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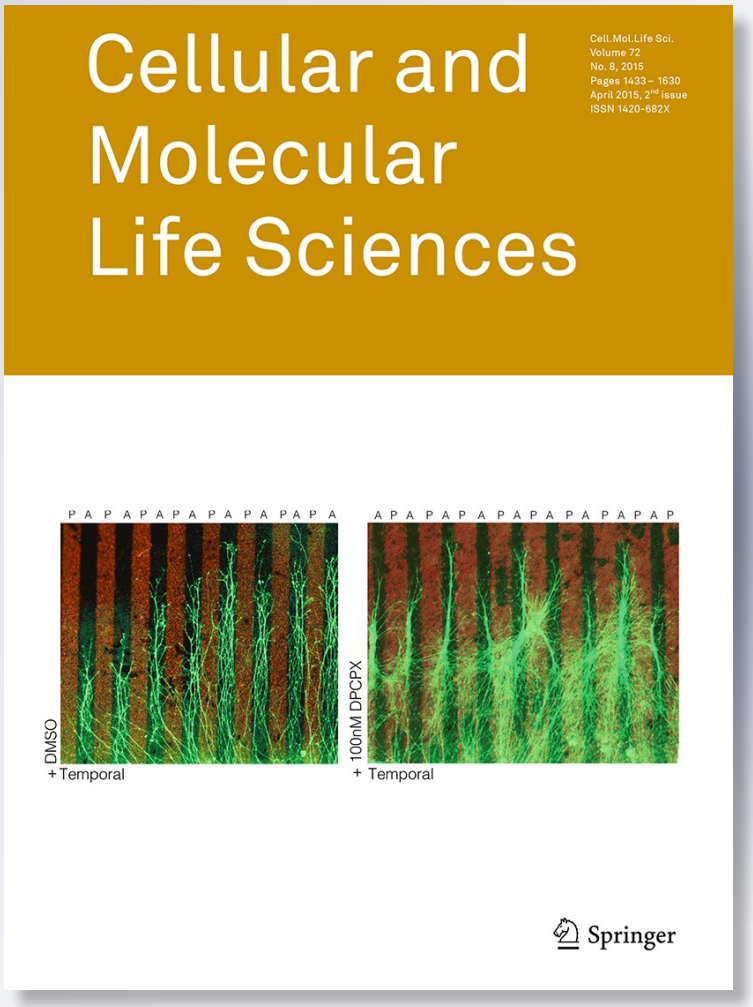

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REVIEW

Nucleoside diphosphate kinases (NDPKs) in animal development

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Abstract In textbooks of biochemistry, nucleoside diphosphate conversion to a triphosphate by nucleoside diphosphate 'kinases' (NDPKs, also named NME or NM23 proteins) merits a few lines of text. Yet this essential metabolic function, mediated by a multimeric phosphotransferase protein, has effects that lie beyond a simple housekeeping role. NDPKs attracted more attention when NM23-H1 was identified as the first metastasis suppressor gene. In this review, we examine these NDPK enzymes from a developmental perspective because of the tractable phenotypes found in simple animal models that point to common themes. The data suggest that NDPK enzymes control the availability of surface receptors to regulate cell-sensing cues during cell migration. NDPKs regulate different forms of membrane enclosure that engulf dying cells during development. We suggest that NDPK enzymes have been essential for the regulated uptake of objects such as bacteria or micronutrients, and this evolutionarily conserved endocytic function contributes to their activity towards the regulation of metastasis.

Keywords Nucleoside diphosphate kinases · NME · Metastasis inhibitor · Endocytosis · Dynamin · Engulfment

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Introduction

Nucleoside diphosphate kinases (NDPKs) were identified more than 50 years ago as ubiquitous housekeeping enzymes that catalyze the phosphorylation of nucleoside diphosphates to nucleoside triphosphates at the expense of ATP [1]. In the human genome, ten paralogous genes encode NDPKs, which belong to two distinct groups based on sequence homology and NDPK activity [2, 3]. Group I genes encode proteins (NME1–4; non-metastatic) with NDPK activity and show high similarity to one another, whereas group II members (NME5–9 and RP2, retinitis pigmentosa 2) are more divergent and display low or no NDPK activity. NDPKs gained initial attention when Patricia Steeg identified a group I NDPK, NME1 (also known as NM23-H1), as the first metastasis suppressor protein [4] (NM23 stands for non-metastatic clone 23); the cDNA of *Nm23* was found to be downregulated in a highly metastatic derivative of a murine melanoma cell line [4]. Mathieu Boissan and colleagues [5] demonstrated the anti-metastatic effect of NME1 in mouse models: *nme1* knockout mice where crossed with a mouse strain prone to hepatocellular carcinoma and the double transgenic mice had a higher incidence of lung metastases. Next, human and murine *Nm23* were shown in cancer cell xenografts to inhibit metastasis without influencing primary tumor growth [6]. The level of NM23-H1 expression was inversely correlated with the metastatic potential for human solid tumors of epithelial origin such as breast, liver, colorectal, ovarian and lung carcinoma and for melanoma [7]. However, in other tumor types, such as ovarian cancers, neuroblastoma and hematological malignancies, upregulated NM23 levels have been detected from patient samples that correlate with a poor prognosis [8–10]. Thereafter, understanding the biological function of the

Nm23 gene family was brought into focus through diverse results, suggesting that the regulation of many physiological processes was associated with this gene family (reviewed in [11]), such as cell migration [12], growth and differentiation [13, 14], signal transduction, transcriptional regulation [15, 16], and apoptosis [17]. Correspondingly, multiple protein interaction partners were reported [12]. These multiple functions assigned to the NM23 family of proteins have thus far been difficult to reconcile because the exact cellular function of NM23 has remained unclear. However, beyond nucleoside diphosphate kinase activity several other molecular activities have been linked to NDPKs, such as histidine-dependent protein kinase (histidine phosphotransferase) [18, 19] and nuclease activity [20, 21], as well as lipid bilayer binding [22–26].

NDPKs are evolutionary highly conserved from yeast to human [3, 27], suggesting critical cellular and developmental functions for members of this protein family. Although, studies in mammalian cell cultures have identified numerous functions and interaction partners of NDPKs, many of these findings have not been directly verified in vivo. Studies on model organisms helped in understanding the biological functions of NDPKs, and could confirm functions derived from in vitro and cell line studies.

In this review, we focus on this issue and give an overview of NDPKs' developmental functions in different model systems such as the slime mold *Dictyostelium discoideum*, the nematode *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, the zebrafish *Danio rerio*, the frog *Xenopus laevis*, and the mouse *Mus musculus*, where NDPKs have now been extensively studied.

Developmental functions of AWD/NM23 in the fruit fly *Drosophila melanogaster*

Early studies: the isolation of *K-pn* and *awd*^{b3} alleles

Amongst genetic model systems, the earliest and most extensive analysis of the homolog of human NM23, *abnormal wing discs* (AWD), was carried out in *Drosophila melanogaster* where the first mutant allele of *awd*, *killer-of-prune* (*K-pn*), was isolated in the 1950s by the famous fly geneticist Alfred Sturtevant [28]. He performed genetic crosses to demonstrate X-linkage of the eye color influencing gene, *prune*. Loss-of-function mutation of *prune* leads to brownish-purple eye color in otherwise normal adult flies instead of the wild-type bright red eye. Sturtevant's seminal observation was that the *K-pn* mutation, which was itself viable, when crossed into viable *prune* loss-of-function mutants, resulted in lethality even in the presence of a single *K-pn* mutant allele. He reasoned that *K-pn* might, therefore, carry a dominant, gain-of-function mutation, which exerts synthetic lethality in the *prune* mutant background. However, the molecular lesion underpinning the *K-pn* allele and the cause of synthetic lethality phenotype were to remain unknown for almost three decades.

