and impairs cell spreading. An interaction of Icap-1 α and Nm23-H2 was originally found using yeast two-hybrid assays, but was confirmed using recombinant proteins and co-immunoprecipitations from CHO lysates transfected with Icap-1 α (Fournier et al. 2002). Confocal microscopy revealed a co-association of both proteins in the lamellipodia and ruffles during cell spreading in vitro, but not in stable focal adhesions. It is noteworthy that Rac/Tiam1 proteins also colocalize with Icap-1 α in lamellipodia, which suggests the possibility of multi-protein complexes. The authors hypothesize that Nm23-H2 may contribute to the functional Icap-1 α pathway.

Plakoglobin—Plakoglobin, also known as γ -Catenin, is a cytoplasmatic protein that binds the intracellular domain of cadherin cell–cell adherence proteins. α -Catenin, also binds actin, linking the cadherin–catenin cell adhesion complex to the actin cytoskeleton (Nelson 2008). Plakoglobin has been associated with tumor and metastasis suppressor activity, although the mechanism of this property is unclear (Kanazawa et al. 2008). Aktary et al. (2010) showed that the stable overexpression of Plakoglobin in SCC9 cells increased the protein levels of both Nm23-H1 and -H2. Using the wild-type cells, as well as Plakoglobin over-expressing cells, they also showed an interaction between Plakoglobin and Nm23 proteins. The authors suggest that Plakoglobin's metastatic suppressor activity may be mediated through the interaction and modulation of the stability, degradation, or other posttranslational mechanisms affecting the levels of Nm23-H1/H2.

Established signal pathways

Kinase suppressor of Ras 1 (Ksr1) in the Map kinase pathway—Ksr1 is one of two known Ksr proteins in mammals, first identified in Ras-dependent functional screens in *Drosophila* and *Caenorhabditis elegans* (Kornfeld et al. 1995). Ksr is thought to be a scaffold for the Map kinase signaling pathway, bringing all of the components together at the membrane to facilitate efficient signaling.

The proline 96 (P96S) and serine 120 (S120G) mutations of Nm23-H1 have previously been shown to abrogate the ability of Nm23 to inhibit motility to a variety of chemoattractants (MacDonald et al. 1996; Zhou et al. 2007). Analysis of these mutants revealed that they affected the histidine protein kinase ability of Nm23-H1 (Freije et al. 1997). A search of proteins that interacted with evolutionarily conserved two-component histidine protein

kinases was undertaken to elucidate potential binding partners that might aid in regulating this function of Nm23-H1. Using an *in silico* approach, Hartsough et al. (2002) found evidence that Ksr shares homology to the *Arabidopsis* two-component histidine sensor kinase interacting protein CTR1. Using two different human cell lines, 293T and MDA-MB-435, transiently transfected Ksr co-immunoprecipitated with Nm23-H1. The consequences of this interaction included the phosphorylation of Ksr on serines 392 and 434 by recombinant purified Nm23-H1. Phosphorylation of Ksr by Nm23-H1 altered the stoichiometry of binding proteins, in particular Hsp90, which resulted in more rapid Ksr degradation and inhibition of the Erk pathway (Salerno et al. 2005). Erk is a well-characterized member of the Map kinase pathway that is involved in signaling for multiple cell properties associated with metastasis, including motility, invasion, and proliferation (Hartsough et al. 2002).

The recent characterization of an antibody to phosphorylated histidine may at last enable more research to be conducted on this type of Nm23-H1 interaction (Kee et al. 2010).

STRAP in the TGF- β signaling pathway—Serine-threonine kinase receptor associated protein (STRAP) is a transforming growth factor beta (TGF- β) receptor-interacting protein, which is thought to stabilize the association of the TGF- β receptor with Smad proteins, altering transcriptional responses. The TGF- β family is well known in cancer biology as being inhibitory to normal cell proliferation but stimulating metastatic tumor cells (Bierie and Moses 2006). Seong et al. (2007) demonstrated that transiently transfected Nm23-H1 interacted with STRAP in co-immunoprecipitation assays; for the reverse, endogenous STRAP interacted with Nm23-H1 in co-immunoprecipitation experiments. A novel feature of this interaction is that mutation of cysteine 145 of Nm23 and cysteines 152 and 270 of STRAP eliminated the interaction, suggesting disulfide bond-mediated conformations were critical; however, none of the well-known Nm23-H1 mutations were tested. Readouts of TGF- β signaling included promoter luciferase assays, which were generally less than two-fold different in the presence and absence of Nm23-H1, and alterations in protein expression, which were minor. In a follow-on report Jung et al. (2007) reported an association of Nm23-H1, STRAP, and p53 using similar techniques which may alter p53-mediated transcription. Analysis of interactions between Nm23 and STRAP proteins at endogenous levels under conditions of TGF- β inhibition or stimulation, is awaited.

