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PATHOBIOLOGY IN FOCUS

NDPKA is not just a metastasis suppressor – be aware of its metastasis-promoting role in neuroblastoma

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NDPK-A, encoded by *nm23-H1* (also known as *NME1*) was the first metastasis suppressor discovered. Much of the attention has been focused on the metastasis-suppressing role of NDPK-A in human tumors, including breast carcinoma and melanoma. However, compelling evidence points to a metastasis-promoting role of NDPK-A in certain tumors such as neuroblastoma and lymphoma. To balance attention on this contrariety of NDPK-A in different cancer types, this review addresses the metastasis-promoting role of NDPK-A in neuroblastoma. Neuroblastoma is an embryonic tumor, arising from neural crest cells that fail to differentiate into the sympathetic nervous system. We summarize and discuss *nm23-H1* genetics and the prognosis of neuroblastoma, structural and functional changes associated with the S120G mutation of NDPK-A, as well as the evidence supporting the role of NDPK-A as a metastasis promoter. Also discussed are the NDPK-A relevant molecular determinants of neuroblastoma metastasis, and metastasis-relevant neural crest development. Because of NDPK-A's dichotomous role in tumor metastasis as both a suppressor and a promoter, tumor genome/exome profiles are necessary to identify the molecular drivers of metastasis in the NDPK-A network for developing tumor-specific therapies.
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Metastasis remains as the major cause of death in cancer patients, accounting for ~90% of cancer mortality. To metastasize, tumor cells need to successfully complete every step in the metastatic cascade, including detachment from the primary tumor, migration and invasion to local tissues, survival in the circulatory and lymphatic systems, and colonization at a distal organ(s) of the human body.

The tumor metastasis field has greatly advanced since the discovery, three decades ago, of the first metastasis suppressor gene, *nm23-H1* (also known as *NME1*).[1] The *nm23-H1* gene encodes nucleoside diphosphate kinase A (NDPK-A, also termed as NM23-H1 and NME1),[2] which belongs to the human NDPK family, currently consisting of ten members. Much of the attention has focused on the metastasis-suppressing role of NDPK-A in human tumors including breast carcinoma and melanoma. However, compelling evidence points to an opposite role for NDPK-A as a metastasis promoter in certain tumor types, such as neuroblastoma and lymphoma. To clarify this dichotomous trait of NDPK-A, this review will address the metastasis-promoting role of NDPK-A in neuroblastoma.

THE CLINICAL RELEVANCE OF NDPK-A TO TUMOR METASTATSIS

After a seminal report by Steeg *et al*,[1] the clinical relevance of NDPK-A in tumor metastasis has been extensively studied. Results from most of these studies are summarized in Tables 1 and 2. A negative correlation between the protein and/or RNA levels of NDPK-A and metastatic potential is displayed by many cancer types, including breast, head and neck, liver and ovarian cancers (Tables 1 and 2). This negative correlation suggests a metastasis-suppressing role for NDPK-A (see the review by Steeg in this issue). For colorectal, gastric and lung cancers, however, the role of NDPK-A in tumor metastasis remains uncertain because of contradictory correlations (Tables 1 and 2). Conversely, a positive correlation between the protein and/or RNA levels of NDPK-A and metastatic potential occurs in neuroblastoma and lymphoma, suggesting a metastasis-promoting role of NDPK-A (Tables 1 and 2).

The dichotomous role of NDPK-A in tumor metastasis is likely due to the unique genetic makeup of different human cancer types. In addition to the different molecules and pathways affected, pediatric tumors such as neuroblastoma

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generally contain fewer mutations than adult tumors such as breast carcinoma, which display 10–20 and 25–130 non-synonymous mutations per tumor, respectively.[3,4] To address this contrariety of NDPK-A, we focus on its metastasis-promoting role in neuroblastoma by starting with the *nm23-H1* genetics unique to this disease.