In the 1980s, the laboratory of Allen Shearn conducted a genetic screen for late larval/pupal lethality to identify genes regulating imaginal disc development [29]. Imaginal discs are epithelial infoldings in *Drosophila* larvae that develop into adult structures (such as legs, antennae, wings, etc.). In this developmental screen, alleles of 12 different disc-defective genes were found [29, 30]. One of them was named *awd* [30], as initially only the paired wing discs appeared to be defective in *awd*^{b3} mutant larvae. Further analysis revealed that other imaginal discs of *awd*^{b3} mutants, including leg and eye-antenna discs, also do not differentiate properly, and show increased cell death (Fig. 1a). Subsequently it turned out that the *awd*^{b3} allele is a genetic null mutation of the gene *awd* as a consequence of P-element insertion into the first exon. In addition, because *awd*^{b3} mutants also had defective ovaries, Dearolf and colleagues [30] performed the following elegant organ transplantation experiments. The *Drosophila* ovaries are composed of cells of both somatic and germline origin. If premature *awd*^{b3} mutant ovaries containing both somatic cells and germ cells were transplanted into wild-type females, the grafted flies remained sterile. However, if isolated germ cell precursors (pole cells) of *awd*^{b3} mutant embryos were introduced into wild-type embryos, this gave rise to fertile flies with normally developed ovaries, which produce gametes. This series of experiments indicated that the ovarian sterile phenotype of *awd* mutants is caused by defects in somatic (follicular) cells of the ovaries. The fact that *awd* activity is required in follicular cells became important in later studies (Fig. 1a, b).

AWD is the homolog of NM23-H1/H2 and displays NDPK activity

awd alleles were isolated and the gene *awd* was cloned cotemporaneously by Patricia Steeg and her colleagues, discovering *Nm23* as the first non-metastatic (i.e., metastasis inhibitor) gene from experiments performed on a highly metastatic murine melanoma cell line [4]. Results obtained from *Drosophila* converged with human studies, and it became clear that AWD is the *Drosophila* homolog of NM23, given that AWD shows 78 % amino acid identity with the two closely related human proteins NM23-H1/H2 [31]. Next, several parallel studies set out to determine the enzymatic 'activities' of NM23/AWD family proteins. First, Allen Shearn, Patricia Steeg and their colleagues found that an antibody originally generated against bovine

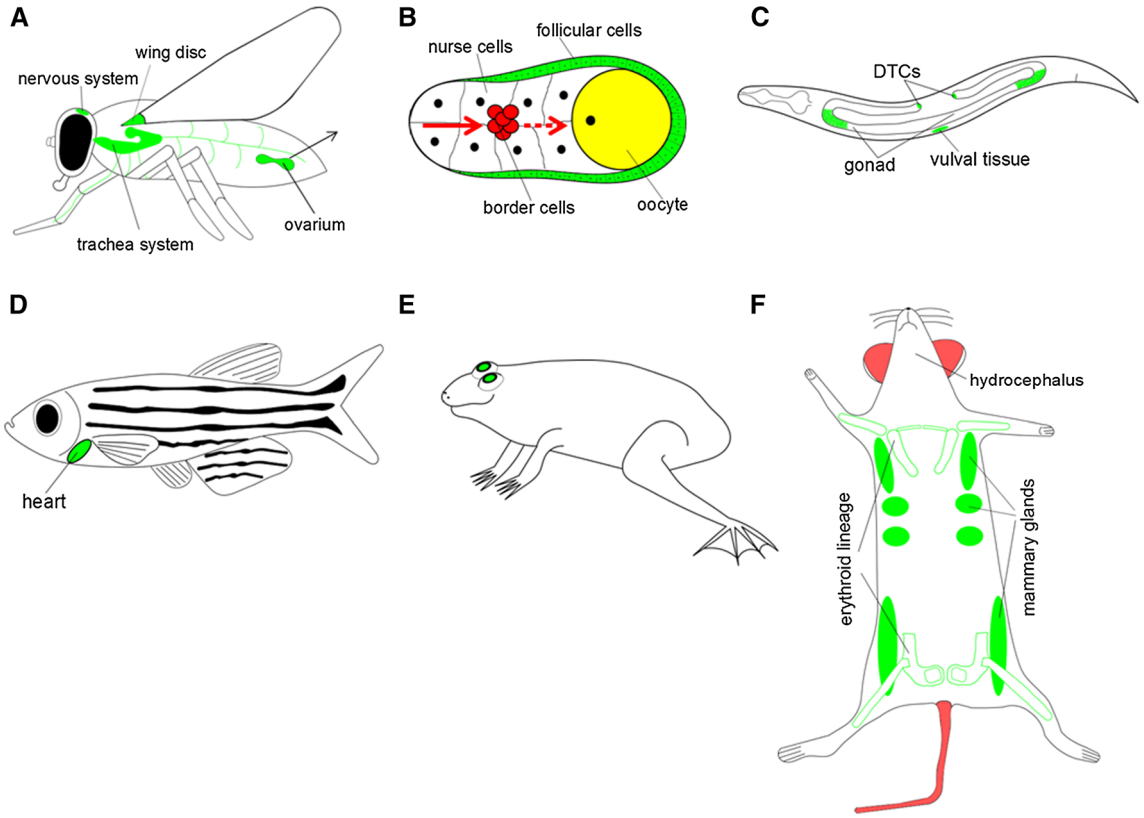

Fig. 1 NDPK-related developmental functions in different model systems. Note that many signaling events, where the functions of NDPKs were observed actually operate during embryogenesis or later developmental stages, but the locations of relevant cells (or their descendants), tissues or organs are shown here schematically in the appropriate adult organism. *Green* labeling indicates the site of NDPK's function. **a** The *Drosophila* NDPK homolog AWD is involved in the development of imaginal discs, also in the wing disc. The absence of *awd* function leads to defects in neurotransmission, ectopic migration of tracheal cells and female sterility. Thus, AWD's function is linked to the nervous system, the developing trachea and the ovarium (detailed in **b**). **b** The fly egg chamber is composed of the germ cell complex (nurse cells and the oocyte), which is surrounded by follicular (epithelial) cells. Throughout *Drosophila* oogenesis, *awd* is expressed in follicular epithelial cells to ensure epithelial integrity. A group of follicular cells delaminating from the epithelium called border cells (marked by *red*) migrate towards the oocyte to form the micropyle. In the border cells *awd* expression is downregulated. **c** In *C. elegans*, NDK-1 functions during vulval and gonadal development, as well as participates in the migration of distal tip cells (DTCs) and engulfment of apoptotic germ cell corpses. **d** Knockdown of *NME2* in zebrafish embryos leads to decreased cardiac contractility and impaired vessel formation. **e** Frog NM23-X4 is needed for retinal development. **f** *Nme1*$^{-/-}$ mutant female mice display nursing disability because of growth retardation of the mammary glands and defects in the final step of mammary duct maturation of the nipple. *Nme2*$^{-/-}$ deficient mice have no obvious defects, but show an altered immune response, reduced Ca^{2+} reuptake in the kidney and impaired pathological angiogenesis. *Nme1*$^{-/-}$/*Nme2*$^{-/-}$ double knockout mice die perinatally due to failure of erythroid lineage development. *Nme7* knockout mice present defects, like hydrocephalus, which can be linked to impaired motility of cilia causing abnormal ependymal flow

nucleoside diphosphate kinases [32] also recognized the fly AWD protein. Correspondingly, *awd* loss-of-function mutant larvae displayed dramatically decreased NDPK levels detected by the bovine NDPK-specific antibody, as compared to wild-type flies [33]. Second, from *D. discoideum* an NDPK-like enzyme was isolated, which showed 60 % sequence identity with AWD [34], and later was used as a source to crystalize hexameric cellular form of NDPK. All these data suggested that AWD, similar to its human and social amoeba counterparts, possess NDPK activity. It was also demonstrated that AWD provides the main source of NDPK activity (98 %) in *Drosophila* embryos [33].