Other pathways

Prune—The overexpression of prune in the MDA-MB-435 cell line has been shown to promote cell motility and high levels of Prune have been shown to correlate with poor prognosis in breast, colorectal, and gastric cancers (D'Angelo et al. 2004; Forus et al. 2001; Kobayashi et al. 2006). Prune has two enzymatic activities, a phosphodiesterase (PDE) and, a more recently identified, exopoliphosphatase activity (PPase); both activities are therapeutic targets (D'Angelo et al. 2004; Tammenkoski et al. 2008). To date, only the Prune-PDE activity has been shown to stimulate *in vitro* cellular motility. No association between Prune-PPase activity and cellular motility has been demonstrated so far. The use of a specific Prune-PDE activity inhibitor, dipyrindamole, reduced the motility of the MDA-

MB-435 cells Prune overexpressing without effecting the migration of the cells overexpressing a mutant form of Prune lacking its PDE activity (D'Angelo et al. 2004).

A phenotypic interaction between Prune and Nm23 was first reported in studies on the orthologue *Drosophila* proteins, where the homozygous *Drosophila* Prune mutants, responsible for the brownish-purple “prune” eye color, did not affect *Drosophila* viability and fertility. In contrast, both the homozygous and hemizygous *Prune* mutants were shown to be lethal in the presence of just a single mutation in the *awd* gene, the Nm23 orthologue (Timmons and Shearn 1996). Prune was initially reported to bind Nm23-H1 using interaction mating assays, in which the P96S (killer of prune) mutation of Nm23 continued to interact, but the S120G mutant protein was impaired (Reymond et al. 1999). Co-immunoprecipitation of Prune and Nm23-H1 was demonstrated in MDA-MB-435 breast carcinoma cells (Garzia et al. 2008). In biochemical and functional assays, Nm23-H1 and Prune also appear to interact: the PDE activity of Prune was enhanced by the presence of Nm23-H1. On the other hand, the Prune exopolyphosphatase activity was markedly inhibited by Nm23-H1, probably through a competition-binding mechanism between Nm23-H1 and the substrates (Tammenkoski et al. 2008). Moreover, it was found that the complex induced a slight increase of Nm23-H1 NDPK activity. A recent review by Galasso and Zollo (2009) showed that the formation of the complex between Prune and Nm23-H1 is associated with the inhibition of Nm23-H1 anti-metastatic properties and an increase in cell motility. The authors postulate that this phenotype is due to two effects: (1) at high levels of Prune expression, all of the Nm23-H1 molecules are involved in the complex formation reducing the possibility of Nm23-H1 to interact with other partners mediator of its anti-motility function. (2) The complex formation enhances the cAMP-PDE Prune activity which has been associated with increased cell motility.

The Prune-Nm23-H1 interaction was impaired by IC261 treatment, a specific inhibitor of Casein kinase I (CKI) isoforms ϵ - δ . Garzia et al. (2008) reported that Nm23-H1 was phosphorylated on Ser 120, 122, and 125 residues by CKI ϵ - δ , and that this phosphorylation was required for the formation of Nm23-H1-Prune complex. Blockage of the CKI-mediated phosphorylation of Nm23-H1 inhibited cellular motility. This could represent a new therapeutic approach if confirmed in vivo.