NM23-H1 GENETICS AND PROGNOSIS OF NEUROBLASTOMA

Neuroblastoma is the most common extracranial tumor of early childhood, accounting for 7% of all pediatric cancers.[5] Neuroblastoma arises from multipotent neural crest cells that fail to differentiate into the sympathetic nervous system.[6] Based on the International Neuroblastoma Staging System (INSS), limited stages (1 and 2) and advanced stages (3 and 4) of neuroblastoma are referred to as localized and metastatic tumors, respectively.[7] The long-term survival rate for advanced stages of neuroblastoma patients is 40–50%.[5]

The genetics of *nm23-H1* in neuroblastoma is more complicated than that in other tumors. An increased *nm23-H1* copy number has been reported in 14% (13 of 95) to 23% (7 of 31) of neuroblastoma patients.[8,9] The *nm23-H1* gene is mapped to chromosome 17q21.3.[10] An increased *nm23-H1* copy number therefore could be due to the gain of a chromosomal segment, 17q21-qter, which occurs in 54–65% of neuroblastoma patients and is associated with poor clinical outcomes.[11–13]

In addition to an increased gene copy number, high levels of *nm23-H1* RNA and/or NDPK-A protein also occur in advanced neuroblastomas, and which are associated with poor prognosis.[8,9,14–16] A high level of NDPK-A can occur in advanced neuroblastomas with or without *MYCN* amplification.[14] *MYCN* amplification is a frequent genetic alteration, occurring in ~20% of patients with advanced neuroblastoma.[6] The ability of MYCN to upregulate *nm23-H1* expression[17] can contribute to a high NDPK-A level in this subset of neuroblastomas. Intriguingly, neuroblastoma patients with *MYCN* amplification display a higher serum NDPK-A level than those without *MYCN* amplification.[18] Serum NDPK-A suggests a secreted form, similar to that reported in myeloid leukemia.[19]

Although *nm23-H1* mutations are rare, a serine 120 → glycine (S120G) mutation of NDPK-A has been reported in 21% (6 of 28) of advanced neuroblastomas, but not in any of 22 limited-stage tumors.[20] This S120G mutation appears to be specific to neuroblastoma as it was not detected in 26 breast carcinoma patients nor in 17 patients with acute leukemia.[20] This mutation can be inherited or occur somatically, and also can occur with or without *nm23-H1* amplification (3–10 copies).[20] Moreover, the S120G mutation of NDPK-A can arise in advanced neuroblastomas with or without *MYCN* amplification.[20] This genetic heterogeneity likely complicates the interpretation of NDPK-A's role in tumor metastasis.

On the basis of a differential expression of genes between favorable and unfavorable (ie, advanced) neuroblastomas, *NME1* (also known as *nm23-H1*), *CHD5* and *PAFAH1B1* genes are proposed as a prognostic signature for risk stratification of neuroblastoma patients.[21]

Table 1 Correlation of *nm23-H1* mRNA level and metastatic potential in human cancers

Cancer types	Patient #	[a]Method	[b]Correlation	References
Bladder cancer	22	NB, SH	None	57
Brain cancer	27	NB	None	58
Breast cancer	27–71	ISH, NB	Neg.	59,60
	153	NB, SH	None	61
Cervical cancer	98	NB	Neg.	62
Colorectal cancer	21–52	NB, PCR	Neg.	63,64
	18–61	NB	Pos.	65,66
	22–43	DIFF-PCR, RPA	None	67,68
Gastric cancer	19–31	NB	Neg.	69,70
	23	NB	None	71
Head and neck cancer	20–78	ISH, RT-PCR, qPCR	Neg.	72,73
Kidney cancer	16	NB, SH	Pos.	57
Leukemia	47	NB, RT-PCR	Pos.	74
Liver cancer	17–30	NB, RT-qPCR	Neg.	75–77
	18	RT-qPCR	Pos.	78
Lung cancer	37	NB	Pos.	79
	10	RT-PCR	None	80
Lymphoma	106	NB	Pos.	81
Melanoma	30–33	NB	Neg.	82,83
Neuroblastoma	75	NB	Pos.	8
Ovarian cancer	48–106	NB, RT-PCR	Neg.	84–87
	45	NB	Pos.	88
Pancreatic cancer	81	ISH, NB	Neg.	89
Peripheral synovial sarcoma	31	RT-PCR+SH	Pos.	90
Prostate cancer	29	NB	Pos.	91
Thyroid cancer	54	RT-PCR	Neg.	92
	39	NB	Pos.	93

[a]DIFF-PCR, differential polymerase chain reaction; ISH, *in-situ* hybridization; NB, Northern blotting; PCR, polymerase chain reaction; qPCR, real-time quantitative PCR; RPA, ribonuclease protection assay; RT-PCR, reverse transcription PCR; RT-qPCR, reverse transcription qPCR; SH, slot blot hydridization.
[b]Positive, negative, or no correlation is indicated as Pos., Neg. or None, respectively.