NDPKs are thought to exert their nucleoside diphosphate kinase (phosphotransferase) activity through a high energy N-phosphate linkage on histidine118 (histidine119 residue in AWD). The Shearn laboratory was the first to demonstrate in vivo that histidine119 is key to one of AWD's functions, as *awd* loss-of-function mutants can only be complemented by a wild-type *awd* cDNA-containing construct, but not by a transgene encoding the H119A allele devoid of the N–P link [35]. Interestingly,

larval lethality of *awd* mutants could be completely rescued by the more ancient human *Nm23-H2*, but not its closely related *Nm23*-H1 isoform that is thought to have arisen by subsequent gene duplication. In the 1990s, it was eventually realized that *K-pn* in fact is an allele of *awd* (and was hence renamed $awd^{K\text{-}pn}$), which carries a proline to serine substitution in position 97 (which corresponds to P96 in NM23-H1/2) that ensures structural integrity of the multimeric forms of NDPK [36, 37]. It is thought that the *K-pn* (P97S) mutation lies in a mutant loop that imparts an increased flexibility but reduced stability to NDPK protein's hexameric quaternary structure. This might enable the protein to interact with additional partners and explain the complexity of the phenotype-mutation axis that differentially perturbs proliferation and differentiation (see *Xenopus* below). This is also consistent with the idea that $awd^{K\text{-}pn}$ might be a neomorphic allele. Although the Prune/NM23-H1 interaction was confirmed in human systems [38, 39], the molecular mechanism of how a single $awd^{K\text{-}pn}$ allele is able to confer lethality to *prune* mutants remains an unresolved issue.

AWD regulates neurotransmission through its endocytic function

Ramaswami's laboratory was interested in identifying genes that regulate neurotransmission at synaptic junctions using forward genetic approaches [40]. In synaptic vesicle recycling and cellular trafficking, endocytosis plays a key role. Pioneering work in Ramaswami's laboratory revealed that AWD/NM23 is an essential component of endocytosis: AWD was identified as a factor required for dynamin-dependent synaptic vesicle recycling [40]. Dynamin is a GTPase functioning in a later stage of endocytosis in eukaryotic cells where it pinches off the nascent vesicle by constricting its neck. *Drosophila* mutants carrying a thermosensitive allele of *dynamin/shibire* are paralyzed at the restrictive temperature due to defective uptake of neurotransmitters at the neuromuscular junctions. Genetic screens were conducted for mutations, which enhance the phenotype of *shibire* (*shi*) mutants. The thermosensitive *shi* mutant flies were further mutagenized at the permissive (lower) temperature, and second-site mutations causing paralysis at lower temperatures were looked for [40]. Remarkably, three alleles of *awd* were isolated in this screen. They confirmed the genetic interaction, using existing *awd* alleles: these mutations also enhanced the *shi* phenotype, but the histidine119 (H119A) mutant could not. Together, these data suggest the existence of a highly specific interaction between *dynamin/shibire* and *awd*.

Very recently, the group of Mathieu Boissan [41] has explored the specific interaction between dynamins and nucleoside diphosphate kinases at the molecular level during membrane remodeling events. Dynamin superfamily proteins are molecular motors, which use GTP as energy source. NDPKs locally fuel GTP towards dynamins, thus these motor proteins are able to work with high thermodynamic efficiency [41].

AWD's function in epithelial tube morphogenesis during trachea development

Using tracheogenesis as a model of tube generation, the laboratory of Tien Hsu was initially interested in characterizing the function of the *Drosophila* von Hippel-Lindau (VHL) tumor suppressor homolog during development of the fly respiratory system [42]. Germline inactivation of the *VHL* gene is associated with an increased risk of renal cell carcinoma [43]. As renal cancer has a tubular origin, and the *Drosophila* trachea is a tractable epithelial tubule system, the fact that dVHL mutants exhibit a tracheal defect is highly interesting. Development of the fly trachea is both linked to matching oxygen demand with supply, and is used to model tubular morphogenesis (also called branching morphogenesis; reviewed in [44, 45]). In brief, during early stages of embryogenesis tracheal placodes (also termed sacs) arise through invagination of specialized clumps of ectodermal cells expressing certain transcription factors. Subsequently, branches are formed from these invaginating placodes. After elongation of branches, some of them fuse to generate an interconnected network of the tracheal tubes. Importantly, immediately post-placode formation, cell division ceases and branching morphogenesis happens as a series of coordinated cell migration events. FGF (fibroblast growth factor)/FGFR (fibroblast growth factor receptor) signaling is a key chemotactic system that drives coordinated tracheal migration: tracheal cells express (transcription-factor-induced) breathless/FGFR, while the target tissues ahead of the advancing tube provide the ligand, branchless/FGF. The combination drives cytoskeletal remodeling of the advancing tracheal tips as they penetrate into an increasingly hypoxic tissue.

The Hsu laboratory reported AWD as an interaction partner of *Drosophila* VHL in a yeast two-hybrid screen; later this interaction was further investigated and VHL, which encodes an E3 ubiquitin ligase, was found to stabilize the AWD protein through its non-canonical function [46, 47]. Detailed analysis of *awd* loss-of-function mutants showed ectopic tracheal migration accompanied by over-accumulation of Breathless/FGFR on tracheal cell surfaces [48]. As the *awd* mutant phenotype was exacerbated in the *shi/dynamin* mutant background [48], it was concluded that AWD regulates tracheal cell motility by modulating FGFR levels through a dynamin-mediated pathway (Fig. 1a). Thus, this study reaffirmed the endocytic function of AWD and suggested that internalization of membrane-bound

FGFR is the mechanism, whereby AWD and dynamin are able to downregulate FGFR levels on tracheal cell surfaces resulting in suppression of directed cell migration (Fig. 2a). A plausible explanation was proposed for NDPK's potential involvement in metastasis inhibition: NDPK/AWD activity facilitates the downregulation of activated growth factor receptors via receptor internalization, whereas the absence of NDPK function causes increased levels of cell surface receptors and results in oversensitised cells to chemotactic signals and elevated metastatic potential.

AWD regulates border cell migration, and is essential for epithelial integrity during *Drosophila* oogenesis

The function of AWD was further investigated in another migrating cell type of epithelial follicular origin, a cluster of co-migrating invader cells found within ovaries, called border cells. Border cell migration during fly oogenesis is considered as an important model of vectorial epithelial cell migration [49], and, in addition, as a model of epithelial-to-mesenchymal transition (EMT) [50]. The *Drosophila* egg chamber is composed of the germ cell complex (nurse cells and the oocyte), which is surrounded by follicular (epithelial) cells (Fig. 1b). These outer lining cells ensure the integrity of the egg chamber and also provide positional information to the developing oocyte. AWD is expressed in these follicular epithelial cells throughout oogenesis (Fig. 1b), but not in the border cells despite their derivation from the same follicular cells [51]. This suggests cell-type-specific regulation of AWD levels. Border cells are a group of 6–10 anterior follicular cells, which delaminate together and move away from the epithelium during oogenesis, invading

