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MINI REVIEW

Extracellular NME proteins: a player or a bystander?

Patrizia Romani^{1,4,5}, Marilena Ignesti^{1,5}, Giuseppe Gargiulo¹, Tien Hsu^{2,3} and Valeria Cavaliere¹

The *Nm23/NME* gene family has been under intensive study since *Nm23H1/NME1* was identified as the first metastasis suppressor. Inverse correlation between the expression levels of *NME1/2* and prognosis has indeed been demonstrated in different tumor cohorts. Interestingly, the presence of NME proteins in the extracellular environment in normal and tumoral conditions has also been noted. In many reported cases, however, these extracellular NME proteins exhibit anti-differentiation or oncogenic functions, contradicting their canonical anti-metastatic action. This emerging field thus warrants further investigation. In this review, we summarize the current understanding of extracellular NME proteins. A role in promoting stem cell pluripotency and inducing development of central nervous system as well as a neuroprotective function of extracellular NME have been suggested. Moreover, a tumor-promoting function of extracellular NME also emerged at least in some tumor cohorts. In this complex scenario, the secretory mechanism through which NME proteins exit cells is far from being understood. Recently, some evidence obtained in the *Drosophila* and cancer cell line models points to the involvement of Dynamin in controlling the balance between intra- and extracellular levels of NME. Further analyses on extracellular NME will lead to a better understanding of its physiological function and in turn will allow understanding of how its deregulation contributes to carcinogenesis.

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Cancer development depends on the interplay between oncogenes and tumor suppressor genes (TSGs). While tumor load can cause morbidity and weight loss, metastasis is the primary cause of patient mortality in the majority of cancers.^{1,2} Therefore the process of metastatic transformation and dissemination of cancer cells are under intensive studies. One particular area of interest involves the function of metastasis suppressor genes (MSGs).

Unlike TSGs, MSGs do not prevent primary tumor growth but inhibit cancer metastatization. The products of MSGs vary in their functions and subcellular locations, and have been detected in different intracellular compartments as well as in the extracellular environment.³

Nme1 was the first identified MSG, whose downregulation was found in metastatic derivative of a murine melanoma cell line.⁴ In human, the *NME* gene family consists of ten related members, some of them displaying the nucleoside diphosphate kinase activity (NDPK).⁵ *NME1* and *NME2* are the most closely related and are the ones most implicated in tumor progression.³ In many clinical cancer samples such as breast cancer, a correlation between a reduced NME level and

metastasis has been found.⁶ However, in other tumors, up-regulated NME levels have been correlated with poor prognosis, especially in neuroblastoma⁷ and some forms of leukemia and lymphoma.^{8,9} Therefore NME proteins are most likely multifunctional, and any therapeutics based on manipulating NME expression levels should consider the tissue context and potential side-effects. In this sense, the recently discovered extracellular activity of NME proteins necessitates closer examination of its pathophysiological relevance.

Although lacking a secretion signal peptide, high levels of extracellular NME proteins have been detected in tissue culture supernatants of a wide range of cancer cell lines and in body fluids of cancer patients. The association of extracellular NME proteins with tumor progression is of prognostic significance in a number of cancer types (see below). However, how these proteins can promote metastasis is still far from our understanding and is complicated by the intricate cellular and cancer stromal processes involved in metastasis.

In this review we first focus on involvement of the extracellular NME proteins as modulators of growth and

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differentiation of embryonic stem cells, and then we point out their putative significance in different tumor cells. In addition, we include our recent data obtained using the *Drosophila* model, on the regulation of extracellular levels of fly NME orthologs.

EXTRACELLULAR NME1 AND NME7 IN STEM CELL PLURIPOTENCY REGULATION

Stem cells are classified as totipotent, pluripotent, or multipotent. As the pluripotent stem cells possess the ability to become any cell type in the human body, they hold the greatest promise for therapeutic use. These cells could be used to replace damaged tissues in organs that have traditionally been thought not to have a significant potential for functional self-repair, such as brain tissue, heart muscle, kidney, and spinal cord.^{10–13} However, recent studies indicate that cultured human pluripotent stem cells are not truly pluripotent, rather they are in a slightly differentiated state called ‘primed,’ as opposed to the true pluripotent ‘naïve’ state.^{14,15} In an attempt to create a growth system that enables maintenance of true pluripotency and self-renewal of naïve stem cells, the Bamdad laboratory has demonstrated that NME acts as a growth factor interacting with its receptor MUC1* (a cleaved form of the full-length MUC1), promoting pluripotency and suppressing differentiation.¹⁶ The transmembrane protein MUC1 is a critical marker for identification of pluripotent stem cells as well as a key mediator of the growth and differentiation of stem cells. In particular, undifferentiated stem cells express a high level of the cleaved form of MUC1 (MUC1*) that retains only 45 amino acids of the original extracellular domain and that has previously only been detected on cancer cells.¹⁷ NME1, which has been shown to be an activating ligand of MUC1* in cancer cells, co-localizes with MUC1* on pluripotent stem cells.¹⁸ As soon as pluripotent cells initiate differentiation, the quiescent full-length form of MUC1 is detected. Thus, the switch from MUC1* to the full-length protein may be considered one of the first signals of the onset of differentiation.

