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EDITORIAL

A critical evaluation of biochemical activities reported for the nucleoside diphosphate kinase/Nm23/Awd family proteins: opportunities and missteps in understanding their biological functions

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The nucleoside diphosphate kinase (NDP kinase or NDPK) protein family has been discovered several times, with implications for the fields of development, signaling, and cancer. In the 1950s, the Nobel laureates, Paul Berg and Hans Krebs, independently identified a biochemical activity that removed the terminal phosphate from a nucleoside triphosphate (NTP), and added it to a nucleoside diphosphate (NDP), the NDPK activity (Berg and Joklik, 1953; Krebs and Hems, 1953). Consequently, the correct biochemical name for the enzyme is NTP/NDP transphosphorylase. NDPK activity was implicated in the regulation multiple aspects of cellular physiology, including nucleotide pools and consequent growth, G-protein signaling, microtubule dynamics, and mutational susceptibility. In the late 1980s, a furor erupted when cancer metastasis, the spread of tumor cells from a primary tumor to a distant site, was linked to the loss of a gene of unknown function, named *nm23* (Steeg et al. 1988). A functional link was established several years later when transfection of *nm23* into a murine melanoma cell line significantly reduced its metastatic spread, without effects on primary tumor size (Leone et al. 1991). Sequencing of the two forms of human Nm23 (Nm23-H1 and –H2) revealed an identity to two NDPKs A and B (Wallet et al. 1990). At the same time, developmental processes in the imaginal discs of *Drosophila* were reported to be regulated by the abnormal wing disc (*awd*) gene, almost 90% identical to human NDPK/Nm23 through evolution (Rosengard et al. 1989). A complex story thus began. Today, it is clear that NDPK-A and NDPK-B belong to a protein family encoded by the NME genes of which ten different genes have been found in man. Phylogenetic analyses identified two distinct subgroups (Bilitou et al. 2009; Desvignes et al. 2009). The first group consists of four genes (*nme1-4*) encoding homologous proteins (NDPKs A–D) with NDPK activity. Group II genes appeared also early in evolution (see (Desvignes et al. 2011; Perina et al. 2011) in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology*). They encode more divergent proteins with low or no demonstrated NDPK activity. Recent reviews presenting different aspects of the proposed activities of NDPK/Nm23/Awd proteins in tumor metastasis are presented in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology* (Andolfo et al. 2011; Bruneel et al. 2011; Hsu, 2011; Marino et al. 2011; Novak et al. 2011; Thakur et al. 2011). With over 50 years of research experience on this protein family between the authors, we have reviewed the reported biochemical activities of Nme proteins with a critical eye, concentrating on the first two members of the NDPK family. Our analysis reveals a list of tantalizing activities that may

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underlie fundamental biological processes, but also long-standing controversies—often published in high-impact journals—that should now be put to rest.

NDPK activity

Table 1 summarizes contributions of the first defined biochemical activity of the family, as an NDPK. X-ray crystallography established the structures of multiple family members, each with an active site containing a histidine serving as a phosphorylated intermediate (Dumas et al. 1992; Chiadmi et al. 1993; Williams et al. 1993; Cherfils et al. 1994; Morera et al. 1995; Strelkov et al. 1995; Karlsson et al. 1996). While the NDPK biochemical activity is straightforward, its biological implications remain obscure. Few measurements of nucleotide pool levels have been reported. Other enzymes have NDPK activity and could potentially modify nucleotide pools independently. In *Drosophila*, the NDPK activity of Awd was necessary but not sufficient for normal fly development (Xu et al. 1996).

One of the most intriguing hypotheses posited was that NDPK would supply GTP to activate either mono- (small) or heterotrimeric (large) G-proteins. NDPK/Nm23 proteins were associated with the Gβγ dimers of heterotrimeric G proteins (Cuello et al. 2003; Hippe et al. 2003), the G_s protein (Kimura and Shimada, 1990), ARF6 (Palacios et al. 2002), Rho (Chopra et al. 2004), the Ras-related protein Rad (Tseng et al. 2001), the Rac regulator Tiam (Otsuki et al. 2001), Menin (Yaguchi et al. 2002), the guanine exchange factor Dbl-1 (Murakami et al. 2008b), and Arf (Randazzo et al. 1991). The evidence for these associations varied by report, including co-immunoprecipitations, effects on G-protein or Nm23 biochemical activities when both were co-expressed, use of neutralizing antibodies to one partner to abrogate a biochemical activity, etc. Only one of these associations has been reproduced several times (Hippe et al. 2003; Hippe et al. 2009; Hippe et al. 2011a), and repeated by an independent group (Kowluru, 2008), i.e., the complex formation of the NDPK-B/Nm23-H2 isoform with Gβγ. The mutual dependence of NDPK-B and Gβγ for biological G-protein function has been demonstrated by