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)—GAPDH is well known as one of the key enzymes involved in glycolysis. Besides its function as a glycolytic enzyme in the cytoplasm, several studies suggest that mammalian GAPDH is also involved in a number of intracellular processes such as membrane fusion, microtubule bundling, DNA replication, and DNA repair (Glaser and Gross 1995; Mironetz et al. 1994; Baxi and Vishwanatha 1995; Meyer et al. 2000). During the last decade, multiple reports have appeared concerning the role of GAPDH in different pathologies including prostate cancer progression (Rondinelli et al. 1997), programmed neuronal cell death (Berry and Boulton 2000), and age-related neuronal diseases, such as Alzheimer's and Huntington's disease (Burke et al. 1996). Engel et al. (1998) reported a new function for GAPDH as a protein partner for Nm23-H1 (and not Nm23-H2) and activator of its protein kinase function. Using SF9 insect cells overexpressing GAPDH, Nm23-H1 and H2, the authors purified His-tagged GAPDH and showed the presence of Nm23-H1 but not H2 in the resulting purified complex.

In the proposed complex, a dimer of GAPDH interacts with a dimer of Nm23-H1, and this interaction stabilizes the normally transient, short-lived oligomeric state of Nm23-H1. In a recent paper, the same authors identified and described Phosphoglycerate mutase, PGM, as a substrate for the Nm23-H1-GAPDH complex histidine kinase activity, leading to activation of PGM (Engel et al. 2004). The inhibition of the PGM phosphorylation by a mimic-peptide resulted in inhibition of MCF7 proliferation suggesting the importance of the glycolytic pathway in tumor growth process. GAPDH was also identified with Nm23-H1 as part of the Oct transcriptional complex discussed above.

Ribosomal protein small subunit 3—(rpS3) is one of the components of the 40S ribosomal subunit present in the eukaryotic ribosome. RpS3 is known to be cross linked with eIF-2 and eIF-3 and is directly involved in translation as well as playing a role in the repair of UV damaged DNA via its endonuclease activity (Westermann et al. 1979; Kim et al. 1995). Using a yeast two-hybrid system, Kim et al. identified Nm23-H1 as interacting with rpS3 and confirmed the interaction by two way co-immunoprecipitation (Kim and Kim 2006). This interaction was mediated by the leucine 190 of rpS3 and the histidine 118 and serine 120 of Nm23-H1. Transfection of either Nm23-H1 or rpS3 into HT1080 human fibrosarcoma cells decreased invasion, but invasion was not additively or synergistically affected by co-transfection of both genes. Similar results were reported for Erk activation and MMP-9 production in HT1080 cells and MCF-7 human breast carcinoma cells, leaving the phenotypic consequences of this interaction unknown.

Aurora-A—Aurora-A/STK15 is one of the centrosomal kinases involved in spindle formation during mitosis. Aurora-A has previously been shown to be an oncogene, and its overexpression has been observed in a number of cancer cell lines, including breast, ovarian, and prostate, in addition to breast cancer tissue where it correlates with worst prognosis (Bischoff et al. 1998; Kimura et al. 1997; Littlepage et al. 2002; Sen et al. 1997). A functional association has been shown between Aurora-A and the tumor suppressor p53 where the two proteins are able to determine a reciprocal inactivation when overexpressed in HeLa cells (Littlepage et al. 2002). In 2002, the co-immunoprecipitation and co-localization of Aurora-A/STK15 with Nm23-H1 in yeast and in normal human fibroblast IMR90 was demonstrated (Du and Hannon 2002). However, the interaction between the two proteins was not found to influence the activity of either.

Conclusions

Many proteins that are important for cellular biology do not function alone; instead, they regulate cellular pathways by forming protein complexes. These protein-protein interactions can have multiple biochemical and phenotypic consequences. Two well-known proteins that are modulated by their interactions with other partners are the tumor suppressors, p53 and Rb. In the case of p53, nearly 30 different binding partners have been described, including oncogenic viral proteins, transcription factors, transcriptional activators, and components of the DNA repair apparatus, all of which serve to either inactivate or regulate the function of p53 as a tumor suppressor (Ko and Prives 1996; Burkhart and Sage 2008). It is probable that functions of Nm23-H1 may also be regulated by a wide variety of binding partners, many of

which are yet to be discovered, and that these partners may prove to be important targets for future therapies.