Smagghe *et al*¹⁹ demonstrated through surface plasmon resonance that differential NME interaction with the extracellular domain of MUC1* is strictly dependent on the NME multimerization state. The updated mechanistic model²⁰ (Figure 1) suggests that early stem cells are able to secrete monomer NME7 that can bind to the extracellular domain of MUC1* and promotes growth and pluripotency. Hence, extracellular NME7 has an important role in maintenance of early stem cell in the naïve status. This is also supported by the evidence that master transcriptional regulators of pluripotency such as SOX2 and NANOG, are able to bind to the promoter of NME7. In addition, Wang *et al*²¹ showed that Nme7 (and Nme6) expression levels are crucial for mouse embryonic stem cell renewal. Carter and colleagues also showed that in late stem cells, the pluripotency

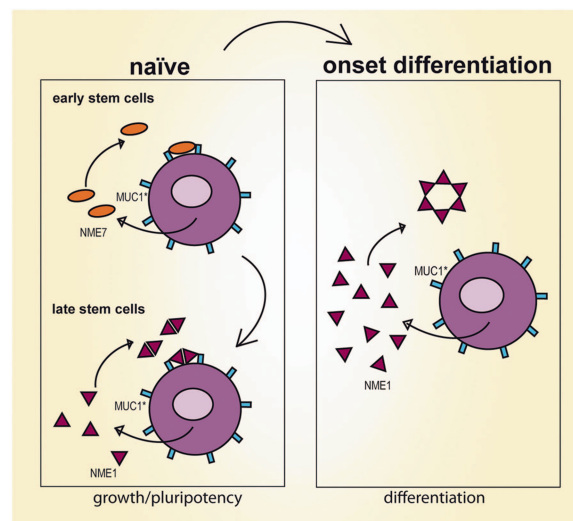


Figure 1 Mechanistic model illustrating how NME7 and NME1 control pluripotency and differentiation in stem cells. Modified from Carter *et al*.²⁰

transcription factor BRD4 (bromodomain and extra-terminal domain family member) suppresses NME7 and upregulates NME1 that in turn replaces NME7 in promoting growth and pluripotency. Studies by the Bamdad laboratory compared the different binding affinities of NME1 multimers to the MUC1* extracellular domain and demonstrated that only the NME1 dimeric form can bind to MUC1*.¹⁹ It is interesting that some NME1 mutants prefer the dimeric state. Of particular interest is the mutant NME1^{S120G}, which is the form mostly expressed in cancer cells. In this context, it is interesting to point out that cancer cells are characterized by their loss-of-ability to limit stem-like self-renewal.^{22,23} NME1^{S120G} in the dimeric form is sufficient to inhibit stem cell differentiation to allow long-term growth and maintenance of pluripotency and to increase expression of naïve markers.

Surprisingly, by manipulating the NME1 multimerization state, Smagghe *et al*¹⁹ also demonstrated that culturing undifferentiated stem cells in the presence of NME1 hexamers rapidly leads to differentiation. Interestingly, the affinity-binding assay demonstrated that NME1 hexameric form is not able to bind MUC1*. The authors hypothesized that once the undifferentiated stem cells reach critical density, they secrete an additional amount of NME1 thus increasing its extracellular level in the microenvironment. At higher concentration extracellular NME1 forms hexamers that do not bind MUC1* and differentiation occurs (Figure 1). It is not certain, however, whether hexameric NME1 can actively promote stem cell differentiation. Nonetheless, the implication is that extracellular NME proteins in different multimeric states exert opposite effects on stem cell differentiation and constitute a sort of ON/OFF switch.

EXTRACELLULAR NME1 IN CENTRAL NERVOUS SYSTEM PATHOPHYSIOLOGY

Q3

Studies in mouse have pointed out a requirement of Nme1 during the development of the nervous system.^{24,25} Most of the studies have focused on the role of intracellular Nme1; however, in 2010 Wright *et al*²⁶ first reported that extracellular Nme1 is able to stimulate neurite outgrowth of explanted embryonic and adult dorsal root ganglia *in vitro*. The authors showed that extracellular Nme1 acts as a positive chemotactic signal that induces neuronal growth cones to move toward substrates with higher Nme1 concentration. It is worth noting that a more moderate but still significant outgrowth of neurites is observed even in the absence of the nerve growth factor. Moreover, the authors also observed enhanced neurite branching when dorsal root ganglia extend on Nme1-coated substrata, and growth cone turning at the boundary between Nme1-coated and uncoated substrata. Such directional movement of growth cones allows neurites to continue to grow in the microenvironment containing Nme1. Since this positive guidance effect of Nme1 occurs in embryonic neurons, it could be hypothesized that the extracellular Nme1 also has a role in the development of the central nervous system (CNS). Indeed, chemotactic signals are broadly used during CNS development and, interestingly, evidence suggests that similar stimuli and subtended signaling pathways could also regulate vascular system development.²⁷ The exact mechanism through which Nme1 drives neurite outgrowth is not defined but the NDPK function seems not necessary, since extracellular kinase-dead mutant Nme1^{H118F} is able to induce the same effect as the wild type protein.