NDPK-A OR NDPK-A^{S120G} ENHANCES NEUROBLASTOMA CELL INVASIVENESS

Metastasis-associated cellular processes include decreased cell adhesion as well as increased cell survival, migration, invasion, and colonization. For simplicity here, these cellular

Table 2 Correlation of NDPK-A protein level and metastatic potential in human cancers

Cancer types	Patient #	[a]Method	[b]Correlation	References
Anal cancer	22	IHC	None	94
Bladder cancer	90-257	IHC	Neg.	95,96
	39-74	IHC	Pos.	97,98
Bone cancer	32	IHC	Pos.	99
Brain cancer	24	IHC	Pos.	100
Breast cancer	63–242	IHC	Neg.	101–104
	44	IHC	None	105
Cervical cancer	8–150	IHC, WB	Neg	62,106–108
	27	IHC	Pos	109
	30–176	IHC	None	110,111
Colorectal cancer	20–185	IHC	Neg.	63,112–116
	52–130	IHC	None	117–119
Endometrial cancer	43	IHC	Neg.	120
	28	IHC, WB	None	121
Esophageal cancer	45–50	IHC	Neg.	122,123
	32	IHC	None	124
Gallbladder cancer	107	IHC	None	125
Gastric cancer	26–101	IHC	Neg.	126,127
	107–413	IHC	Pos.	128,129
	19–177	IHC	None	69,130–132
Head and neck cancer	20–231	IHC, WB	Neg.	72,73,133–141
	28–40	IHC	None	142,143
Kidney cancer	85	IHC	Pos.	144
	95	IHC	None	145
Liver cancer	12–92	IHC, WB	Neg.	76,146–150
	27–33	IHC	None	151–153
Lung cancer	32–147	IHC	Neg.	154–156
	104–134	IHC	Pos.	157,158
	27–88	IHC, WB	None	159–161
Lymphoma	106–262	ELISA, FC, IHC	Pos.	81,162–164
Melanoma	32–49	IHC	Neg.	165,166
	36–138	IHC, WB	None	62,167,168
Neuroblastoma	40–217	2D, ELISA	Pos.	14,18
Ovarian cancer	50–127	IHC	Neg.	84,86,103,169,170
	24	IHC	Pos.	171
Pancreatic cancer	81	IHC	Neg.	89
	31–47	IHC	Pos.	172,173
	73	IHC	None	174
Prostate cancer	59–80	IHC	Neg.	175,176
Retinoblastoma	73	IHC, WB	Neg.	177
Skin cancer	60–68	IHC	None	178,179
Vulvar cancer	68	IHC	Neg.	180
Thyroid cancer	30–115	IHC	Neg.	103,181–183
	49	IHC	None	184

[a]2D, two-dimensional gel electrophoresis; ELISA, enzyme-linked immunosorbent assay; FC, flow cytometry; IHC, immunohistochemistry; WB, Western blot.
[b]Positive, negative, or no correlation is indicated as Pos., Neg. or None, respectively.

processes are collectively termed cell invasiveness. It is noteworthy that cell proliferation and death are generally considered as tumorigenesis- and not metastasis-associated processes.

For NDPK-A to be a bona fide metastasis promoter in neuroblastoma, it is expected to increase the invasiveness but not the proliferation of neuroblastoma cells. This is indeed the case for human neuroblastoma NB69 cells that express ectopic NDPK-A or NDPK-A^{S120G}.[22] NDPK-A or NDPK-A^{S120G} readily increases the invasiveness of NB69 cells, as measured by serum-independent survival, cloning efficiency, cell migration, and colony formation on soft agar.[22] On the other hand, ectopically expressed NDPK-A or NDPK-A^{S120G} does not affect the proliferation of NB69 cells under normal growth conditions.[22] A similar migration-enhancing effect of NDPK-A or NDPK-A^{S120G} is also observed in another human neuroblastoma cell line, SH-SY5Y (unpublished data). These two neuroblastoma cell lines do not exhibit *MYCN* amplification, which therefore excludes the possibility of interference by MYCN in the cell invasiveness-enhancing role of NDPK-A.

