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PATHOBIOLOGY IN FOCUS

NM23/NDPK proteins in transcription regulatory functions and chromatin modulation: emerging trends

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NM23/NDPK proteins have been studied for their metastasis suppressor role but the molecular pathways involved in this process are not very vivid. Nucleotide binding and kinase activities of NM23 proteins implicated in anti-metastatic effects have been widely studied. In addition to these, transcriptional regulation adds another arm to the versatility of NM23 proteins that together with the other functions may contribute to better understanding of underlying mechanisms. In this review we discuss emerging reports describing the role of NM23 proteins in gene regulation and chromatin modulation in association with other factors or on their own.

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The metastasis suppressor gene *NME/nm23/NDPK* was discovered in 1988 by Steeg *et al*,[1] from the analysis of murine melanoma K-1735 cells based on its differential expression with respect to metastatic potential. The first identified member of the *nm23* metastasis suppressor gene family has been extensively studied ever since. Later, in 1991 a close homolog of NM23-H1 that shared >88% sequence identity was found and referred to as NM23-H2.[2] Subsequently 10 other homologs of this family were discovered across several other species.[3] *nm23* gene homologs are found in almost all organisms including eukaryotes, eubacteria as well as archaea,[4] the only exception being the Mycoplasma taxon.[5] NM23 are multifunctional, ubiquitously distributed hexameric histidine kinases that catalyze phosphate-transfer from nucleloside triposphates to diphosphates via a phosphohistidine intermediate following a ping-pong mechanism.[6–10] They are involved in regulating several fundamental cellular processes such as cell proliferation, apoptosis, G-protein signaling, and DNA repair.[11–13]

Among the human members of the *nm23* gene family *nm23-H1* and *nm23-H2* are widely studied as metastasis suppressors and discussed in several reviews.[14–17] The phylogenetic analysis of NM23 family members divided them into two distinct groups.[3,12] Group I includes NM23-H1-H4—enzymatically active proteins that are 58–88% identical, whereas group II includes proteins that are enzymatically inactive and more divergent, ie, with 22–44% identity.[3,12] Members of the NM23 family are known to be involved in multiple DNA-associated functions including nucleotide binding, nucleoside triphosphate synthesis, cleavage of DNA strands through nuclease activity as well as transcription.[12] The kinase-related activities of NM23 proteins including their role in metastasis suppression have been discussed in multiple reviews.[6–11] Although studies about the role of NM23 in gene regulation and chromatin-associated changes are relatively more recent, this review will focus on these aspects.

NDPK/NM23 PROTEINS: EVIDENCE SUPPORTING ROLE IN TRANSCRIPTION

Several lines of evidence implicate the role of NM23 proteins in transcriptional regulation of gene expression. These include first, studies such as those substantiating nuclear localization of NM23 proteins, although they have been reported to lack any nuclear localization signal.[18] Not only NM23-H1 and H2 the more frequently studied members of the NM23 family but also *NM23-H4* was reported to localize in the nucleus.[19] Several reports describe nuclear localization of NM23-H1 and NM23-H2 showing cell cycle-dependent transport of NM23-H1 and H2 proteins from cytoplasm to the nucleus during interphase.[18,20,21] In addition, Fujita *et al*[19] also showed nuclear localization of NM23-H4, which was previously understood to be found mainly within mitochondria. Second, multiple reports describe binding of NM23-H1/H2 proteins to the nuclease-hypersensitive element (NHE) region within

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Table 1 NM23/NDPK proteins carry characteristics of conventional transcription factors