Extracellular Nme1 has also been implicated in the short-term response to damage in an *in vitro* traumatic brain injury model (TBI). Loov and colleagues co-cultured primary neurons, astrocytes and oligodendrocytes to simulate brain environment and analyzed, through mass-spectrometry, proteins present in the culture medium of injured and uninjured neurons. By comparing these proteomes, they found that Nme1 was recovered as one out of 53 proteins exclusively present in the culture medium of injured neurons.²⁸ This specific release after injury suggests a potential neuroprotective or regenerative function of extracellular Nme1. On the other hand, their data imply that neurons, astrocytes or oligodendrocytes do not secrete Nme1 in physiologically healthy conditions. However, whether the extracellular Nme1 indeed plays an active role in neuron repair, or is a passive occurrence resulting from cell death, is not yet clear.

An established model for global brain insult is the *post-mortem* cerebrospinal fluid (CSF)²⁹ that has been successfully used to identify potential markers for neurodegeneration. Interestingly, NME1 is increased in CSF within 6 h *post-mortem* with respect to control CSF,²⁹ suggesting the possibility that it could be a marker for neurodegenerative diseases. NME1 has also been identified as a highly sensitive

and specific marker of stroke, whose plasma levels rise in the very early time frame of 3-6 h after the onset of symptoms.³⁰ In this regard, the work from the Meloni laboratory³¹ in two different ischemia-related injury models (excitotoxicity mediated by L-glutamic acid and *in vitro* ischemia through oxygen and glucose deprivation) is noteworthy. They showed that exogenous Nme1 treatment of primary rat cortical neuronal culture 24 h before and during the injuries enhances neuronal survival, thus exerting a neuroprotective activity. These authors speculated that this effect could be mediated through the binding of Nme1 to a putative receptor thus triggering signaling pathway activation. The involvement of Muc1 in this system is not likely because of the absence of expression of this glycoprotein in the brain. The authors also proposed an indirect mechanism of Nme1 intervention for neuroprotection involving an enhancement of extracellular ATP or GTP levels leading to activation of purinergic receptor and triggering of neuroprotective pathways. It is possible that NME proteins might protect ADP and ATP from degradation by ectonucleotidases, but this mechanism would require one NME protein molecule for one nucleotide, which is highly inefficient.³² If as proposed, NME1 and NME2 can act as suppliers for extracellular ATP, this requires the classical transphosphorylase activity of NME proteins, which necessitates a constant source of NTPs and ADP to maintain ATP in effective concentrations. It has been shown that the amount of such nucleotides in the extracellular space is limited.³³ This will also restrict the efficiency of extracellular NME proteins to generate nucleotide triphosphates even if more extracellular NME proteins are present. As such, these potential mechanisms require further validation.

Besides the hypothesized physiological roles in CNS development and regeneration, extracellular NME1 has also been linked to neuroendocrine tumor development such as neuroblastoma. Neuroblastoma is an embryonic malignancy arising from neural crest-derived progenitor cells, manifesting in pediatric patients. Interestingly, increased intracellular NME1 level has been linked to poor prognosis, contradicting its role as a metastasis suppressor.⁷ Moreover, serum level of NME1 also displays a direct correlation with poor outcome in neuroblastoma patients.³⁴ Therefore intra- and extracellular NME1 may be pro-oncogenic in this setting, although it is not yet clear whether the extracellular NME1 has any pathophysiological function in this tumor type.

EXTRACELLULAR NME IN ACUTE MYELOID LEUKEMIA

Acute myeloid leukemia (AML) is an aggressive malignancy characterized by an uncontrolled clonal expansion of immature myeloid blast cells with an impaired capacity to differentiate.³⁵ Proliferation of these cells in the bone marrow and blood affects normal hematopoiesis causing anemia, neutropenia and thrombocytopenia.