Compared with the wild type, ectopically expressed NDPK-A^{S120G} level is lower but more potent in enhancing the invasiveness of NB69[22] and SH-SY5Y cells (unpublished data). A lower level of NDPK-A^{S120G} is not due to protein instability because a similar half-life is observed between the mutant and its wild type.[23] This indicates that S120G may be a gain-of-function mutation.

A gain-of-function for S120G mutation is further observed in human cancer cell lines, in which NDPK-A behaves as a metastasis suppressor. NDPK-A^{S120G} increases, whereas the wild type inhibits, the migration of human breast cancer MDA-MB-435 cells.[24] In human prostate carcinoma DU145 cells, the S120G mutation abrogates the ability of NDPK-A to inhibit cell colonization and invasion.[25] It seems reasonable to speculate that the genetic background unique to neuroblastoma, breast, and prostate carcinomas might dictate the role of wild-type NDPK-A in cell invasiveness. However, an apparent gain-of-function of S120G mutation renders NDPK-A with a better ability to enhance cell invasiveness regardless of tumor origins.

NDPK-A OR NDPK-A^{S120G} PROMOTES NEUROBLASTOMA METASTASIS

Approximately 50% of human neuroblastomas originate from the adrenal gland,[26] which serves as an ideal orthotopic site for a xenograft animal model of neuroblastoma metastasis. A fluorescent orthotopic xenograft model developed in SCID mice not only recapitulates human neuroblastoma, but also allows sensitive detection of GFP-labeled primary and metastatic tumors in mice.[27] In this orthotopic xenograft model, NDPK-A- or NDPK-A^{S120G}-expressing NB69 cells increase both the incidence and colonization of neuroblastoma metastasis in animal lungs without significantly affecting primary tumor development.[22] Compared with the wild-type, NDPK-A^{S120G} is more effective in promoting neuroblastoma metastasis in mice, consistent with their abilities in cell invasiveness.[22] The lymphatic system appears to be one route for neuroblastoma cell dissemination because of accumulation of GFP-labeled NB69 cells in the inguinal lymph node of xenograft mice.[27]

As the xenograft mouse model is difficult to use for monitoring the behaviors of moving tumor cells, a xenograft zebrafish model has been developed, which is able to show that NDPK-A or NDPK-A^{S120G} enhances the ability of NB69 cells to extravasate the fish tail vein (unpublished data). The extravasation-enhancing ability in xenograft zebrafish is consistent with the metastasis-promoting ability of NDPK-A or NDPK-A^{S120G} in xenograft mice.[22] Extravasation is essential for migrating tumor cells to gain access to other organs, an end point that is difficult to measure in the xenograft mouse model. Because of economic, physiological, and real-time observational advantages of zebrafish, this xenograft model will facilitate mechanistic and therapeutic studies of extravasation regulated by NDPK-A.

STRUCTURAL AND FUNCTIONAL CHANGES OF NDPK-A^{S120G}

The molecular mechanism by which NDPK-A or NDPK-A^{S120G} contributes to neuroblastoma metastasis remains unknown. Nevertheless, this mechanism is likely associated with S120G-associated structural and functional changes. Phosphotransferase activity is a well-established function of NDPK, including NDPK-A.[28] Histidine 118 (H118) is an active site, and the H118-phosphorylated intermediate is essential for the transfer of the terminal phosphate from a triphosphate nucleotide (eg, ATP) to a diphosphate nucleotide (eg, UDP) via a 'ping-pong' mechanism.[28,29]

Among all the NDPK family members from different organisms, the S120 residue is highly conserved. The NDPK-A^{S120G} recombinant protein displays ~50% lower phosphotransferase activity than the wild-type recombinant protein *in vitro*.[23] The same mutation when introduced to NDPK of *Dictyostelium discoideum* results in an 80% loss of the activity.[30] The reduction of NDPK-A^{S120G} activity is caused by the instability of its phosphorylated intermediate, as there are no defects in the phosphate incorporation of the H118 residue nor in the phosphate transfer from NDPK-A^{S120G} to UDP.[23] In human neuroblastoma tissues, a decrease in the phosphotransferase activity of NDPK-A^{S120G} apparently is compensated for by other NDPK family members, such as *nm23-H2*-encoded NDPK-B.[23,31] Therefore, function(s) other than the phosphotransferase activity of NDPK-A^{S120G} likely account for its metastasis-promoting role in neuroblastoma.