NM23 proteins and nuclear localization	
NM23-H1 and H2	NM23-H1 and NM23-H2 localize to nucleus mainly in interphase[18,20,21]
NM23-H4	Increased nuclear localization was demonstrated on SIRT1 mediated acetylation of NM23-H4[19]
NM23 proteins with DNA-binding potential	
NM23-H2	NM23-H2 binds *c-myc* promoter purine-rich sequence GGGTGGG[22,23]
NM23-H2 and NM23-H1	Involvement of Arg34, Asn-69, and Lys-135 residues in DNA binding[25]
NM23-H1 and NM23-H2	Both the isoforms demonstrated to interact with *PDGF-A* promoter[24]
NM23-H1 and NM23-H2	Occupancy on several gene promoters *CCR5, CD11b, p53, WT1, ING1*[66]
NM23-H2	Interaction with G-quadruplex DNA in *c-myc* promoter[48]
NM23-H2 and NM23-H1	DNA binding with telomeric ssDNA repeats [26,27]
Maize ZmNDPK1	Interaction with G-quadruplex DNA[54]
NM23 proteins interact with other regulatory proteins	
NM23-H1	Interaction with EBNA3C and regulation of *MMP-9,*[37] *COX2,*[38] *alpha V integrin,*[39] *Necdin;*[40] interaction of mouse NM23β with YB-1 inhibits *gelatinasA* expression[33]
NM23-H2	Interaction with ERβ and activation of downstream genes[31,32,56]
NM23-H1	Interaction with STRAP and repression of TGFβ downstream signaling;[41,42] interaction with ERα and repression downstream genes[29]
NM23-H2	Interaction with CNBP activates *c-myc;*[30] interaction with APP regulates Alzheimer's disease progression;[57] NME2-PIWIL2 interaction upregulates *c-myc*[55]

the promoter of the oncogene *c-myc* and *pdgf-a,* resulting in transcriptional activation of *c-myc* and repression of *pdgf-a.*[22–24] Consequently, the Arg34, Asn-69, and Lys-135 amino-acid residues were reported to mediate NM23-H1 and NM23-H2 DNA binding.[25] Interestingly, apart from binding at gene promoters interaction with telomeric DNA was also noted.[26,27] Third, apart from direct DNA interactions, NM23 proteins were also reported to regulate expression of multiple genes through other transcription factors and regulatory proteins. For example, NM23-H1 was reported to associate with the Epstein–Barr virus nuclear antigen 3C EBNA3C, estrogen receptor alpha (ERα) and other factors[28,29] NM23-H2 was shown to associate with several factors including the cellular nucleic acid binding protein such as CNBP,[30] estrogen receptor beta (ERβ)[31,32] thereby activating *c-myc* expression (these are discussed in detail below). Table 1 summarizes these aspects for ready reference.

NM23-H1 AND TRANSCRIPTION REGULATION THROUGH DNA BINDING

The first report of transcription regulatory role was from Kaetzel's group in 2002 demonstrating NM23-H1 mediated transcriptional repression of the oncogene *pdgf-a* in HeLa cells.[24] NM23-H1 was shown to recognize both, a 5′ distal S1 nuclease-hypersensitive silencer element and a proximal regulatory NHE of the *pdgf-a* promoter. Interestingly, nuclease activity of NM23-H1 within the promoter of *pdgf-a* was shown to cleave the 3' end of both the pyrimindine- and purine-rich strands independent of single or double stranded conformation.[24] In 2002, NM23β (rat homolog of NM23-H1) was shown to regulate *gelatinase A* by binding with the GGGTTT-repetitive sequence within the 40 bp enhancer response element 1 (RE-1) of *gelatinase A*. Overexpression of *nm23β* in glomerular mesangial cells was found to out-compete the Y-box protein-1 from the RE-1 binding site resulting in repression of *gelatinase A.*[33] Recently, NM23-H1 was reported to induce expression of the extracellular matrix protein fibronectin mRNA and protein in M14 and 1205LU melanoma cell lines. These results were further confirmed in clinical biopsies of normal skin, benign nevi and also in primary melanomas.[34]