The central feature of AML is the failure of differentiation. Okabe-Kado and colleagues identified secreted Nme2 in murine M1 leukemic cell line that impaired myeloid

differentiation.^{36,37} This activity was independent of its kinase function.³⁸ *NME1* and *NME2* were also found highly expressed in AML cells and the levels of *NME1* gene expression was correlated with poor prognosis in AML.^{39–41}

The intracellular amount of *NME1* was inversely correlated with hematopoietic maturation,^{42,43} suggesting an anti-differentiation function. The finding that *NME1* is elevated in the serum of AML patients⁴⁴ while no high concentration of this protein is present in normal healthy plasma,⁴⁵ suggests a role of extracellular *NME1* in AML. Therefore elevated expression of *NME1* appears to be oncogenic in AML.

Data on the extracellular functions of NME proteins on AML cells came from using recombinant NME proteins (reviewed in Okabe-Kado *et al*⁴⁶ and Lilly *et al*⁴⁷). Okabe-Kado *et al*⁴⁸ using recombinant NME proteins on cultured primary AML cells demonstrated that extracellular *NME1* promoted the survival and growth of AML cells at a concentration equivalent to the level found in AML patients. This activity was independent of the *NME1* NDPK function,

since the recombinant mutant *NME1*^{H118F} protein could promote survival and growth of AML cells. The pro-survival activity was also reported for the recombinant *NME2* protein.⁴⁸

Lilly *et al*⁴⁹ by analyzing cells expressing the AML blast marker CD34⁺ and the myeloid differentiation marker CD11b⁺ demonstrated that *NME1* does not bind the most immature blasts in AML mononuclear cell preparations, but it binds to more mature CD34^{lo}/CD34⁺ (CD34^{low}/negative) and CD11b⁺ cells. Conditioned medium (CM) by more mature cells that had been stimulated by *NME1* enhances the survival of purified blast cells. These findings suggest that *NME1* indirectly promotes survival and growth of AML blasts by acting on the more mature cells in the clone, and that a cross-talk between cell populations occurs within the tumor clone. However, the receptor responsible for the *NME1* pro-survival effect on AML cells is still not known, and it has been shown that MUC1* is not involved.⁴⁹ How extracellular *NME1* promotes survival and growth of AML cells is still unclear, but the response of AML cells to *NME1* was associated with the production of a number of cytokines. An increased levels of granulocyte macrophage colony stimulating factor (GM-CSF), interleukin-1 β (IL-1 β), and interleukin-6 (IL-6) have been found in CM of AML cells treated with *NME1*.^{49,50} The cytokines GM-CSF and IL-1 β are known to promote AML growth.⁵¹ Lilly *et al*⁴⁷ used KG1a CD34⁺ AML cell line, in which the high *NME1* mRNA level is consistent with the one observed in primary CD34⁺ cells of AML samples. They demonstrated that *NME1*-containing CM and IL-1 β could activate the nuclear factor- κ B (NF- κ B) that was shown to be constitutively active in AML stem cells.⁵² Altered activity of NF κ B has been found in different types of cancer and is correlated with the cancer cell resistance to chemotherapies and radiation.^{53–56} 40% of AML patients have shown constitutively active NF- κ B⁵² that allows leukemia cells to escape apoptosis and to proliferate. Based on their data the authors also suggest that CD34⁺ AML cells secrete GM-CSF after the release of IL-1 β from *NME1* stimulated mature CD34^{lo}/CD11b⁺ cells. Recently Lilly *et al*⁴⁷ proposed a model on the function of *NME1* in AML (Figure 2). The high expression of *NME1* in the AML blast cells generates an elevated level of extracellular *NME1*, which binds to more mature myeloid cells promoting the secretion of a number of cytokines including IL-1 β and IL-6. The role of IL-6 in AML is poorly understood; however, there is evidence indicating that high IL-6 levels trigger constitutive activation of the STAT3 pathway, which has been detected in 20–45% of AML samples.^{57,58} According to the model proposed by the authors, the secreted IL-1 β and IL-6 cytokines will promote the survival of blast cells, by triggering survival pathways such as STAT3 and NF- κ B, as well as enhancing the expression of other cytokines such as GM-CSF.

The overall findings on the extracellular *NME1* activity on AML suggest that *NME1* could be strongly involved in AML progression, not just a passive correlate of prognosis.