Table 1 Nucleoside diphosphate kinase (NDPK) activity of NDPK/Nm23/Awd

Features:

- Catalyzes the γ-phosphorylation of nucleoside 5'-diphosphates to corresponding triphosphates, via a phosphohistidine intermediate:

  (1) $N_1TP + NDPK \leftrightarrow N_1DP + NDPK\text{-}P$

  (2) $NDPK\text{-}P + N_2DP \leftrightarrow N_2TP + NDPK$

- Found as tetramers and hexamers; crystal structures reported.
- Found in all subcellular compartments
- Postulated to maintain nucleotide pools, activate G-proteins, induce a mutator phenotype, regulate microtubule dynamics.

Concerns:

- Displays little specificity for different nucleotide bases (Mourad and Parks, 1966, Dumas et al., 1992).
- NDPK activity of vector- versus Nm23 transfectants comparable (MacDonald et al., 1993, Otero et al., 1999).
- Other enzymes have overlapping functions in maintaining nucleotide pools (Lu and Inouye, 1996, Zhang et al., 2002).
- Direct G-protein activation by NDPK-mediated GTP formation from bound GDP retracted (Randazzo et al., 1992).
- Mutator activity dissociated from nucleotide pool levels (Nordman and Wright, 2008).

knock-down experiments in zebrafish, as well as in fibroblasts obtained from NDPK-A and NDPK-B double-deficient mice (Hippe et al. 2009; Hippe et al. 2011a). The requirement of the NDPK-B/Gβγ complexes for the formation of signal transducing caveolae reported in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology* (Hippe et al. 2011b) provides a plausible explanation to understand these data.

Nevertheless, all hypotheses assuming a direct activation of monomeric GTPases or heterotrimeric G-protein α subunits by NDPK should be regarded with great care. The Arf case is instructive. Originally published in *Science*, NDPK/Nm23 was reported to act as an NDPK to phosphorylate GDP bound to the small G-protein Arf, providing a novel mechanism of G-protein activation independent of nucleotide exchange (Randazzo et al. 1991). The same research group retracted this hypothesis after further research using multiple purified G-proteins (including the heterotrimeric G-protein transducin (G_t), Ha-Ras p21, and Arf). In these latter experiments, dissociated GDP was the substrate for the NDPK and converted into GTP even on thin layer chromatography plates (Randazzo et al. 1992). Thus, the G-protein activation occurred by nucleotide exchange. Note that the special provision of GTP appears at odds with the limited specificity of NDPK for any particular NDP as well. Given the wealth of the associations of NDPK B and heterotrimeric G-proteins, an alternative biochemical mechanism has been advanced based on the histidine protein kinase activity of NDPK, discussed below.

The interaction of NDPK/Nm23 proteins with microtubules has also been controversial. NDPK/Nm23/Awd proteins co-purified with microtubules in some (Jacobs and Caplow 1976; Biggs et al. 1990; Lombardi et al. 1995; Pinon et al. 1999) but not all studies (Melki et al. 1992; Roymans et al. 2000), and were postulated to provide GTP for polymerization. Defects in mitosis presumably resulting from impaired interaction leading to microtubule failure were debated, as was transphosphorylation. Collectively, we view the data to date as unsupportive of any specific provision of NTPs via the NDPK activity without evidence for an additional explanatory mechanism.