All known eukaryotic NDPKs, including NDPK-A, exist in a hexameric quaternary structure via assembling identical dimers.[2,32] However, NDPK-A^{S120G} affects the subunit assembly and results in 17% dimeric structures, which is approximately sixfold higher than the wild type, but only when disulfide bonds are reduced.[23] This indicates the susceptibility of the NDPK-A^{S120G} structure to the intracellular redox state. Moreover, NDPK-A^{S120G} reduces its enzyme stability when subjected to heat and urea,[23,33] and exhibits a protein-folding defect.[33] This folding defect can be corrected when NDPK-A^{S120G} is phosphorylated by ATP or by phosphoramidate.[34] When forming a complex with ADP, no significant structural changes are observed between NDPK-A^{S120G} and the wild-type.[35]

In addition to affecting the subunit assembly, the S120G mutation changes the interaction of NDPK-A with other cellular proteins. NDPK-A^{S120G}, in contrast to the wild type, interacts with the 28-kDa protein[23] but not with PRUNE.[36] NDPK-A^{S120G} also appears to indirectly alter protein-protein interaction. For example, the S120G mutation abolishes the ability of NDPK-A to suppress desensitization of the muscarinic potassium current.[37] Extracellular recombinant NDPK-A^{S120G} is more efficient than the wild type in supporting the colony formation of undifferentiated human embryonic stem cells.[38]

NDPK-A-RELEVANT MOLECULAR DETERMINANTS OF NEUROBLASTOMA METASTASIS

For NDPK-A to be a metastasis promoter in neuroblastoma, certain interacting proteins of NDPK-A[39] may be the molecular determinants of neuroblastoma metastasis. Data from genome- and exome-wide sequencing studies are useful for identifying these molecular determinants. It has been reported that recurrent mutations affect pathways such as focal adhesions, Rac/Rho, RAS-MAPK, and YAP in advanced and relapsed neuroblastomas.[3,40–42] Among these pathways, the Rac/Rho pathway is pertinent to the current knowledge of the NDPK-A network.

Rac, Rho, and Cdc42 are well-studied members of the Rho GTPase family, which is a part of the Ras superfamily.[43] Rho GTPases regulate cytoskeletal rearrangement, essential for cell migration, invasion, and neuritogenesis,[43] and are relevant to neuroblastoma metastasis.

High-frequency recurrent mutations of *Tiam1* have been detected in one but not a second study of advanced neuroblastomas due to a low mutation frequency.[3,44] As an interacting protein of NDPK-A,[45] Tiam1 functions as a Rac1-specific guanine nucleotide exchange factor[46,47] and

participates in neuritogenesis, cell invasiveness, and tumor progression.[48] In addition to several genes involved in neuronal growth cone stabilization, Tiam1 and other regulators of the Rac/Rho pathway are also mutated, implicating defects in neuritogenesis.[3]

Data of chromosomal aberrations in advanced neuroblastoma are also useful for identifying molecules and pathways in the NDPK-A network. The loss of heterozygosity of 1p36 occurs in 23–35% high-risk neuroblastomas.[6] One of the genes is located on 1p36 is *Cdc42*, and its gene product interacts with NDPK-A.[49] In *MYCN* non-amplified neuroblastomas, overexpressed NDPK-A binds to Cdc42 and prevents the induction of neuronal differentiation.[50] In advanced neuroblastomas with *MYCN* amplification, MYCN inhibits neuritogenesis by downregulating *Cdc42* expression.[50]

METASTASIS-RELEVANT NEURAL CREST DEVELOPMENT

Neuroblastoma originates from multipotent neural crest cells committed to the lineage of sympathetic neurons. At the end of the first trimester in humans, neural crest cells are induced to undergo an epithelial-to-mesenchymal transition (EMT), delaminate from the neural tube and migrate through surrounding tissues before arriving at their final destination for terminal differentiation.[51] Neural crest development thus shares common mechanisms with tumor metastasis, including EMT, migration, and invasion.[52]