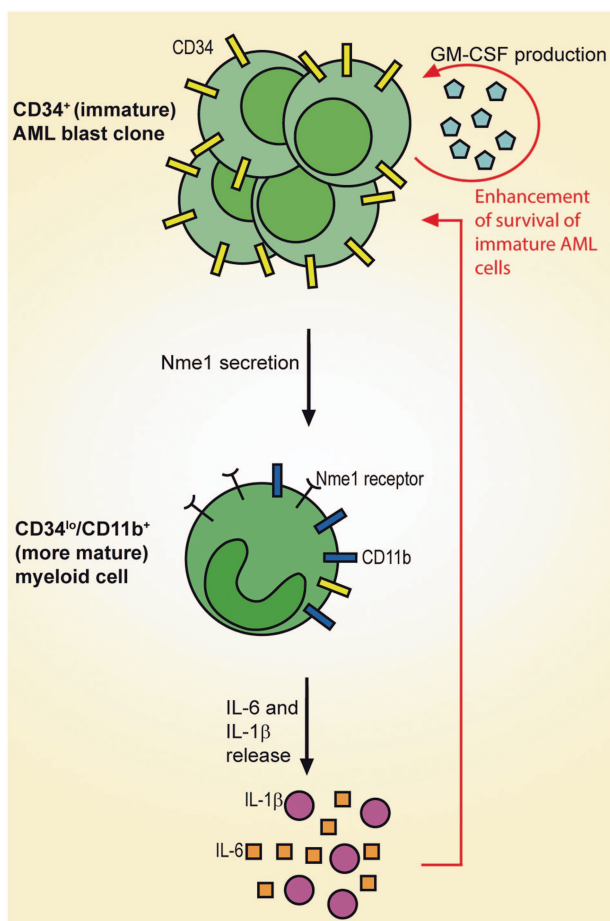


Figure 2 Model of the role of *NME1* in the promotion of immature AML blast survival and growth. The interplay between subpopulations of AML cells at different maturity stages is mediated by the secretion of small molecules, including *NME1*. This generates a positive feedback loop that prompts malignant blasts to expand. Modified from Lilly *et al*.⁴⁷

Moreover, of particular interest is that in addition to AML, high levels of NME1 were found in the serum of patients with malignant lymphomas,^{45,59–61} suggesting that the extracellular levels of this protein have a relevant role in clinical outcome in diverse hematological malignancies.⁴⁶

EXTRACELLULAR NME1 AND NME2 IN BREAST CANCER

The presence of NME proteins in the extracellular environment of breast cancer cells has been investigated. Several *in vitro* studies have shown the presence of NME proteins in breast cancer cell CM. Anzinger *et al*⁶² have shown that the MDA-MB-231 metastatic human breast carcinoma cell line secretes NME2. The Buxton laboratory has also provided additional evidence of extracellular NME proteins in different tumor cell lines developed from women with metastatic breast cancer and from primary ductal carcinoma. These tumor cell lines are metastatic in the murine xenograft model.⁶³ Western blot and NDPK activity analyses showed that NME1 and NME2 are present as soluble enzyme in growth medium of these tumor cell lines. These extracellular NME proteins promote endothelial cell migration and proliferation. The *in vivo* validation of NME1/2 presence in the extracellular environment has been obtained through western blot analyses of serum from women affected by different breast cancers.⁶⁴

Recently, Izadpanah and Pucci-Minafra laboratories have performed proteomic profiling of extracellular vesicles secreted by the highly metastatic MDA-MB-231 breast cancer line and shown the presence of NME1 and NME2 in extracellular vesicles.^{65,66}

Several studies have investigated the functional role of secreted NME and a possible role in purinergic signaling has emerged. Extracellular ATP has key roles as a neurotransmitter and as a signaling molecule.⁶⁷ Once released, it is rapidly metabolized by various types of ectonucleotidases.⁶⁸ Analyses of purine metabolism in the human bloodstream highlighted the existence of NDPK activity.³² Previous analyses also showed that the extracellular NDPK activity regulates extracellular nucleotide levels on the cellular surface in many cell types.³³ Since the nucleotide amount in the extracellular space is limited,³³ as discussed earlier the role of NME in modulation of ATP signaling by counteracting ectonucleotidases activity may depend on localized concentration of extracellular nucleotides.

Extracellular ATP/ADP and adenosine exert their biological roles by activating P2 and P1 receptor types, respectively.⁶⁶ The P2Y family of ATP/ADP selective receptor is composed of heterotrimeric G protein-coupled receptors and includes eight different subtypes. The P2Y1 and P2Y2 receptors, in response to extracellular ATP, mediate proliferation in the vast majority of tumor types. Furthermore, activated P2Y1/2 receptors in turn transactivate the vascular endothelial growth factor type 2 receptor (VEGFR-2).^{69,70} This receptor transduces the vast majority of angiogenic and permeability effects of VEGF and thus has a crucial role in physiological as well as

pathological conditions.⁷¹ Interestingly, many *in vitro* studies have shown that NME2 transactivates VEGFR-2.^{63,69,72,73} Yokdang *et al*⁶³ have shown that treatments of human endothelial cell cultures with purified NME2 activates VEGFR-2 in a P2Y dependent fashion as pretreatment with MRS2179, a P2Y1-specific antagonist, blocked VEGFR-2 activation. Moreover, *in vitro* experiments have also shown that purified NME2 is able to promote P2Y1-dependent migration of endothelial cells.