Transcriptional regulation of c-*myc* and other genes

Table 2 provides a timeline of the major findings supportive of the role of NDPK-B/Nm23-H2 as a c-*myc* transcriptional regulator. Also published in *Science*, a "95%" purified recombinant NDPK-B/Nm23-H2 protein was reported to bind the nuclease hypersensitive element (NHE) in the c-*myc* promoter (Postel et al. 1993). Purification of the protein was only shown on Coomassie-stained gels, known to be less sensitive than a silver stain. The story of NDPK-B/Nm23-H2 evolved when the c-*myc* NHE was reported to fold into a quaternary structure, a G-quadraplex; NDPK-B/Nm23-H2 was postulated to either cleave the DNA or remodel it to change the quadraplex structure, resulting in increased transcription (Postel 1999; Postel et al. 2000a). The arguments against this activity have been numerous and broad. Briefly, regulation of c-*myc* transcription by NDPK/Nm23 was not observed using traditional promoter-driven fusion protein transcriptional assays used throughout the *myc* field (Michelotti et al. 1997), NDPK/Nm23 was reported to bind to any single-stranded DNA without specificity (Hildebrandt et al. 1995; Agou et al. 1999), and the DNA cleavage activity postulated for remodeling of the quadraplex was a contaminant (Dexheimer et al. 2009). Another paper on the G-quadraplex binding activity of NDPK/Nm23 was published in a high-ranking journal and later retracted (Grand et al. 2004). We view the c-*myc* transcriptional activity as a likely artifact, and lament that this is the activity most connected with the NDPK/Nm23

Table 2 Transcriptional activation of *c-myc* and other genes by NDPK/Nm23/Awd

Features:
• In 1993, Postel et al. (1993) identified NDPK-B/Nm23-H2 as the PuF transcription factor binding the nuclease hypersensitive element (NHE) at positions −142 to −115 of the *c-myc* promoter.
• DNA binding and transactivational activities were maintained in a NDPK catalytically inactive Nm23-H2 H118F mutant (Postel and Ferrone, 1994). Other amino acids in NDPK (Arg 34, Asn-69, Lys 135), all charged, were identified as important to DNA binding (Postel et al. 1996).
• NDPKs A and B/Nm23-H1 and –H2 were reported to repress the transcriptional activity of the *pdgf* promoter (Cervoni et al. 2003).
• The *c-myc* NHE was reported to form a G-quadraplex structure which suppresses c-myc transcription. NDPK-B/Nm23-H2 was postulated to remodel this structure, either by an intrinsic DNA endonuclease activity or by DNA unwinding (Postel et al. 2000a).
Concerns:
• For the identification of the *c-myc* promoter transcriptional activity, bacterially produced NDPK-B/Nm23-H2 was only demonstrated to be pure on Coomassie stained gels (Postel et al. 1993).
• NDPK-B/Nm23-H2 bound to both single stranded and duplex portions of the *c-myc* NHE (Postel et al. 2000b, Postel et al. 2002). NDPK/Nm23 proteins bound to single stranded DNA in a non-sequence specific manner (Hildebrandt et al. 1995, Agou et al. 1999).
• Nm23-H2 did not function as a *c-myc* transcription factor using multiple promoter activity constructs (Michelotti et al. 1997, Chae et al. 1998).
• The *c-myc* expression of control and Nm23 transfected cell lines was unchanged.
• A manuscript describing the interaction of NDPK/Nm23 with a *c-myc* quadraplex structure was retracted (Grand et al. 2004).
• Further purification of recombinant NDPK/Nm23 resulted in a separation of the DNA nuclease activity from the protein, implying a contaminant (Dexheimer et al. 2009).

protein family among the scientific readership. The data highlight the continuing problem of the purity of recombinant NDPK/Nm23 preparations for biochemical activities; this is an extraordinarily sticky set of proteins.

Other DNA-based activities?

The laboratory of Edith Postel also postulated that the DNA cleavage activity of NDPK/Nm23 protein could be important to DNA repair, and underlie the "mutator" phenotype observed in bacteria with *ndk* (homolog of *ndpk*) deletions (Lu et al. 1995). In a *PNAS* paper, NDPK was reported to be a DNA repair nuclease whose substrate was uracil misincorporated into DNA (Postel and Abramczyk 2003). While not formally retracted, a subsequent manuscript from the lab identified this DNA-based activity as a uracil-DNA glycosylase contaminating the recombinant NDPK preparation (Goswami et al. 2006).