To date, multiple binding partners for Nm23-H1 that may be involved in its metastasis suppressor ability have been reported (Fig. 1). Extreme caution is suggested in the interpretation of the data as Nm23 proteins are notoriously “sticky”, leading to non-specific interactions in vitro. Where possible, we have attempted to describe the primary data supporting an interaction; techniques such as yeast two-hybrid, co-immunofluorescence and even protein pull-downs should be interpreted with great caution. Two-way co-immunoprecipitations using proteins at endogenous expression levels constitute top-tier evidence and occur in only a minority of studies. Some of this may be due to a lack of specific antibodies to the endogenous proteins, forcing the use of tagged constructs. The phenotypic consequences of an interaction and the effect of known mutations constitute other important lines of evidence.

One of the most important conclusions from this review is that Nm23-binding proteins reported to date largely self-assemble into groups. At least three Rho GEFs were identified, suggesting the functional importance of this interaction. The phenotypic consequences of the Nm23-GEF interactions are poorly understood to date, and would benefit from biochemical and cell biology studies in both tumor metastasis as well as development. Another self-assembling category included the transcriptional complexes, of which Nm23 proteins belong to at least five. Two of the transcription factor interactions were discovered as being downstream of Epstein–Barr latent protein expression. It is tempting to hypothesize that changes in gene expression associated with Nm23 inhibition of metastasis may be generated via its participation in specific transcriptional complexes. Herein, we suggested that the Nm23-H1–SET interaction may also belong to this category, given the known transcriptional functions of SET and the controversy surrounding a proposed DNA endonuclease function for Nm23-H1. A variety of cytoskeletal proteins bound Nm23, although a unifying network of interactions has yet to emerge. Our lab is developing preliminary data identifying additional cytoskeletal-related Nm23-H1-binding proteins that may help to generate a coherent regulatory network. The other self-assembling small group constituted regulators of well-known signaling pathways, including the Erk Map kinase and TGF- β signaling pathways.

One function of Nm23-H1, visible in the compiled data, is to bind up the “bad guys”, proteins that promote invasion and metastasis. Included in this list is Prune, EBV latent proteins, and Rad. In other cases, Nm23-H1 promoted functional anti-metastatic pathways through still poorly understood mechanisms. Protein–protein binding also influenced the subcellular localization of Nm23, i.e., the interaction with SET, ER α , EBNA1–3C promoted the translocation of Nm23-H1 from cytoplasmic compartment to the nucleus; the interaction with ARF6-GTP recruited Nm23-H1 to the membrane. Protein–protein associations also altered the activity of the binding protein, i.e., the complex formation with Nm23-H1 increased the activity of Prune, Rad, and dynamin, inhibited the activity of Rac1/Tiam and Dbp1, or promoted the degradation of Krs.

One of the advantages of a relatively mature field of study is the availability of multiple model systems, mutations, and assays that can be used to further identify and understand Nm23 protein–protein interactions. Work on this protein family is now ongoing in the fields of development, signaling, cancer, and other diseases, and can be marshaled to provide a multi-disciplinary approach to these protein–protein networks.

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Fig. 1.

A proposed model showing the interactions of Nm23-H1 with cellular pathways. The depicted tumor cell is polarized and moving upwards in response to a chemoattractant, while still maintaining a cell–cell interaction to a stromal cell below it. **a** The leading edge of the cell showing a representation of the focal adhesion structure, including pathways involved in cell motility that could be modulated by Nm23-H1 as shown in **b**. The leading edge is driven by actin polymerization-mediated protrusions that point in the direction of cell movement. The JNK, Rho, and Map kinase pathways are involved in cell signaling inducing motility

towards an external stimulus. **b** Depiction of the trailing edge of the cell showing a link between Nm23-H1 anti-motility properties and the regulatory effect on its described binding partners. Motility-associated pathways can be inhibited by the interaction of their constituents to Nm23-H1. The binding of Nm23-H1 to Dbp1 can modulate the Rho pathway, while Tiam1 binding can inhibit JNK and association with Ksr1 can regulate the Map kinase pathway. Nm23-H2 can interact with Icap-1 α inhibiting its association with the FAK complex. **c** Cell–cell interaction between the polarized cell and another nearby stromal cell. Nm23-H1 can bind to Vimentin, leading to a localized thickening of the intermediate filaments. In addition, it can bind to the Plakoglobin complex in the area of cell–cell contact