Previous evidence obtained in the Buxton laboratory has already suggested a possible role of NME2 in supporting tumor formation by acting on extracellular ATP level.⁷⁴ These *in vitro* experiments have shown that NME2 secreted from MDA-MB-435 human cancer cell line induces tubulogenesis of human cardiac endothelial cells. Significantly, they have also shown that MRS2179 treatment attenuates this *in vitro* pro-angiogenic effect. However it should be noted that the origin of MDA-MB-435 cell line has been determined to be melanoma; therefore this cell line should not be considered a model for breast cancer.⁷⁵

Recently, Buxton laboratory has shown interesting results in an orthotopic xenograft model of breast cancer obtained through transplantation of MDA-MB-231 human breast cancer line in the mammary fat pad of SCID (severe combined immunodeficiency) mice.⁶⁴ Monitoring of tumor growth and metastatization in this model has been possible thanks to the expression of the *luciferase 2* gene in the MDA-MB-231(Luc+) transplanted cells. Besides to confirm the presence of NME1/2 proteins in the bloodstream, the analyses of this human breast cancer xenograft have shown that increased tumor growth, monitored through *in vivo* imaging of bioluminescence, is correlated with enhanced level of circulating NME1/2. Furthermore, treatment of this orthotopic breast cancer model with the NDPK/VEGFR-2 inhibitor ellagic acid^{76,77} or with MRS2179⁷⁸ reduced both primary tumor growth and metastasis to the lung.

Taken together, the extracellular NME proteins in CM of cell culture *in vitro* and in serum of patients or mouse cancer model seems to exert oncogenic effects.

NME IN THE DROSOPHILA MODEL SYSTEM

The *abnormal wing discs (awd)* is the unique *Drosophila* ortholog of the NME1 and NME2 proteins, sharing 78% of amino acid identity with human counterparts.⁷⁹

Genetic studies unraveled an essential requirement of *awd* gene function at different stages during *Drosophila melanogaster* development. Indeed, *awd* is necessary for the development of the imaginal discs,⁸⁰ the larval nervous system,^{24,80,81} the lymph gland⁸⁰ (the fly hematopoietic organ) and the embryonic vascular system.⁸² Moreover, *awd* function during oogenesis has also been extensively explored.^{83–85} These studies support the notion that Awd fulfills a function as an endocytic mediator. In particular, *awd* genetically interacts with the well-characterized endocytic marker *Rab5*⁸⁵ and with the internalization mediator *shibire*