GENE REGULATION BY NM23-H1 IN COLLABORATION WITH OTHER FACTORS

A substantial body of literature suggests regulation by NM23-H1 that is executed through interaction with other proteins or regulatory factors. Interaction of NM23-H1 with the Epstein–Barr virus protein nuclear antigen 3C (EBNA3C) showed increased NM23-H1 nuclear localization and altered NM23-H1 transcriptional activities that influenced both cellular and viral gene expression. Interestingly, this interaction negatively impacts the function of NM23-H1 in suppressing migration

of breast carcinoma and Burkitt's lymphoma cells in vitro.[28,35,36] In 2005, Kuppers *et al*[37] further showed NM23-H1 interaction with EBNA3C resulted in upregulation of matrix metalloproteinase 9 (MMP-9) expression through association with Ap1 and NFκB-binding sites on *MMP-9* promoter. This interaction reversed the anti-migratory effect of NM23-H1. NM23-H1 and EBNA3C cooperation was also noted to transcriptionally upregulate cyclooxygenase 2 (*COX-2*) expression by co-binding to target sequences of transcription factors NFκB and CRE in the *COX-2* promoter. NM23-H1 upregulated *COX2*, whereas EBNA3C alone had no effect on *COX-2* expression but contributed to increased *COX2* expression when coexpressed with NM23-H1.[38]

In addition to these, reports also showed that NM23-H1 and EBNA3C regulate alpha V integrin expression independently as well as synergistically by binding different transcription factors. EBNA3C binding to the transcription factor Sp1 upregulates alpha V integrin. On the other hand, NM23-H1 binding to GATA-1 resulted in repression of alpha V integrin. Again, NM23-H1 and EBNA3C when coexpressed negated the repression of alpha V integrin expression mediated by NM23-H1.[39] Additional work from the same group showed NM23-H1-EBNA3C interaction to be important for transcriptional suppression of the cellular regulatory factor *Necdin*. Interestingly, *Necdin* downregulation rescued downstream suppression of the vascular endothelial growth factor (VEGF) promoter and thereby abrogated anti-angiogenic effects.[40]

Protein–protein interactions through NM23-H1 and its impact on gene regulation were also evident from association of NM23-H1 with ERα, which enhanced binding of ERα with the estrogen response element (ERE). This study demonstrated that NM23-H1 silencing in U2 osteosarcoma and MDA-MB231 breast cancer cells resulted in downregulation of an ERE-harboring reporter plasmid, supporting transcription regulation of ERE-harboring progesterone receptor, *Bcl2, cyclin-D1* and cathepsin through NM23-H1 However, other ERE-containing genes like pS2 remained unaltered by NM23-H1 levels.[29]

Interaction of NM23-H1 with the serine threonine kinase receptor associated protein (STRAP) was reported to transcriptionally repress *PAI-1, p21* and *SMAD7* together with activation of *CDK4* and *cyclin-D1*. These together were shown to repress the downstream TGFβ signaling.[41] The same group also reported NM23-H1-STRAP association, following genotoxic stress, with p53 using cystein residues in the central DNA-binding domain (DBD) of p53. Through this interaction NM23-H1-STRAP was found to regulate p53-mediated transcription of apoptosis and cell cycle-related proteins[42]

NM23-H1—ROLE IN GLOBAL GENE REGULATION

In addition to studies showing involvement of NM23-H1 in gene regulation either directly or in association with other factors several global studies revealed differential mRNA expression following altered NM23-H1 expression. Microarray analysis performed on oral squamous cell carcinoma cells CAL27 after subjecting them to NM23-H1 overexpression observed 241 genes with change of more than or equal to twofold, 103 of these genes were downregulated, whereas 138 showed upregulation.[43] The altered genes were primarily related to cell adhesion, invasion, and metastasis including TGFβ signaling. In another study on transfection of breast cancer MDA-MB-435 cells with *nm23-H1*, 197 genes were found to be significantly upregulated, whereas the expression of 1961 genes were downregulated.[44] Functional significance of the altered genes showed them to cluster into six categories namely, invasion and metastasis, apoptosis and senescence, signal transduction and transcription factors, cell cycle and repair, adhesion, and angiogenesis.