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Table 1

Nm23-H1-binding proteins and the biochemical consequences of the interaction

Protein	Assay	Cell line (s)	Interaction phenotype (s)	Reference
Tiam1	Co-IP	293T, MDA-MB-435	Inactivation of Rac1 pathway	Osuki et al. 2001
Lbc ^a	Co-IP	COS7	Inactivation of RhoA	Iwashita et al. 2004
Dbl-1	Two-hybrid, GST pull-down, Co-IP	Yeast, 293T	Cdc42 inactivation and inhibition of cytoskeleton reorganization	Murakami et al. 2008a, b
ARF6	Co-IP	MCF-7, MDCK II, NIH3T3	E-cadherin-endoctocytosis with consequent increase of cell motility	Palacios et al., 2002
Rad	GST pull-down, Co-IP	Recombinant protein, human muscle lysate, K-1735	Rac activation and consequently inhibition of Ras pathway	Zhu et al. 1999
EBNA-1-3C	Co-IP	293T	Regulation of EBNA-1-3C-mediated transcription	Subramanian et al. 2001
ERα	Co-IP	MCF-7	Negative regulation of ER-mediated transcription	Curtis et al. 2007
RORα	Two-hybrid, GST pull-down	Yeast, recombinant protein	Unknown	Paravicini et al. 1996
Oet-1	Co-IP, IF	T24	Unknown	Zheng et al. 2003
SET	GST pull-down, IF, Co-IP	Recombinant protein, K562	Apoptosis	Fan et al. 2003
Vimentin	GST pull-down, Co-IP	Recombinant protein, frog heart muscle liver lung, C6	Reduced cytoskeleton flexibility	Otero et al. 1997 Otero, 2000
Tubulin	IF, Co-IP	B16, MDA-MB-231, MCF-7 C2C12	Microtubule depolymerization	Pinon et al. 1999 Lombardi et al. 1995
Icap-1α ^a	Co-IP	Recombinant protein, CHO	Unknown	Fournier et al. 2002
Plakoglobin	IF, Co-IP	Plakoglobin-expressing SCC9, MCF-10-2-A, MCF-7, SW620, MDCK	Increase of Nm23-H1 stability	Aktary et al. 2010
Ksr1	Co-IP	293T, MDA-MB-435	KSR phosphorylation and degradation	Hartsough et al. 2002
STRAP	GST pull-down, Co-IP	293T, Hep3B, SK-N-BE(2)C	Cell cycle arrest and p53-dependent apoptosis	Seong et al. 2007
Prune	Co-IP	MDA-MB-435	Inhibition of Nm23-H1 NDKP and metastasis suppressor activity	D'Angelo et al., 2004 Timmons and Shearn 1996
GAPDH	GST-pulldown	Recombinant protein	Increase Nm23-H1 stability	Engel et al. 1998
rps3	Two-hybrid, Co-IP	Yeast, HT1080	Inactivation of Erk pathway	Kim and Kim 2006
Aurora-A	coIP, IF	Yeast, IMR90	Unknown	Du and Hannon 2002

Co-IP co-immunoprecipitation, IF immunofluorescence, 293T human renal epithelial cell line, B16 mouse melanoma, C2C12 mouse skeletal muscle cell line, C6 rat glioma cell line, CHO Chinese hamster ovary cell line, Hep3B human hepatoma cell line, HT1080 human fibroblast cell line, IMR90 human fibroblast cell line, K-1735 murine melanoma cells, K562 human immortalized myelogenous leukemia cell line, MCF-10-2-A human non-tumorigenic epithelial cell line, MCF-7 human breast adenocarcinoma cell line, MDA-MB-231 human breast carcinoma cell line, MDA-MB-435 human breast carcinoma/melanoma cell line (Rae et al., 2007), MDCK Madin Darby canine kidney cell line, SCC9 human squamous carcinoma cells, SK-N-BE(2)C human neuroblastoma cell line, SW620 colon carcinoma cell line, T24 human bladder carcinoma cell line, NIH 3T3 mouse embryonic fibroblast cell line

β has been reported to have an interaction with Nm23-H2 but not with Nm23-H1

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