NDPK-A is highly expressed in the first-trimester placenta in humans, whereas it is downregulated in second- and third-trimester placentas.[53] The high level of NDPK-A seen in advanced neuroblastoma indicates deregulation of *nm23-H1* expression. If *nm23-H1* deregulation occurs during embryogenesis, it will arrest neural crest cells in less differentiated, yet highly migratory and invasive stages, leading to more aggressive neuroblastoma. Ectopically expressed NDPK-A or NDPK-A^{S120G} inhibits neuronal differentiation of NB69 cells upon induction with retinoic acid.[22] Such an arrest of the neural crest may occur indirectly via the ability of NDPK-A to bind the *c-myc* promoter and reduce its transcription (unpublished data), considering that c-Myc is required for neural crest specification.[54,55] Alternatively, NDPK-A-mediated reduction of *c-myc* transcription possibly increases neuroblastoma metastasis because c-Myc suppresses the metastasis of human breast carcinoma.[56]

Understanding the developmental functions of NDPK orthologs in different organisms (see review by Ćetković *et al* in this issue) will clarify the underlying mechanisms of tumor metastasis.

CONCLUDING REMARKS

A prognostic signature, consisting of *nm23-H1* and two other genes, has been proposed for risk stratification of neuroblastoma patients. NDPK-A is encoded by *nm23-H1* and acts as a metastasis promoter in neuroblastoma (eg, an embryonic tumor), unlike its metastasis-suppressing role found in many adult tumors such as breast and prostate carcinoma.

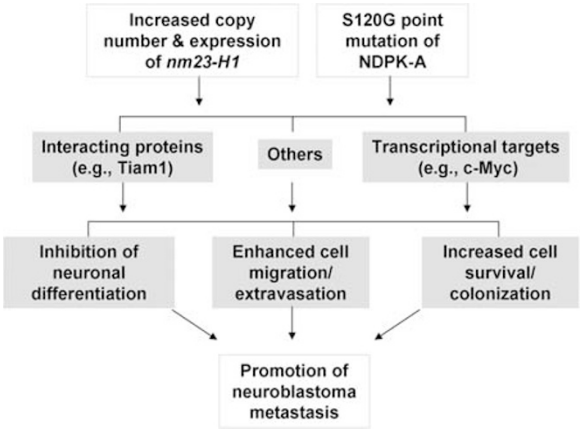

Figure 1 A current understanding of NDPK-A in promoting neuroblastoma metastasis. NDPK-A is encoded by the *nm23-H1* gene. Overexpression or S120G mutation of NDPK-A found in patients with advanced neuroblastoma promotes neuroblastoma metastasis by inhibiting neuronal differentiation, enhancing migration/extravasation, and increasing survival/colonization of neuroblastoma cells *in vitro* and *in vivo*. NDPK-A promotes neuroblastoma metastasis likely via its interacting proteins such as Tiam1, and/or its transcriptional targets such as c-Myc, in addition to other yet-to-be-determined molecular mechanisms.

Overexpression and the S120G mutation of NDPK-A, likely driving forces of neuroblastoma metastasis (Figure 1), occur in patients with advanced neuroblastoma. Relative to the wild type, NDPK-A^{S120G} is more effective in promoting cell invasiveness and metastasis of neuroblastoma *in vitro* and *in vivo*. NDPK-A^{S120G} appears to be a gain-of-function mutation because it increases, whereas the wild-type suppresses, the invasiveness of breast and prostate carcinoma cells. An apparent gain-of-function of the S120G mutation of NDPK-A is likely caused by a protein-folding defect, which affects its protein-protein interactions.

The molecular mechanism(s) by which NDPK-A promotes neuroblastoma metastasis remains elusive. Future studies will be facilitated by identifying molecules and pathways that are frequently altered in advanced neuroblastoma. Because developmental and metastatic processes share common mechanisms, understanding the functions of NDPK orthologs in neural crest development will shed light on neuroblastoma metastasis.

A promising targeted therapy for neuroblastoma is being developed based on a permeable peptide that disrupts the interaction of NDPK-A and PRUNE (reviewed by Ferrucci *et al* in this issue). This targeted therapy is, unfortunately, not useful for treating neuroblastoma patients harboring the S120G mutation because there is no interaction between NDPK-A^{S120G} with PRUNE. Frequent disruption of Rho GTPases signaling pathways in advanced neuroblastoma suggests potential therapeutic strategies for preventing neuroblastoma metastasis. Because of NDPK-A's dichotomous role in tumor metastasis as both a suppressor and a

promoter, tumor genome/exome profiles are necessary to identify the molecular drivers of metastasis in the NDPK-A network for developing tumor-specific therapies.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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