Horak *et al*[45] compared the expression profiles following NM23-H1 overexpressing with cells overexpressing NM23-H1 mutants P96S and S120G that are incapable of inhibiting motility and invasion in breast cancer cells. This study reported nine genes (*MET, FZD1, PTN, SMO L1CAM, NETO2, CTGF, MMP-2,* and *EDG2*) downregulated by the wild-type NM23-H1 that remained unaltered in case of NM23-H1 mutants. A later transcriptome analysis in L9981 lung cancer cells showed similar observations.[46] L9981-*nm23-H1* stable cells characteristically exhibited lower cell proliferation, increased apoptosis and a dramatic loss of tumor cell metastasis. This study emphasized the finding that genes like *E-cadherin*, *b-catenin* and *TIMP-1* were upregulated, whereas *MMP-2, CD44v6*, and *VEGF* were repressed on NM23-H1 overexpression, suggesting altered expression of these genes to be significant in the anti-metastatic function of NM23-H1.

DNA BINDING BY NM23-H2 AND TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION

Transcriptional activation of the *c-myc* oncogene by NM23-H2 was shown in human as well as murine cells, in cervical, lung carcinoma, and Burkitt lymphoma by multiple research groups.[16,22,47,48] Furthermore, it was found that NM23-H2 associates with the *c-myc* promoter NHE, constituting an asymmetric repetitive sequence that is cytosine-rich in one strand and guanine-rich in the other—an arrangement conducive to formation of specific DNA secondary structures called G-quadruplexes.[23,49–51] Later, it was shown that NM23-H2 interacts with the *c-myc* promoter in a manner that was dependent on formation of the G-quadruplex structure.[48] Despite these, reports have cast doubt on transcriptional activity of NM23-H2 because of non-specific binding to single-stranded purine-rich sequence.[52,53] On the other hand, it is possible that NM23-H2 association to DNA depends on the structure adopted by such sequences rather than mere sequence. In 2015, in maize nucleoside diphosphate kinase1 (*ZmNDPK1)* the first plant G-quadruplex-binding protein was reported. It is a close homologue of NM23-H2, which was shown to bind folded G-quadruplex structures with higher affinity as compared to unfolded

G-rich DNA. The G-quadruplex-binding activity of ZmNDPK1 was demonstrated to be independent of its nucleotide binding and kinase activities.[54]

GENE REGULATION BY NM23-H2 IN ASSOCIATION WITH OTHER FACTORS

Recently, a NM23-H2-interacting protein was reported in relation to *c-myc* regulation, which supported earlier studies of NM23-H2-G-quadruplex binding in the *c-myc* promoter. Piwi-like RNA-mediated gene silencing 2, PIWIL2 interaction with NM23-H2 was found to upregulate *c-myc* expression by facilitating the association of NM23-H2 with the G-quadruplex motif.[55] In addition to these, CNBP was shown to interact with NM23-H2 and regulate *c-myc* expression. It was further demonstrated that CNBP binds to the G-quadruplex structure formed in the NHE region within the *c-MYC* promoter and while CNBP repressed c-*MYC*, CNBP-NM23-H2 interaction resulted in upregulation of *c-MYC* expression.[30]

As noted for NM23-H1, NM23-H2 also mediated negative regulation of *pdgf-a* transcription by interacting with its silencer sequence (5′-S1 nuclease-hypersensitive site) along with basal NHE. On transient transfection of NM23-H2 negative regulation of the *pdgf-a* basal promoter was found in HepG2 cells.[24] In addition to these, Rayner *et al*[31] demonstrated association of NM23-H2 with the ERβ.[32] Interestingly, in contrast to NM23-H1, which associates with estrogen receptor alpha and functions as a repressor in a estrogen dependent manner, NM23-H2 was found to activate gene expression.[31,32,56]