Given the outcome of these studies, other DNA-based activities are met with healthy skepticism. At least two other DNA-based activities have been proposed. The laboratory of Judy Lieberman reported in *Cell* that granzymes released by cytotoxic T-lymphocytes activate a DNase which is NDPK-A/Nm23-H1, which in turn induces apoptosis and is inhibited by the Set protein (Fan et al. 2003). Low Nm23-H1 expression in metastatic tumor cells was hypothesized to facilitate escape from apoptosis (Chakravarti and Hong 2003). Again, the issue of NDPK/Nm23 purification is critical. The Set complex was isolated by affinity purification and gel filtration, but no silver-stained gel to demonstrate purity was shown. Recombinant Nm23 was prepared using ammonium sulfate precipitation, hydroxyapatite, ATP agaraose, and DEAE columns; a silver-stained gel was not shown. Silencing of Nm23-H1 but not Nm23-H2 reduced DNA nicking, and overexpression of Nm23-H1 increased DNA nicking. More recently, overexpression of a related Sei1 candidate oncogene was reported in esophageal cancers, and was proposed to interact with Set and Nm23-H1 to induce genomic instability (Li et al. 2010). Our view of the evidence is that it is partial; it is clear that NDPK/Nm23 protein is part of the relevant protein complex; however, the possibility of a contaminant binding NDPK/Nm23 with DNase activity remains. Furthermore, in multiple Nm23-H1 transfections, an increase in tumor cell apoptosis was not observed.

The laboratory of David Kaetzel reported a 3′-5′ DNA exonuclease activity for NDPK-A/Nm23-H1 (Ma et al. 2004). Co-elution of NDPK-A/Nm23-H1 and a 3′ exonuclease activity was demonstrated on hydroxylapatite and gel filtration columns; it is stated that Coomasie- or silver-stained gels were used to assess purification but the latter was not shown. The conclusion that this enzymatic activity belongs to NDPK-A/Nm23-H1 is however supported by mutation data. A K12Q mutation of NDPK-A/Nm23-H1 diminished its exonuclease activity. Mutation of the NDPK/Nm23 catalytic histidine abolished its NDPK but not exonuclease activity. The K12Q mutation also altered the protein's elution pattern on columns, but the exonuclease activity continued to copurify with NDPK-A/Nm23-H1. In a follow-up study, an E5A mutation was reported to diminish the exonuclease activity of NDPK-A/Nm23-H1 without effects on its NDPK or histidine protein kinase activities (Table 3). This mutation reduced the metastasis suppressor function of NDPK-A/Nm23-H1 in vivo but, surprisingly, not motility suppression in vitro (Zhang et al. 2010).

Protein–protein interactions

One of the most widely reported biochemical activities for NDPK/NM23/Awd proteins is their association with other proteins, thereby affecting the function of one or both of the pair. Protein–protein interactions involving NDPK/Nm23/Awd proteins are detailed elsewhere in this volume but include a number of oncogenic, viral, and cytoskeletal proteins. These data, to the extent that the interactions are specific, suggest the hypothesis that

Table 3 The histidine protein kinase (HPK) activity of NDPK/Nm23/Awd proteins

Features:
• Unlike serine and tyrosine kinases, involves the formation of a histidine phosphorylated intermediate, which then transfers the phosphate to the substrate protein.
• Ubiquitous in bacteria where two-component signal transduction pathways regulate responses to environmental stimuli. NDPK/Nm23 protein substituted for the histidine kinase component in the EnvZ and CheA two-component pathways (Lu et al. 1996).
• NDPK/Nm23 proteins functioned as HPKs for Aldolase C (Wagner and Vu, 2000), ATP citrate lyase (Wagner and Vu, 1995), the potassium channel $K_{Ca}3.1$ (Srivastava et al. 2006), and $\beta\gamma$ subunits of heterotrimeric G-proteins (Cuello et al. 2003). A counteracting phosphatase (PHP-1) has been identified (Wieland et al. 2010).
• Phosphorylation of serine residues by Awd (Inoue et al. 1996) and NDPK/Nm23 (Engel et al. 1995) was reported; NDPK/Nm23 phosphorylated serine residues on the Kinase suppressor of ras (Hartsough et al. 2002).
• The HPK activity of wild type and mutant NDPK-A/Nm23-H1s was correlated with its tumor motility suppressing activity (Freije et al. 1997, Wagner et al. 1997).
Concerns:
• The active pocket of NDPK/Nm23/Awd for its NDPK activity on x-ray crystallography appears too small to accommodate a protein substrate.
• Little is known about HPKs due to technical limitations.