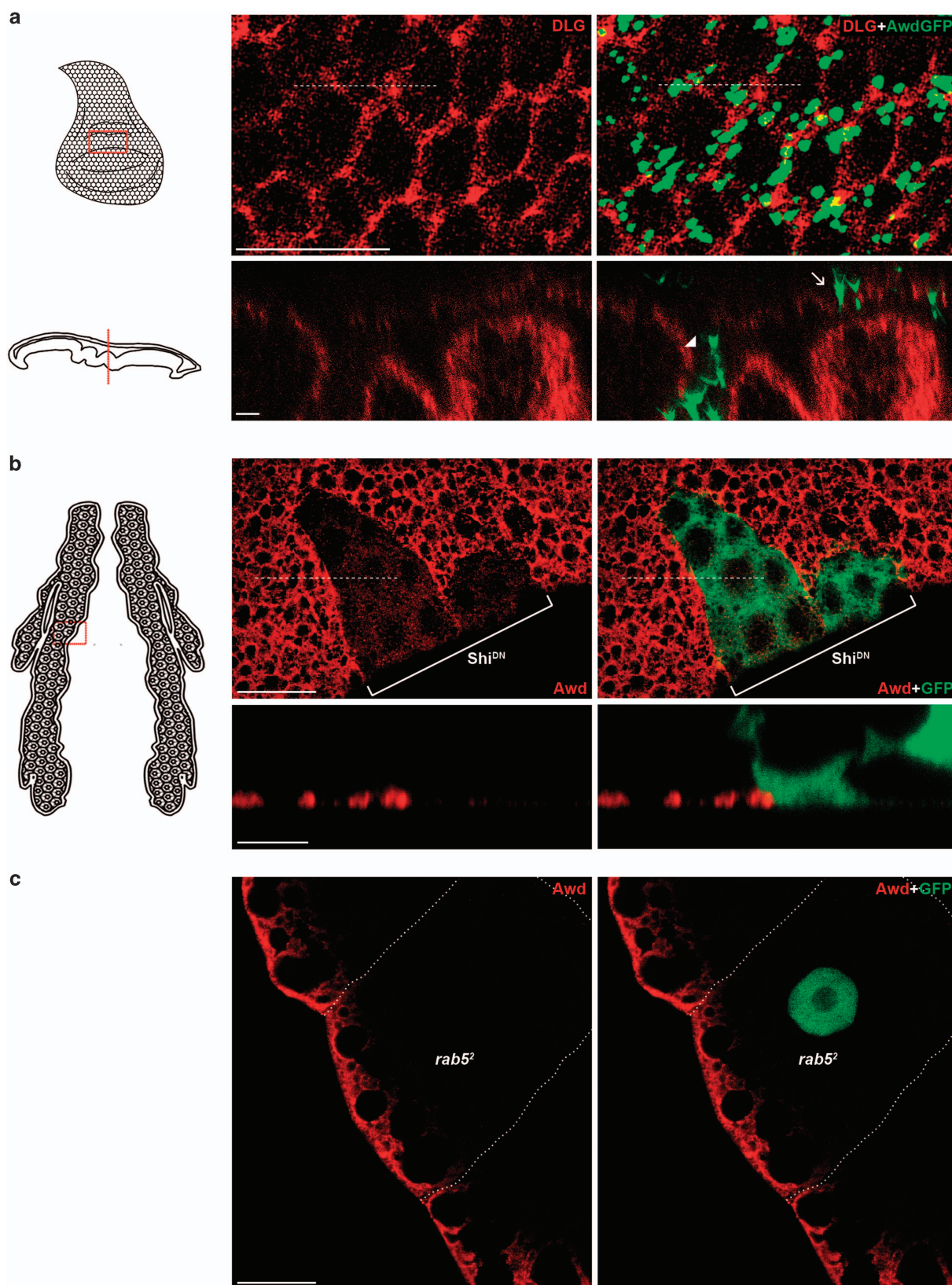


Figure 3 Intra- and extracellular localization of Awd in *Drosophila* larval tissues. (a) Left panel, schematic drawing of the *Drosophila* wing disc (frontal view, up and z-stack, below). The red box and the red line indicate the position of the x–y section and the x–z section, respectively. Middle and right panels, confocal analysis of peripodal membrane and columnar cells stained for the DLG apical marker. In these panels, the dotted white lines indicate the position of confocal z-stack. By using the *UAS-Gal4* system,⁹⁶ we targeted the expression of *UAS-Awd-GFP* (green) transgene in peripodal cells under the control of the *Ubx-Gal4* driver. Awd-GFP vesicles are detected in peripodal cells (arrow) and also in the lumen that separates these cells from the columnar epithelium (arrowhead). (b) Left panel, schematic drawing of the *Drosophila* fat body organ. Middle and right panels, immunofluorescence analysis of fat body tissue stained for Awd (red). By using the FLP-out/*Gal4* technique,⁹⁷ we induced clones of GFP positive adipocytes overexpressing *Shi^{DN}*, a dominant negative form of *Shi*.⁹⁸ The white brackets enclose the clone. The white dotted lines in the x–y section indicate the position of the x–z projection magnification showed in lower panels. In *Shi^{DN}* clones, the intracellular Awd level is reduced. Scales are 20 and 5 μ m for the x–y and x–z planar images, respectively. (c) Immunofluorescence analysis of fat body tissue stained for Awd (red). By using the MARCM technique⁹⁹ we obtained cell clones (easily identifiable thanks to the presence of nuclear GFP) homozygous for *rab5²*, an amorphic allele of *rab5*. The white dotted lines encircle a *rab5²* mutant adipocyte. There is no change of the Awd level in the *rab5²* mutant clone. Scale is 20 μ m.

(*shi*),⁸⁶ the fly homolog of the human Dynamin1 (DNM1). Beside its intracellular functions, interestingly, proteomic studies showed that Awd can be released into larval hemolymph, the unique *Drosophila* extracellular fluid,⁸⁷ and into the extracellular environment by micro-vesicles in fly cell lines,⁸⁸ suggesting potential extracellular roles of this NDPK. Recently, we showed that the balance between intracellular and extracellular Awd and NME1 is controlled by *Shi* and DNM1 in *Drosophila* and human cell lines.^{89–91} We studied Awd localization in the *Drosophila* wing disc primordium that is composed of a columnar proliferating epithelium in continuum with a peripodal membrane (Figure 3a). In this tissue Awd is strictly localized in the apical region that faces the lumen, in vesicle-like aggregates that can be secreted by the wing disc epithelium to reach the hemolymph. By using transgenic lines expressing the GFP-tagged Awd, we observed that the wing disc is not the only tissue able to secrete Awd into the hemolymph. This finding suggests that the Awd balance between the outside and inside of the cell is highly dynamic. The extracellular Awd level is controlled by the Dynamin function since the extracellular protein level is enhanced in *Drosophila* larvae bearing a *shi* loss-of-function mutation (*shi^{ts}*). We also demonstrated that the relationship between extracellular NME1 and DNM1 exists in mammalian cells. By knocking down *dnm1* gene expression in normal colon (NCM) and colon carcinoma cancer (HT-29) cells, we observed a significant increase of extracellular NME1.