In 2013, regulation of the alzheimer associated amyloid-β peptide (*APP*) was found to be through interaction of NM23-H2 to the proximal regulatory element (PRE) within the *APP* promoter. Presence of the 30 nucleotide PRE sequence was reported to make *APP* regulation vulnerable to epigenetic modifications. This report further implicated an increased risk of Alzheimer's disease on any interference with NM23-H2's role in regulation of *APP*.[57]

GENOMIC STUDIES ON NM23-H2-MEDIATED GENE REGULATION

In 2014, gene expression profiling along with promoter occupancy of NM23-H2 in lung carcinoma A549 cells was reported.[58] Authors found occupancy of NM23-H2 within promoters of 346 genes related to focal adhesion, nucleosome remodeling, transcriptional regulation, apoptosis, Notch signaling pathway, p53, and Wnt signaling pathways. Of these, 64 gene targets also showed altered expression, either up or downregulation, on changing NM23-H2 levels in the cell. Several targets, for example, adenomatous polyposis coli (*APC*), Rho-related GTP-binding protein B (*RHO B*) and *connective tissue growth factor* (*CTGF*) were found to be under direct regulation of NM23-H2. The study thereafter focused on NM23-H2-mediated transcriptional repression of the focal adhesion factor *vinculin* (*VCL*). NM23-H2 was shown to bind a 12-mer motif located 262 bases upstream of *vinculin* transcription start site.[58]

In a later more extensive study, genome-wide chromatin-immunoprecipitation followed by sequencing (ChIP-seq) was performed in A549 cells and NM23-H2 target sites were checked before and after induction of *nm23-H2*.[59] A 12-mer consensus motif was identified for NM23-H2 binding, which was present in $>70\%$ of the ChIP-seq peaks—a motif that was similar to the one reported by Postel *et al* within the *c-myc* promoter NHE several years earlier.[23,58] This further revealed 2005 and 11017 peaks in endogenous and induced states respectively. On analyzing the altered gene expression profile 1679 genes were found to be differentially expressed in NM23-H2 altered conditions, 781 genes were upregulated, whereas 898 were downregulated. Of these 1679 gene targets, 1235 genes were found to have at-least one NM23-H2-binding site within 10 kb of the transcription start site.[59]

A study that described genes differentially expressed in drug resistance cancer cells implicated NM23-H2 as a factor that altered expression of several well known genes involved in epithelial to mesenchymal transition.[60] Based on this analysis it was tested and found that NM23-H2 repressed several mesenchymal cell markers (such as *SNAI2, VIM, FN1, TIMP-1, ITGA5*) while inducing expression of epithelial cell markers (*OCLN, CDH1, DSP, PPDE2*), thereby promoting mesenchymal to epithelial transition of breast cancer cells (MDA-MB-231). Furthermore, the study reported NM23-H2 levels to be important for preventing drug resistance in cancer cells. Overall, this also supported function of NM23-H2 as a metastasis suppressor.[60]

NM23-H1 AND NM23-H2-MEDIATED CHROMATIN LEVEL CHANGES

Interaction of NM23-H1 with the chromatin remodeling SET complex was reported in 2003 by Fan *et al*.[61] The SET complex is a multimeric 270–420 KDa complex, comprising HMGA, SET, Ape-1, and pp32, generally linked to the chromatin-associated processes of nucleosome assembly, replication, and DNA repair.[61] On activation by granzyme A, NM23-H1 as a part of this complex was shown to induce DNase activity thereby inducing cellular apoptosis through chromatin degradation.[61] In another study, both NM23-H1 and NM23-H2 were found to be part of a multi-component OCA-S (Oct-1 co-activator complex in S phase) complex in transcriptional activation of the histone 2B gene in a S phase-dependent manner.[62] Although both NM23-H1 and NM23-H2 were found to have occupancy on histone 2B gene promoter, their exact role within OCA-S was not clear. This further implicated the significance of NM23 proteins in chromatin modification through regulation of a core histone protein.