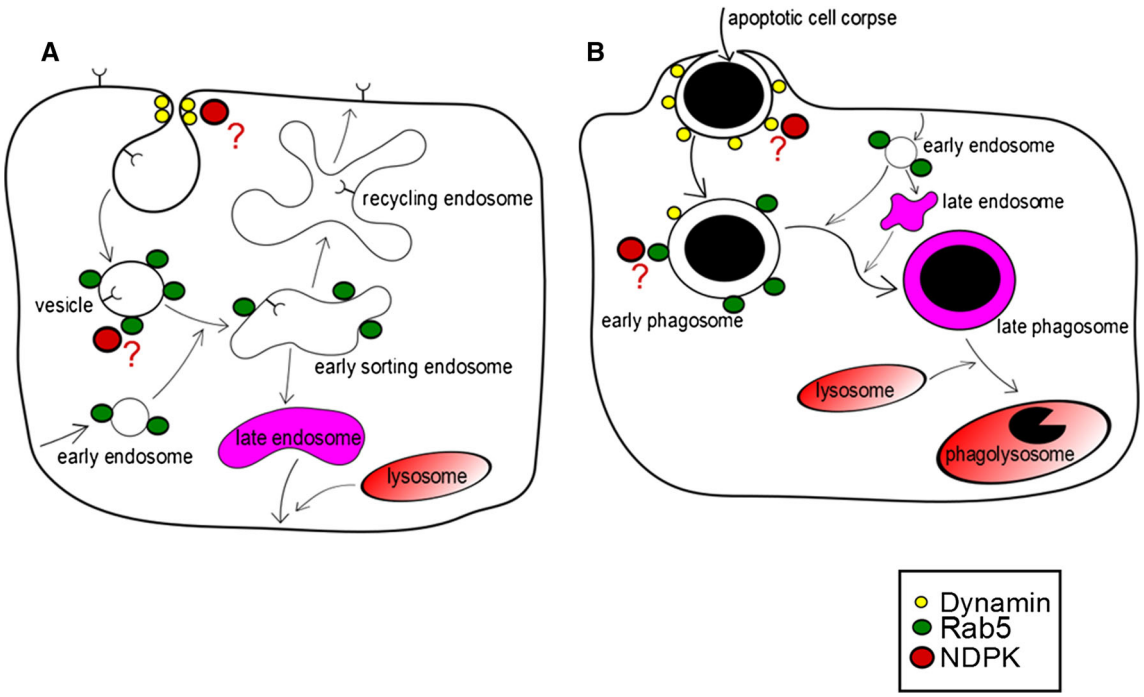

Fig. 2 NDPKs in endocytosis and vesicular trafficking. Dynamin is necessary for membrane remodeling events. NDPKs (*Drosophila* AWD and *C. elegans* NDK-1) show a genetic interaction with dynamin and are proposed to supply GTP for dynamin. In both cases (**a**, **b**), the release of endocytic vesicles/phagosomes from plasma membrane is related to dynamin's membrane fission activity, and then Rab5 GTPase promotes the fusion of these vesicles/early phagosomes with early endosomes. **a** During *Drosophila* development levels of cell surface receptors levels on migrating cell types (for example, FGFR on tracheal cells) are regulated through receptor internalization. Dynamin's membrane fission activity is required for the release of receptor-containing endocytic vesicles from plasma membranes. Rab5 then promotes the fusion of these vesicles with early endosomes. AWD is proposed to act in these processes: it interacts genetically with dynamin and its activity is required for Rab5 function in early endosome formation. Early endosomes communicate with both lysosomes and recycling endosomes: their cargo will be either degraded in lysosomes or recycled back to the plasma membrane. **b** In *C. elegans*, DYN-1/dynamin is involved in both engulfment and clearance of apoptotic cells. NDK-1/NDPK shows a genetic interaction with DYN-1. During apoptotic corps removal, the dying cell (such as a germ cell in the worm), also called apoptotic corps is first engulfed by a neighboring cell (gonadal sheath cell). DYN-1 organizes vesicle transport and promotes fusion of early endosomes to ensure the extension of pseudopods. Recruitment of the early endosomal marker RAB-5 also relies on DYN-1. DYN-1 is also essential for the degradation of apoptotic corpses, through its role in phagosome maturation: DYN-1 is necessary for recruitment and fusion of endosomes and lysosomes to phagosomes. The exact relationship of NDK-1/NDPK, DYN-1/dynamin and the GTPase RAB-5 is not yet known

through the adjacent nurse cells within the ovary until they reach the anterior of the oocyte. Border cells, on arrival at their final position, have an essential function in forming the opening of the micropyle, a structure necessary for future sperm entry. Border cell migration is regulated by the *Drosophila* PDGF (platelet derived growth factor)/VEGF (vascular endothelial growth factor) signaling (the PDGF homolog ligand, Pvf comes from the oocyte and is received by the Pvr receptor on the border cells) [52, 53]. The JAK (Janus kinase)/STAT (signal transducer and activator of transcription) pathway is also involved [54], and other factors beyond the scope of this review such as integrins and EGF are also important for border cell movement. Nallamothu and colleagues showed that AWD, expressed in follicular cells, functions as a negative regulator of cell migration because it downregulates the cell surface receptors Pvr and Domeless, receptors of the PDGF and JAK/STAT pathways, respectively, most probably through receptor internalization in cooperation with Shi/dynamin, similar to the regulation of FGFR levels in tracheal cells discussed above. Thus, to drive border cell migration, AWD expression is downregulated in border cells (Fig. 1b). Conversely, ectopic expression of AWD causes stalled migration of border cells [51].

AWD is expressed and functions in the follicular cells of the egg chamber during oogenesis (Fig. 1b). This expression pattern of AWD is consistent with the work of the Shearn laboratory (discussed earlier), as the ovarian sterile phenotype of *awd* mutants originates from defects of the follicular epithelial cells. The Hsu laboratory demonstrated that AWD is required to maintain epithelial integrity [55]. Lack of AWD and excess AWD activity both disturb epithelial integrity. In the absence of AWD activity, accumulation and spreading of adherens junction components, such as *Drosophila* E-cadherin, beta-catenin and alpha-spectrin, were observed, which resulted in disintegrated epithelial structure (piling up of epithelial cells). Overexpression of *awd* also perturbs epithelial characteristics, but in a different manner. Epithelial cells overexpressing *awd* lose adherens junction components on their surface and undergo a morphological change: they start to adopt a spindle-like shape instead of the epithelial polygonal morphology. This morphological change is a hallmark of EMT, indicating that overexpression of AWD has a proto-oncogenic effect in follicular epithelial cells. Together, these data are also consistent with the idea that an optimal level of AWD is needed to appropriately regulate the turnover of adherens junction components through its endocytic controlling function towards multiple receptors. Consistently, a subtly regulated turnover of dynamin is also necessary to maintain epithelial integrity [56, 57].

AWD is required for Notch signaling in follicular and wing disc cells

Next, the Hsu laboratory linked AWD's function to the Notch pathway [58]. Endocytosis and endosomal trafficking are required for proper processing of Notch signaling and intracellular trafficking in both *Drosophila* follicle cells and imaginal discs. In homozygous *awd* mutant follicular cells and wing disc cells, which were generated by genetic mosaic techniques, Notch signaling was found to be defective. The authors observed that loss of *awd* function blocks Notch signaling by altering the receptor processing after the S2 cleavage step that normally liberates an intracellular Notch signal to the nucleus. Defective AWD causes Notch accumulation in early endosomes, but acting beyond dynamin on this occasion. Thus, another (a later) endocytic step at which AWD is necessary has also been identified: AWD functions in early endosome maturation by influencing Rab5 function. This adds further complexity as follows: AWD, a GTP-GDP balancing enzyme is linked to GTPase function as both dynamin and Rab5 proteins are GTPases. However, it must be noted that they are two very different GTPases with different biochemical properties. Rab5 is a small GTPase with high affinity for GTP and a low basal GTP hydrolysis rate whereas dynamin is a large atypical GTPase with very low affinity for GTP and a high basal GTP hydrolysis rate.

The worm NDK-1: the *C. elegans* group I NDPK promotes activation of the Ras/MAPK cascade and participates in DTC migration and apoptotic engulfment

NDK-1/NM23H1/H2 is required for full activation of Ras signaling in the nematode *C. elegans*

In the genome of *C. elegans*, three NDPK paralogs were identified by BLAST searches [27]. The open reading frame (ORF) *F25H2.5* shows high sequence similarity to group I NDPKs, therefore it was named *ndk-1* (*C. elegans* ortholog of *nucleoside diphosphate kinase-1*; [59]) whereas the other two paralogs (ORFs *F55A3.6* and *Y48G8AL.15*) belong to the more divergent group II. Functions of the single group I NDPK homolog NDK-1 in Ras signaling have recently been characterized by our laboratory [59]. *ok314* is a deletional allele of *ndk-1*, which removes the entire ORF. 50 % of *ndk-1*(*ok314*) homozygous mutants die as embryos, but the remainder develop into sterile adults, which display a protruding vulva phenotype (Fig. 1c).