NDPK/Nm23 proteins may suppress tumor metastasis, in part, by binding and inactivating signaling pathways promoting aggressiveness. The contribution of this type of inactivating interaction has not been fully explored for the many G-proteins previously described. Several of the reported interactions overcame suppression of in vitro motility or in vivo metastasis by NDPK/Nm23, including Dbl-1 (Murakami et al. 2008a) Prune (Reymond et al. 1999; D'Angelo et al. 2004) and the Epstein–Barr latent viral proteins (Subramanian et al. 2001; Murakami et al. 2005), while an interaction of NDPK-A/Nm23-H1 and the TGF-β receptor interacting protein Strap regulated Nm23-H1 regulation of proliferation (Seong et al. 2007).

An example of these interactions, the Prune (Pn) protein, provides insight into the validation needed. In *Drosophila*, mutations in *pn* are responsible for the brownish-purple "prune" eye color. Null *pn* mutations did not affect *Drosophila* viability or fertility. In contrast, both the homozygous and hemizygous *pn* mutants were lethal in the presence of a single copy of a gain-of-function mutation in the *awd* gene (Biggs et al. 1988; Timmons and Shearn 1997). This *awd* mutation is analogous to the P96S "killer of prune" mutation studied in NDPK/Nm23. A mechanistic understanding of NDPK/Nm23/Awd-Pn interaction was resolved in mammalian cells when a direct interaction was revealed by two-way co-immunoprecipitation assays using endogenous levels of protein expression (Reymond et al. 1999). Further refinement of this interaction identified the region on NDPK/Nm23/Awd responsible for the interaction, serines 120, 122, and 125 (Garzia et al. 2008). These mutations, alone or in combination, impaired the formation of NDPK/Nm23/Awd-Prune complex. NDPK-A/Nm23-H1 S120 is highly conserved throughout evolution and undergoes serine phosphorylation by casein kinase I (CKI), which is essential for the formation of the NDPK-A/Nm23-H1–Pn complex (Garzia et al. 2008). As reported in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology*, a naturally occurring mutant of NDPK-A at S120, the S120G mutant, has the tendency to aggregate into amyloid structures (Georgescauld et al. 2011). In addition to CKI, casein kinase II (CKII) might phosphorylate S120 in nm23-H1, too. This phosphorylation might be important in the regulation of the interplay of NDPK with AMP-activated protein kinase (see (Annesley et al. 2011) in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology*) or its histidine kinase substrates, both hypothetically involved in the regulation of the cystic fibrosis transmembrane conductance regulator (see (Venerando et al. 2011), in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology*).

Nevertheless, due to the sticky nature of NDPK/Nm23/Awd proteins, we view suspiciously data based on co-immunofluorescence or two hybrid analyses only. Supporting data should use endogenous protein levels, identify the binding site, and demonstrate an alteration in function.

Histidine protein kinase

Histidine protein kinases (HPKs) are well known in bacteria and other lower organisms where they form two or more component signal transduction pathways, a major form of response to environmental stimuli. Briefly, an environmental stimulus induces the histidine phosphorylation of a sensor histidine kinase, which transfers the phosphate to an aspartate on a response regulator, often a transcription factor capable of altering gene expression and consequently cellular function. In more complex systems in lower eukaryotes, a phosphorelay occurs: The first histidine phosphoryl group is transferred to an aspartate of a single domain response regulator and, subsequently, to a second histidine residue of a phosphotransferase that finally phosphorylates an effector moiety on a second aspartate. The presence and function of HPKs in mammalian cells is poorly studied, owing to the lack of suitable anti-phosphohistidine antibodies. In theory, the NDPK activity of NDPK/Nm23/Awd proteins acts as the reversible first step in a HPK reaction:

1. NDPK+NTP ⟷ NDPK-histidine phosphate+NDP, the NDPK equation.
2. NDPK-histidine phosphate+substrate ⟷ NDPK+phosphorylated substrate.

Questions arise over the nature of the phosphorylated residue in the substrate, which in experimental systems has included high-energy histidines and aspartates as well as lower energy (and therefore irreversible) serines.