Larval hemolymph composition is mostly determined by the secretory and endocytic activities of adipocytes, polyploid cells composing the *Drosophila* larval fat body (scheme in Figure 3b). The fat body is a large endocrine organ that fulfills several functions resembling those performed by the mammalian liver. We showed that in adipocytes Awd protein is highly expressed (as also suggested by high-throughput *awd* expression data⁹²) and it is mostly localized at the pericellular region. Interestingly, in adipocytes with impaired *Shi* activity, Awd intracellular level is greatly reduced (Figure 3b). This result is in accordance with the previously described enhancement of Awd amount in the hemolymph of *shi^{ts}* larvae and allows us to hypothesize that extracellular Awd is internalized by cells through a *Shi*-dependent mechanism. In

order to further characterize Awd endocytic route, we analyzed the role of Rab5, the best-known early endosomal marker. We show that Awd trafficking into adipocytes is independent of Rab5 activity since its loss-of-function does not alter the intracellular level of Awd (Figure 3c).

The endocytic route NME1 follows during its internalization could have potential implications for cancer research. Indeed, Lim and colleagues⁹³ presented an anti-metastatic therapy based on systemic delivery of a modified NME1 protein with improved cell-permeability. Their data support the notion that extracellular NME1 internalization by tumor cells inhibits metastasis-associated phenotypes *in vitro*, including cell migration, Matrigel invasion, ability to adhere to different substrates and ability to induce vascular endothelial cells to form angiogenic tube. Moreover, these authors also showed that treatment of tumor-bearing animals with the cell-permeable NME1 enhances their survival score and impedes the onset or stimulates the clearance of lung metastasis.

CONCLUDING REMARKS AND PERSPECTIVE

NME proteins have multiple cellular and molecular functions, some of which may contribute to their proposed anti-metastatic activities.⁹⁴ Moreover, these proteins may play different tissue-specific functions acting in various tumors and this could be the case also for the extracellular NME activity. As we highlighted in this review, growing evidence on the high extracellular NME levels in various tumor cohorts and in stem cell maintenance point out its possible function as pro-metastatic factor. However, it is not clear whether the elevated level of extracellular NME proteins indicates an active process used by the cancer cells to modulate the microenvironment, or it is a byproduct of tumor progression.

The most extensively studied extracellular NME protein functions are in the context of hematopoietic cells or pathophysiological conditions, including AML and cultured peripheral blood mononuclear cells. In these hematopoietic systems, extracellular NME proteins appear to inhibit differentiation, as is the case in stem cell maintenance. In other tumors, elevated NME proteins have been found in serum or body fluid in neuroblastoma and breast cancer

patients. In breast cancer, extracellular NME proteins were shown to induce angiogenesis.

In light of their potentially metastasis-promoting effects, it is critical to know how wide-spread the presence of extracellular NME proteins is in other tumor patients. Currently the mechanism of NME protein secretion is unknown. It should be an important issue to address since an active secretory mechanism will support a pathophysiological role for the extracellular NME proteins. It would also be important to survey a range of different cancer cohorts to establish whether serum levels of NME proteins correlate with cancer prognosis using proteomic approaches.

Looking ahead, these are important to know before the implementation of therapeutics that elevate the expression of NME proteins based on their function as metastasis suppressor. However, besides the high relevance that the NME levels could serve as a prognostic and therapeutic targets of a specific tumor, the mode of action of the extracellular NME in normal and tumor cells is a challenging issue to be addressed. It has been shown that the NME NDPK activity is not required for the survival and growth of AML clones. Furthermore, in these cells NME1 may bind to an unknown receptor, different from the embryonic stem cells where it binds to Muc1* receptor. On the other hand, the oncogenic function of extracellular NME proteins in breast cancer requires their NDPK activity to modulate the purine levels.

The work on *Drosophila* model and in normal and colon carcinoma cells have suggested the involvement of Dynamin (therefore endocytosis) in internalizing extracellular NME proteins. Interestingly, these internalized extracellular NME proteins mediate the canonical metastasis suppressive function.⁹⁰ From these data we may speculate that a mechanism balancing the intra- and extracellular NME levels in normal cells may be misregulated in cancer cells leading to a secretion of high levels of NME.

Taken together, the characteristics of extracellular NME proteins seem to mirror their intracellular counterparts, in terms of the complexity of physiological and molecular functions. Recently, it has been proposed that the myriad of intracellular NME functions could be explained by a model that NME proteins act as a scaffold, which exerts different functions in different subcellular locations based on the macromolecular partners (including proteins and lipids).⁹⁵ It is tempting to speculate that the action of extracellular NME proteins follows the same rule.

In conclusion, the weight of the accumulating evidence seems to favor the notion that extracellular NME proteins do play important roles in pathophysiological conditions. Nonetheless, to clarify many of the unanswered questions, amenable *in vivo* models need to be established, in which a class of NME mutations should be created that specifically eliminates the extracellular NME. This will require the elucidation of the secretory mechanism. Future studies should focus on this point.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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