First, the vulval phenotype of viable *ndk-1*(–) mutants was examined. Four signaling pathways including the RTK

(receptor tyrosine kinase)/Ras/MAPK (mitogen activated protein kinase), Notch, Wingless/Wnt and synMuv (for synthetic Multivulva) molecular systems are involved in the development of the hermaphrodite reproductive organ, the vulva, which is an intensively studied example of animal organogenesis [60]. The EGFR/Ras/MAPK pathway plays an essential role during vulval development, as an EGF ligand emanating from a specific cell of the somatic gonad, called the anchor cell (AC), provides an inductive signal for a subset of epidermal blast cells, the six vulval precursor cells (VPCs, also called P(3–8).p). At the early L3 larval stage, the three VPCs closest to the AC, P(5–7).p, receive the inductive signal, which activates the EGFR/Ras/MAPK pathway in these cells (reviewed in [60, 61]). P6.p, the VPC closest to the AC, adopts the primary vulval fate due to LET-23/EGFR activation at high levels. In the neighboring VPCs, P5.p and P7.p, LET-23/EGFR activation occurs at lower levels, and these VPCs adopt the secondary vulval fate as a result of lateral signaling mediated by the LIN-12/Notch pathway. Each induced VPC divides three times, generating 22 daughter cells at the L4 larval stage. These cells undergo cell migration events, and fuse into 7 toroids to form the adult vulval structure. Mutations causing reduced EGFR/Ras/MAPK signaling lead to a Vulvaless phenotype, whereas increased Ras signaling causes a Multivulva phenotype (multiple ventral protrusions, reviewed in [61]). Protruding vulva (Pvl) mutants display a single protrusion, which is caused by eversion of the vulval tissue [62].

Using specific markers which label individual vulval cell types, it was demonstrated that the number of vulva cells in *ndk-1*(*ok314*) mutants is decreased, as compared with the 22 cells observed in the wild type. Misspecification of both primary and secondary vulval cell fates occurred in *ndk-1*(*ok314*) mutants [59]. In addition, one of the markers examined, *egl-17*/*FGF*, is a known MAPK target [63], showing reduced expression in P6.p granddaughter cells in the *ndk-1* mutant background, suggesting that Ras/MAPK signaling is dampened in the absence of NDK-1. Next, the epistatic relationships of *ndk-1* with Ras pathway components were established to further understand the mechanisms by which NDK-1 influences Ras/MAPK signaling. Epistasis analysis revealed that *ndk-1* affects vulval patterning downstream of *lin-45*/*Raf* but upstream of *mek-2*/*MEK* and *mpk-1*/*MAPK* [59]. Concordant with these results, a dramatic decrease was detected in activated phospho-MAPK levels in somatic tissues of *ndk-1* knockouts, whereas total MAPK levels were unchanged, as compared with wild-type animals. These data suggest that NDK-1 is necessary for full activation of MAPK signaling in somatic tissues of the worm. Because the scaffold proteins KSR-1/KSR-2 (kinase suppressor of Ras) are indispensable for MAPK activation [64], and because several studies on human cell lines have linked NM23's function to KSR scaffolds [65, 66], *ndk-1*(–);*ksr-1*(–) and *ndk-1*(–)*ksr-2*(–) double knockouts were generated to test potential genetic interactions between *ndk-1* and *ksr* genes. In these double mutants, *ndk-1* single-mutant phenotypes were enhanced. For example, embryonic lethality of homozygous *ndk-1*(–) mutants (50 %) became fully penetrant in *ndk-1*(–) *ksr-2*(–) double mutant background. NDK-1 was also shown to directly interact with worm KSR-2 and murine KSR-1 by in vitro pulldown assays.

Thus, a previously proposed link between an NDPK protein and KSR scaffolds [65, 66] was confirmed in vivo [59]. However, as NDK-1 is necessary for proper MAPK activation, one can conclude that it promotes the activation of Ras/MAPK signaling. In human studies, however, NDPKs were shown to attenuate Ras/ERK signaling through phosphorylation of KSR scaffolds [65, 66]. Moreover, cancer invasion induced by NM23-H1 silencing was reverted by a pharmacological inhibitor of the Ras/ERK pathway, further showing an inverse relationship between NDPK activity and Ras/ERK activation in human [67]. Structural differences in worm and mammalian KSRs might lead to altered regulation of these scaffold proteins [68, 69]. Furthermore, the mechanisms of Raf activation, which is proposed to occur through Raf dimerization [70], might not be evolutionary conserved as the nematode possess only a single B-Raf homolog whereas in mammalian systems, 3 Raf isoforms are present [69].

NDK-1 regulates distal tip cell migration and apoptotic engulfment

Defects in NDK-1 functions also cause abnormal gonadal development in *C. elegans* [59]. The viable homozygous *ndk-1*(–) mutant worms are sterile. The development of their germ cells is arrested at the mitotic phase. In addition, gonad arms in these mutants display an abnormal shape [71]. Wild-type *C. elegans* hermaphrodites possess two U-shaped gonad arms whose morphogenesis is directed by specialized leader cells called distal tip cells (DTCs), which are located at the distal edges of either arm [72]. During larval development, DTCs migrate in response to three-dimensional attractive and repulsive cues to properly form mirror image U-shaped gonad arms. DTC migration provides a tractable system to monitor genes influencing guided cell migration [72]. In a transgenic strain expressing a translational fusion NDK-1 reporter, NDK-1 accumulation was detected in the DTCs [71]. Consistent with this NDK-1 expression profile, an incomplete DTC migration was observed in *ndk-1* loss-of-function mutants; DTCs stopped their migration prematurely in the third (or dorsal) phase of migration, resulting in a "J-shaped" gonad arm [71] (Fig. 1a). In *C. elegans*, integrins also act in the third

phase of DTC migration [72, 73]. Therefore, an epistasis analysis was performed with genes involved in integrin-mediated signaling (members of the CED-10/Rac signaling pathway such as *vab-3*/*Pax6*, *ina-1*/*alpha-integrin*, *unc-73*/*Trio*, *mig-2*/*RhoG*, *ced-5*/*Dock180*, *ced-12*/*Elmo*, *ced-10*/*Rac*) and with *abl-1*/*ABL* and *abi-1*/*ABI* which have been shown to act in parallel to the CED-10 pathway [74]. Epistasis analysis revealed that NDK-1 functions downstream of CED-10/Rac and in parallel to ABI-1/ABI in DTC migration [71].

In multicellular organisms, unnecessary or dangerous cells are often eliminated by type I programmed cell death, apoptosis. Dying cells are removed by neighboring cells or specialized cell types, which engulf the apoptotic cells and destroy their debris to avoid inflammation. Pioneering studies in *C. elegans* identified and extensively characterized genes of the killing step of programmed cell death and also genes participating in cell corpse engulfment [75].

Apoptotic engulfment and DTC migration are analogous processes as both require precise reorganization of cytoskeleton and regulation of membrane trafficking/recruiting to extend cell surfaces [76, 77]. The CED-10 pathway and signaling through *abi-1*/*abl-1* regulate both engulfment and migration processes [74, 78], whereas the parallel acting CED-1 signaling participates only in engulfment. The CED-10 pathway is linked to cytoskeleton remodeling, whereas the CED-1 pathway recruits membranes to extend the surface of the engulfing cell.

The above data prompted us to examine whether apoptosis is affected in *ndk-1*(–) mutants. Indeed, an increased number of apoptotic cell corpses were measured in the germline of *ndk-1*(*RNAi*) animals and in *ndk-1*(*ok314*) embryos, as compared to wild-type animals [71]. Furthermore, NDK-1 accumulation was detected in sheath cells around dying germ cell corpses (Fig. 1a). Gonadal sheath cells are specialized engulfing cells, which eliminate neighboring germ cell corpses [79]. Analysis of the number of apoptotic corpses in *ced-10*(–), *ndk-1*(–) single mutants and *ndk-1*(–); *ced-10*(–) double mutants led to the conclusion that NDK-1 functions downstream of CED-10/Rac, i.e., in the process of apoptotic engulfment as well. In addition, a genetic interaction was observed between *ndk-1* and *dyn-1*/*dynamin* (e.g., single mutants show phenotypic similarities, moreover *ndk-1*; *dyn-1* double mutants are not viable). *dyn-1*/*dynamin* is a downstream effector of the parallel acting CED-1 pathway, and its *Drosophila* homolog, *shibire*, shows a well-known genetic interaction with *awd*.

Together, these results suggest that NDK-1 plays a role in DTC migration and engulfment of apoptotic cells by influencing the CED-10 pathway. Moreover, NDK-1 interacts with the CED-1 pathway through DYN-1 in engulfment through DYN-1. Thus, this study is the first to link an NDPK to apoptotic engulfment [71] (Fig. 1c) and consistent with a large body of data confirming that NDPK and dynamin are ubiquitous functional partners across model systems.