More evidence supporting involvement of NM23 in chromatin remodeling was reported in 2014. In a genome-wide ChIP-seq study, NM23-H2 was used as a candidate for testing whether transcription factor binding on target gene

promoters influence nucleosome occupancy in its vicinity.[59] Using lung cancer A549 cells, authors showed that on induction of NM23-H2, 70% of the putative NM23-H2 binding sites earlier occupied by nucleosomes became nucleosome-free. Perhaps more importantly, it was also shown that these newly vacated sites were occupied by NM23-H2, in the induced cells, resulting in altered expression of the target gene.[59]

In another study, a yeast two-hybrid screen found NDPK-D (NM23-H4) associates with the NAD^+ dependent histone deactylase SIRT1. Here it was also shown that deacetylation by SIRT1 enhances the nuclear localization of NM23-H4. Since no evidence of any specific intra-nuclear functions of NM23-H4 has been revealed as yet it would be interesting to explore its role, if any, in gene regulation particularly due to its interaction with the nucleosome remodeler SIRT1.[19]

TRANSACTIVATION DOMAIN IN NM23 PROTEINS: DIFFERING VIEWS

In the light of transcriptional roles attributed to NM23 proteins, particularly NM23-H1 and H2, the differing views on whether they harbor transactivation domain(s) is of interest. NM23-H2 was first implicated in *c-myc* transactivation in 1995, but a distinct transactivator domain was not defined.[22] In 1997, presence of a typical transactivation domain was negated by Michelloti *et al*,[63] when following reporter assays that fused a DBD with wild-type NM23-H2, authors did not observe any transactivation. In the following year, Chae *et al*[64] reported presence of transactivation domain from studies where human *nm23-H1* gene constructs were transfected in yeast. They co-transfected a fusion protein containing GAL4/LEXA DBD with a truncated versions of NM23-H1 and noted that the *C*-terminal residues of NM23 displayed clear transactivation potential, whereas no transactivation was observed through the *N*-terminal residues. Presence of a C-terminal transactivation domain was further substantiated later when transactivation activity of *C*-terminal residues of NM23-H1 (amino-acid 109–152) was found in yeast, HeLa, and COS cells.[65] Interestingly it was noted that further extension of the domain (including amino acids from 58–152) lead to a loss of transactivation. Authors also tested the role specific mutants—C-terminal residues such as P96S, S120G/S120A (known to inhibit anti-metastatic effect of wild-type NM23-H1) and H118F (NDP Kinase mutant of NM23-H1). Interestingly, only H118F showed reduction of transactivation activity suggesting a link between NDP kinase activity and transactivation potential NM23-H1. In another study, reporter assays in 293T cells using NM23-H1 fused to Gal4 DBD authors found increase in reporter expression.[28]

EMERGING TRENDS AND FUTURE PERSPECTIVE

Research on regulation of gene expression by NM23 proteins themselves or in collaboration with other interacting partners was primarily discussed here. This, in addition to, the emerging work on involvement of NM23 in chromatin-related processes projects interesting line of work that has received relatively less attention. Also, several genome-wide studies have revealed the global impact of NM23-H1/H2 on a wide array of cellular processes including cell development, differentiation, and proliferation.

We particularly take note of the work that may link the inherent NDP kinase activity of NM23 to its role in gene expression based on the study that found loss of transactivation potential in the mutant NM23-H1 (H118F) devoid of kinase activity.[65] Another interesting theme that may open up new avenues comes from the implicated involvement of NM23-H2 in modulating the state of regulatory chromatin through nucleosome repositioning within gene promoter.[60] Based on these, future work on the influence of the NDP kinase activity in chromatin modifications, and mechanisms of how this impacts global gene regulation could be of much interest and impact.

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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