What is the evidence that NDPK/Nm23/Awd proteins possess HPK activity? (1) NDPK clones can substitute for histidine protein kinases in bacterial two-component systems (Lu et al. 1996). (2) NDPK-A/NM23-H1 but, to a lesser extent, its P96S and S120G mutants, phosphorylated an aspartate on aldolase C in vitro; this aspartate cannot autophosphorylate, ruling out the provision of a phosphate for autophosphorylation by the NDPK activity (Wagner and Vu 2000). In addition, the phosphorylation of another metabolic enzyme, ATP citrate lyase by NDPK-A/NM23-H1 (Wagner and Vu 1995) has been reproduced by others (Klumpp et al. 2003; Wieland et al. 2010) (3) An intriguing pathway involving the βγ subunits of heterotrimeric G-proteins was uncovered, in which NDPK-B/Nm23-H2 phosphorylated the histidine 266 in Gβ (Cuello et al. 2003). Since the phosphorylated Gβ can only use the high-energy phosphate to transfer to a GDP (Wieland et al. 1993), the reaction in essence could account for the GTP specificity in

NDPK-B/Nm23-H2 stimulation of G-protein function. By re-expression of NDPK-B/Nm23-H2 or its catalytic inactive H118N mutant in a combined NDPK-A and NDPK-B null background, it has been demonstrated that this phospho-transfer regulates basal, receptor-independent G-protein activation (Hippe et al. 2011a). (4) NDPK-B/Nm23-H2 phosphorylated the $K_{Ca}3.1$ potassium channel on histidine 358, facilitating its activation (Srivastava et al. 2006). Activation of this channel mediates K^+ efflux building up a negative membrane potential, required to establish a favorable electrochemical gradient for Ca^{2+} influx. $K_{Ca}3.1$ channels are important for diverse physiological responses in a variety of cell types, including osmotic and volume regulation in red blood cells, mitogen-dependent activation of T-lymphocytes, Cl^- secretion of exocrine epithelial cells, and control of proliferation of T-and B-lymphocytes, vascular smooth muscle cells, and some cancer cell lines. The importance of its NDPK-B dependent regulation for T-lymphocyte function in vivo has recently been demonstrated in NDPK-B deficient mice (Di et al. 2010). A review on the role of HPK and its counter regulator phosphohistidine phosphatase in the regulation of insulin secretion in the pancreatic β cell can be found in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology* (Kowluru et al. 2011). (5) Using homology to a eukaryotic two-component pathway in *Arabidopsis*, the kinase suppressor of ras (Ksr) was hypothesized to be a HPK substrate. NDPK-A/Nm23-H1 phosphorylated Ksr on two serine residues (Hartsough et al. 2002). Ksr is a scaffold for the Erk Map kinase pathway central to proliferation and other activities. Phosphorylation of Ksr by NDPK-A/Nm23-H1 resulted in altered Hsp90 binding to the scaffold with consequences for its stability and Erk activation (Salerno et al. 2005). Finally, the potential relevance of the HPK activity to the metastasis suppressive activity of NDPK-A/Nm23-H1 was demonstrated in two studies where mutations that decreased its HPK activity correspondingly abrogated the ability of the protein to inhibit tumor cell motility in vitro (Freije et al. 1997; Wagner et al. 1997).

The potential problems with adoption of HPK as a relevant mechanism of action for NDPK/Nm23/Awd proteins lie in its structure determined by crystallization. The active site for its NDPK activity forms a small cleft and thus regularly fits nothing more than a nucleotide. Although at least some of the phosphorylated histidine residues, e. g., His266 in Gβ (Cuello et al. 2003), stick out of the protein surface, it is difficult to imagine that protein substrates can fit into the cleft and gain access to the intermediately phosphorylated histidine of NDPK isoform. HPK status co-crystals or NMR studies will address these problems. Alternative explanations for this paradox are still lacking. However, it is noted that NDPK/Nm23 proteins can assume other structures, for instance, a molten globule conformation (Lascu, 2006). Unless, for example, co-crystallization efforts with a peptide or a protein substrate solve this mystery, we consider the HPK activity of NDPK/Nm23/Awd proteins a tantalizing but incompletely understood biochemical mechanism.

Conclusions

It is undisputed that NDPK/Nm23/Awd family of proteins have more biological functions than originally anticipated from the primary enzymatic activity. The biochemical mechanisms underlying these biological functions have been the subject of a multitude of hypotheses, some published in high-profile journals and widely disseminated. Several well-known hypotheses concerning NDPK/Nm23/Awd function relied on impure protein preparations and other types of potentially inadequate evidence and should be discounted. Emerging evidence has identified novel biochemical functions and interactions. Stringent biochemistry, coupled with developmental and molecular cellular biology, including cellular compartment definition, mutational analysis, and animal model proof of concept (see (Boissan and Lacombe 2011) in this issue of *Naunyn-Schmiedeberg's Archives of Pharmacology*), will undoubtedly enhance the discovery and validation of NDPK/Nm23/Awd functions in normal and pathological states.

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