NM23-X4 function in *Xenopus* retinal development

Cyclins control kinases that in turn regulate the cell cycle, but are themselves controlled by a family of inhibitor proteins, the cyclin-dependent kinase inhibitors (CDKIs) [80]. During frog retinal development, stem cells generate the neuronal precursor cells that exit the cell cycle immediately before their terminal and cell-fate determination step [81, 82]. Next, these cells differentiate to determine the final composition of mature retinal cells of different subtypes. Thus, the terminal cell cycle before cell specification of pluripotent precursors is when CDKI proteins become key players in determining a given cell's fate irrespective of the nature of the final retinal cell types (i.e., irrespective of whether that cell will become neuronal or glial).

The emerging concept is that at a given time during development of the retina, once a set of factors (determinants) have accumulated to a threshold [83], this specifies which amongst many of the final retinal components are to be preferred. CDKI family members promiscuously enhance that role in phenotypic choice. For example, in mouse retina, deletion of Math5 [84], a driver of retinal ganglion cell fate, upregulates one CDKI protein, which then creates a glial alternative (Muller cell), i.e., non-neuronal cell fate. Such effects are driven by interaction with the N-termini of such CDKI family members, although their C-termini are equally important but play a different role, in which they interact with both the actin cytoskeleton and microtubular structures to drive differentiation. In summary, there exists promiscuity of action in these CDKI molecules towards a given cell fate depending on other factors (the cell context) [85].

To further understand the context relationship between cell cycle exit from G1, subsequent cell-fate determination and the contemporaneous regulation of CDKI components, Shin-Ichi Ohnuma and colleagues [86] tested the hypothesis that there may be other proteins that bind to the N-terminus of this regulator [14]. They used a bacterial 2-hybrid screen of the N-terminus of the CDKI p27XiC1. Since p27XiC1 is a low abundance protein that is normally rapidly degraded, they also inhibited the proteasome to raise CDKI levels finding that frog NM23-X4, X3, the human NM23-H1 and NM23-H4 all interacted with this CDKI (and with only some of the three human CDKI-equivalents, suggesting specificity of actions). Overexpression of NM23-X4 (less for other isoforms) all inhibited the pro-gliogenesis proclivity of p27XiC1, but inactive

NM23 mutants that could not transfer p-histidine were unable to act as CDKI inhibitors. Interestingly, the *K-pn*-equivalent mutant, a mutant lying remote from the catalytic site but important for the quaternary stability of NDPK multimers, inhibited just as well as the wild type, but this mechanism of action may not be dependent on CDKI(s) per se because overexpression of NM23-X4, when the CDKI was pre-suppressed, retained the glial promoting effect [85, 87]. Interestingly, overexpression of NM23 in the absence of CDKI potentiates gliogenesis, suggesting that NM23 has also ability to modulate gliogenesis in a CDKI independent manner (Fig. 1e).

The *Dictyostelium* NDPK is implicated in macro- and micropinocytosis

To further explore the relationship between NDPK, cell migration, responses to nutrient supply, and substrates available to the developing organism, Fisher and colleagues applied the tractable social amoeba *Dictyostelium*, exploiting its unparalleled variety of cell types and cell morphology that are all amenable to genetic manipulation [88, 89]. This soil amoeba can exist either as single cells that each engulf bacteria/soluble nutrients or can form multicellular aggregates (primitive pre-cadherin epithelial coated slugs) that undergo directional, i.e., cue-driven vectorial mobility [88, 89]. When induced to starve by either mitochondrial depletion or by inducing a false starvation signal generated by stably expressing a constitutively overactivated form of the cellular fuel sensor, AMP-activated kinase (AMPK), the directional cue in response to light (but not the speed) of the motile slug form was abolished [89]. The consequent 'blind' random slug motion implied a sensor defect. However, light cue-dependent vectoring was restored by concomitant NDPK knockdown, suggesting that NDPK is a latent but positive regulator of an energy depleted state. Slugs use the front of their rounded transparent cell bodies to focus light, and this primitive 'lens' refracts light towards the back of the slug body as a means to navigate from dark soil to the light at the soil surface. At the soil surface, the aggregate slug can differentiate into a multicellular stalk and head structure that will make spores at its distal tip, to be carried in the wind to complete its life cycle [90]. Importantly, vectorial motion to light was not the only perturbed function during induced starvation. As with the retinal fate, during starvation cell differentiation was also profoundly altered with structural anomalies (abnormal head and stalk of the fruiting body), and once again, concomitant NDPK knockdown reversed the mal-differentiation observed in the starved state to an outwardly normal morphology [89]. Importantly, NDPK knockdown alone in the fully fed state could neither alter vectorial motion nor change gross cell morphology during differentiation. This interesting observation is consistent with the roles of NDPK in hypoxia responsiveness, nutrition and cell-fate specification [81]. Next, these authors studied nutrient uptake in single *Dictyostelium* cells either plated onto a flat-bed on top of bacterial lawns as a food source (macropinocytosis assay) or, by placing these single cells in a liquid environment, exposed to micronutrient broths of soluble factors such as amino acids (axenic culture) [88]. In axenic culture, NDPK knockdown alone now created a phenotype (despite substrate availability) whereby endocytosis and exocytosis of recycling vesicles budding from the plasma membrane were both unexpectedly accelerated, and yet these NDPKs knocked down single cells failed to retain sufficient imbibed nutrients and consequently displayed slower growth. Since NDPK/AWD is a very important regulator of GTP supply to enhance dynamin function (*shi* function, in flies) [48], the authors concluded that nutrient retention within the single cell interior was perturbed by lack of NDPK. Their measured reduction in the mass of nutrient accumulation implies a deficit in nutrient transfer from the plasma membrane to the machinery within the cell interior that utilizes imbibed nutrients. However, in the case of single cells exposed to two-dimensional bacterial lawns, the exact opposite was observed whereby NDPK knockdown increased the rate of phagocytic bacterial uptake (and hence enhanced growth rate). Thus single cells confined to a two-dimensional surface use a process that is negatively regulated by NDPK whereas single cells in suspension culture use NDPK to balance nutrient entry by micropinocytosis against cell growth. Once again, context is key for NDPK function.

NDPK knockout mice point to roles of different NME proteins in erythroid lineage development, $CD4^+$ T cell activation, cardiac contractility and ciliar functions

The mouse genome encodes 8 NDPKs, which constitute 2 groups similar to human: NM23-M1-M4 isoforms belong to group I NDPKs, whereas group II is composed of M5–M8 isoforms [91]. Mouse and human orthologs of *Nm23* family genes are strongly conserved: the structure of the orthologous genes, their expression pattern in different tissues and primary structure of the encoded proteins are all very similar. Human NM23-H1 and NM23-H2 show 94 and 98 % sequence identity with their mouse counterparts, respectively. Active homo- and heterohexamers are composed of NM23-M1 and NM23-M2 subunits, and the M1 and M2 isoforms are responsible for 80 % of the cell's NDPK activity [92].

Constitutive knockout mice have been generated for *Nm23-M1* (also known as *Nme1*; [93]), *Nm23-M2* (also

known as *Nme2*; [94]), a double knockout of *Nme1* and *Nme2* [95], and finally, *Nm23-M5* (*Nme5*) and *Nm23-M7* (also known as *Nme7*; [96]). Mice deficient for either NME1 or NME2 are able to develop until adulthood without gross developmental defects, however, *Nme1*$^{-/-}$ mice obtained by Arnaud-Dabernat and co-workers [93] display a mild hypotrophy. Further studies on *Nme1*$^{-/-}$ knockout mice have shown that mutant females cannot feed their pups due to growth retardation of the mammary glands and defects in the final step of mammary duct maturation of the nipple leading to duct obstruction [97] (Fig. 1f).

Experiments using *Nme1* knockout mice in the laboratory of Marie-Lise Lacombe brought the strongest evidence for the metastasis suppressor effect of NME1. When *Nme1*$^{-/-}$ mice were crossed with mice prone to develop liver tumors (ASV mice expressing the SV 40 large T antigen in the liver leading to hepatocellular carcinoma), the double transgenic mice showed a higher incidence of lung metastases compared to the single mutant and the single transgenic strains [5]. Interestingly, in *Nme1*$^{-/-}$ mice, subjected to partial hepatectomy, liver regeneration was not affected, suggesting either that NME1 is not involved in this process or another NDPK paralog compensates for the absence of NME1 during liver regeneration [67].

Nme2$^{-/-}$ mice were generated by the laboratory of Ed Skolnik [94]. These knockout mice are phenotypically normal at birth and have a normal life span. The absence of NME2 influences the function of the immune system: although T and B cell development is normal in these mice, K^+ channel KCa3.1 activity and cytokine production are defective in T helper (Th)1 and 2 cells. The Skolnik group showed in a previous study that activation of the K^+ channel KCa3.1 is required for Ca^{2+} influx and the subsequent activation of B and T cells, therefore the proper function of NME2 is critical for activation of $CD4^+$ T cells. KCa3.1 channel activation occurs by phosphorylation: a histidine residue located in the carboxyl terminus of KCa3.1 is directly phosphorylated by NME2 through its histidine kinase activity [98]. Interestingly, the KCa3.1 channel was recently linked to invasion in glioma, where high KCa3.1 expression is associated with poor patient prognosis [99]. Moreover, KCa3.1 channel activation is also implicated in proliferation of vascular smooth cells, linking NDPK dependent activation of the channel to atherosclerosis and vessel re-stenosis.

Nme1$^{-/-}$/*Nme2*$^{-/-}$ double knockout mice were generated by Edith Postel and colleagues [95] by targeted deletion of both adjacent genes on chromosome 17q21.3. *Nme1*$^{-/-}$/*Nme2*$^{-/-}$ newborn mice are three to five times smaller than wild-type mice, display hematological phenotypes including severe anemia, impaired maturation of erythrocytes, and die perinatally. These pleiotropic phenotypes show that NME1 and NME2 functions are necessary for erythroid lineage development (Fig. 1f), and it is suggested that the absence of both NM23 isoforms leads to insufficient triphosphonucleoside precursors for DNA and RNA synthesis in these double mutant mice. Tissue-specific targeted deletion is clearly needed to understand this issue further.

Mouse embryo fibroblasts (MEFs) from *Nme1*$^{-/-}$/*Nme2*$^{-/-}$ mice and zebrafish have been used by Tom Wieland and colleagues to investigate the role of NME2 in heterotrimeric G protein signaling. The authors found that NME2 forms a complex with the G beta-gamma complex (Gβγ) [100] and directly passes its high energy phosphate onto Gβ. The high energetic phosphate on Gβ induces G protein activation without the need for receptor-ligand interactions on the cell surface [100, 101]. Thus, histidine phosphorylation of Gβγ dimers by NME2 can activate G proteins independently from GPCRs. In vitro experiments suggested that this phosphorelay can regulate Gs protein activity in cardiomyocytes, thereby stimulating cAMP synthesis and cardiac contractility through β-adrenergic receptor signaling [102, 103].

In mouse embryo fibroblasts (MEFs) derived from non-viable *Nme1*$^{-/-}$/*Nme2*$^{-/-}$ knockout mice, the absence of NME1/2 in membranes was accompanied by a gradual loss of Gβ1 and Gβ2 as well as a reduction in basal cAMP levels. Re-expression of human NME2 in KO MEFs rescued the loss in G protein content and restored basal cAMP levels [104]. The Wieland laboratory showed that heart failure is associated with similar defects and in zebrafish, where morpholino-mediated knockdown of NME2, as well as Gβ1, both resulted in dramatically reduced cardiac contractility (Fig. 1d). They suggested that decreased α-subunit abundance alters adenylyl cyclase-regulating Gs and Gi protein subfamilies [104]. These defects were also rescued by injection of zebrafish *Nme2* mRNA into morphants.

In a novel study, the Wieland laboratory identified the role of NME2/NDPK B in angiogenesis using *Nme2* deficient mice and zebrafish *nme2* morphants. They found that NDPK B depletion in endothelial cells disturbs VEGFR-2 cycling and impairs the formation of endothelial adherence junctions [105].

The expression of group II genes was described in axonemal structures of cilia and flagella [2, 106]. Constitutive *Nme5* and *Nme7* knockout mice were generated and described by Vogel and co-workers [96, 107, 108]. *Nme7* knockout mice present defects like situs inversus (a congenital condition characterized by left–right transposition of thoracic and visceral organs and associated vasculature) and hydrocephalus [108] (Fig. 1f). Both situs inversus and hydrocephalus can be linked to impaired motility of cilia.

Motile flagella and cilia normally create fluid flow over cell surfaces. Impaired ependymal flow contributes to the development of hydrocephalus, whereas the randomization of left–right body asymmetry is due to dysfunctional nodal cilia during early embryogenesis. All these data suggest that NME7 is involved in the biogenesis and function of motile cilia. Vogel and colleagues also reported that *Nme5*$^{-/-}$ mice display a pathological phenotype highly suggestive of primary ciliary dyskinesia [107].

Discussion

NDPK-related molecular activities in animal models

Nucleoside diphosphate kinase enzymes display multiple molecular functions, such as nucleoside diphosphate kinase, histidine kinase (histidine phosphotransferase), and nuclease activities, as well as DNA and lipid bilayer binding. Developmental studies on mice and zebrafish reinforce the histidine kinase activity of NME2 [19]. Activation of CD4^{+} helper T cells is impaired in the immune system of *Nme2*$^{-/-}$ mice due to the lack of NME2-mediated phosphorylation of the KCa3.1 channel [94]. Recently, in the kidney, in the process of urinary Ca^{2+} excretion a Ca^{2+}-permeable channel, the transient receptor potential-vanilloid-5 (TRPV5) channel was found to be phosphorylated and thus activated by NME2 [109]. Experiments performed on fibroblasts of *Nme1*$^{-/-}$/*Nme2*$^{-/-}$ knockout mice and zebrafish show that NME2 influences heterotrimeric G protein signaling through phosphorylation of Gβγ dimers, thereby stimulating cardiac contractility [104]. Data on the activation of heterotrimeric G proteins and different ion channels point to a role of NDPK as a histidine phosphotransferase (histidine kinase).

NME1 is ascribed to regulate the outcome of Ras/MAPK signaling through phosphorylation of a MAPK scaffold, KSR. Inactivation of KSR1 by NME1 occurs through phosphorylation of Ser392 of this scaffold protein [65]. Thus, KSR1 is also a target of NM23-H1's histidine kinase activity, which was demonstrated in different cell lines [65, 66]. In *C. elegans*, a genetic interaction was observed between NDK-1/NDPK and the worm KSRs [59]. However, NDK-1 exerted an opposite effect on the outcome of Ras signaling compared to data obtained from cell lines, probably due to altered regulation of KSRs [69]. Discrepancy between results obtained from mammalian cell lines and from the worm might be resolved if we better understand the function, structure and regulation of KSR scaffolds.

In *Xenopus*, the cyclin-dependent kinase inhibitor p27Xic1 is a cell-fate determinant of retinal gliogenesis and neurogenesis. The frog NME4 homolog NM23-X4 functions as an inhibitor of p27Xic1-mediated gliogenesis in retinal development. NM23-X4 directly binds to p27Xic1, and this binding is dependent on amino acid residues (e.g., His148 and Ser150 of NM23-X4), which are critical for kinase activity [14].

Data from *Dyctiostelium*, *Drosophila* and *C. elegans* suggest that NDPKs participate in the processes of endocytosis and phagocytosis (reviewed in [51, 71, 88] and see below in this work). The endocytic role of AWD and NDK-1 is linked to the large GTPase dynamin. In human cells it was shown that in the NM23–dynamin interaction the nucleoside diphosphate kinase activity of NDPK is important to generate GTP from GDP and to ensure the energy supply of dynamin [41].

NDPKs as signal integrators of nutrient supply

Developmental studies on the slime mold *Dictyostelium* indicated a genetic interaction between AMPK and NDPK [89], knockdown of NDPK suppressed the phenotypic defects caused by AMPK hyperactivity. AMPK signaling acts as a main sensor of cellular energy status, and also influences processes related to cellular energy levels, including autophagy and pre-conditioning to hypoxia. Moreover, activation of AMPK with drugs such as metformin has been linked to lower incidences of certain cancers [110]. In recent experiments on human bronchial epithelial cells, AMPKalpha1 and NDPK-A (NM23-H1) were found to bind each other, and catalytically active NDPK-A was essential in the transmission of an inhibitory AMPK signal towards an energy consuming process, ion transport [111]. Here, AMPK retards the activity of the cystic fibrosis transmembrane conductance regulator, CFTR, an ion channel whose dysfunction causes cystic fibrosis [112]. This CFTR inhibitory signal requires functional NDPK-A. The authors also mutated a key residue close to the active site in NDPK at Ser120, a site that is mutated in the neonatal form of neuroblastoma (S120G) [113]. They found that the S120 mutation also abrogated the inhibitory AMPK signal, as observed for the catalytically dead H118F mutant [111].

It may be pertinent that the fidelity of NDPK and AMPK interaction requires an intact S120 on NDPK that is mutated in a developmental form of neuroblastoma. One unifying idea from Lascu and colleagues is that the S120G mutation affects the quaternary structure of the NDPK hexamer; e.g., it generates an unstable molten globule form of NDPK that cannot form the highly active hexamer [114, 115].

Another study in *Drosophila* performed by Onyenwoke and colleagues [116] also established a functional relationship between AMPK and NDPK. The

researchers identified AWD/NDPK as a direct target of AMPK. The regulation of NDPK by AMPK is mediated through a phosphoserine switch on S120 residue of AWD. These authors propose that under unfavorable conditions such as starvation, AMPK inhibits ATP consumption of NDPK to conserve energy. Phosphorylation of S120 residue of NDPK blocks its functions, thus leading to inactivation of its anti-metastatic function in neuroblastoma.

Together, the relationship of NDPK and AMPK is not yet perfectly understood in different model systems, but studies in *Dictyostelium* and *Drosophila* confirm the existence of a genetic interaction between these factors. In the fruit fly, NDPK activity is directly regulated by AMPK through a phosphoserine switch but the exact mechanism remains to be determined.

Data from *Drosophila*, *C. elegans* and *Dictyostelium* converge on NDPKs' role in endocytosis and phagocytosis

In *Drosophila*, *awd* function has been extensively studied and associated strongly with endocytosis. *awd* interacts genetically with *shi/dynamin* to promote endocytosis in various tissues. In neurons, for example, *awd* facilitates dynamin-mediated neurotransmitter uptake at neuromuscular junctions. AWD also functions in migrating cell types to ensure proper migration of tracheal cells and border cells by modulating appropriate receptor levels on cell surfaces through receptor internalization. AWD-mediated endocytosis also contributes to ensure epithelial integrity of follicular cells in the egg chamber by regulating the level of adherens junctions' components. Recently, the endocytic function of AWD has been shown to be indispensible for Notch signaling. In addition, the authors suggested that AWD is required for Rab5 function during early endosome maturation.

Evidence for dynamin-NDPK interaction was also observed in *C. elegans*. The phenotypes of NDPK-defective *ndk-1*(–) and dynamin-defective *dyn-1*(–) single mutants are quite similar in the worm; the late-embryonic lethality with persistent cell corpses. Furthermore, *ndk-1*(*ok314*); *dyn-1*(*ky51*) double mutants are lethal indicating a genetic interaction between the two genes in *C. elegans* as well. NDK-1 has also been linked to apoptotic engulfment [71], which is mediated by two partially redundant pathways, the CED-10 and CED-1-mediated molecular systems. The large GTPase DYN-1/dynamin, which plays a pivotal role in various membrane-related cellular processes [117], is a downstream component of the CED-1-mediated pathway. Cells dying by apoptosis are eliminated in every eukaryotic system either by neighboring cells or by specialized cell types. Failure of this process in mammals results in inflammation [118], which favors tumor progression in certain circumstances [119]. Removal of apoptotic cells occurs in two steps: first, the dying cell is surrounded and engulfed by a so-called engulfing cell; second, the apoptotic cell (often called a corpse in the worm) is then degraded within the engulfing cell by the lysosomal system [120] (Fig. 2b). Detailed studies on *dyn-1* revealed its function in both steps of apoptotic cell removal [121, 122]. During the engulfment phase, DYN-1 ensures the rapid expansion of phagocyte membrane along the surface of dying cells by organizing vesicle transport and promoting fusion of early endosomes to extending pseudopods [121, 122] (Fig. 2b). DYN-1 is also essential for the degradation of apoptotic cells through its fundamental role in phagosome maturation: DYN-1 is necessary for recruitment and fusion of endosomes and lysosomes to phagosomes [121, 123, 124] (Fig. 2b). Recruitment of two endosomal markers, RAB-5 and RAB-7, also relies on DYN-1 activity [122–124]. RAB-5 is important for promoting the maturation of phagosomes containing apoptotic cells [124, 125].

Studies in *Dictyostelium* reinforce the role of NDPKs in phagocytosis: pinocytosis, phagocytosis and exocytosis were first linked to NDPKs in this model [88].

Recently, it was shown that NDPK supplies GTP for dynamin's functions [41]. Thus, we propose that data from *Drosophila* concerning receptor internalization and results related to *C. elegans* apoptotic engulfment imply a general role of NDPKs in endocytosis and vesicular trafficking (Fig. 2a, b). Dynamin acts as a main organizer of membrane remodeling events. The membrane fission activity of dynamin is essential for the release of endocytic vesicles from plasma membranes. Rab5 then promotes the fusion of these vesicles with early endosomes. Endosomes play a key role in endocytic pathways, as they communicate with both lysosomes and recycling endosomes. During receptor internalization, cell surface receptors are taken up into endocytic vesicles from where they are either targeted to the lysosome for degradation or recycled back to the plasma membrane through recycling endosomes, thereby regulating surface receptor levels (Fig. 2a). AWD is involved in this process in migrating cell types such as tracheal and border cells according to data obtained from *Drosophila* (Fig. 2a).

In *C. elegans*, DYN-1/dynamin is involved in both engulfment and clearance of apoptotic cells. The GTPase Rab-5 plays an essential role in phagosome maturation as it promotes fusion between early (sorting) endosomes and phagosomes (Fig. 2b). It is known that Rab5 function is dependent on dynamin activity during apoptotic cell corps removal. The exact relationship of NDK-1/NDPK, DYN-1/dynamin and the GTPase Rab5 should be further investigated during engulfment and phagosome maturation.

Commonality of developmental roles of NDPKs across model systems

As detailed above, NDPKs' functions are related to nutrient supply, energy consumption and receptor internalization through endocytosis. These processes have great importance in tumor progression as well, as they are implicated in the development of tumor vasculature. Hypoxic conditions favor the development of tumor vasculature [119] driven by VEGF/FGF signaling pathways. The multifunctional tumor suppressor VHL is linked to hypoxia sensing, as it is able to degrade the α-subunit of hypoxia-inducible factor (HIF-α) under normal physiological conditions through its E3 ligase-related function [126]. Conversely, loss of VHL function results in increased HIF-α expression and VEGF production, which in turn leads to angiogenesis [127].

VHL binds to AWD/NM23, and this evolutionary conserved interaction has been recently characterized in detail in flies and cell lines [128]. VHL and NM23 are associated with early endosomes during endocytosis of activated FGFR1, and the loss of VHL disrupts the association of NM23 and endosomes [128]. This work proposed that VHL, this time through its non-canonical function, acts as an adaptor molecule that brings NM23 to the endosome.

Together, we propose that signals sensing hypoxia through VHL and signals sensing cellular energy status through AMPK converge on NDPKs. This unifying concept awaits confirmation but fits with proposed relationships between NDPK and hypoxia sensing, and links NDPKs to nutrition and cell proliferation [81].

Clarifying the link between phagocytosis, ion transport, AMPK signaling and NDPK function requires further studies, but combined data already obtained suggest that actual NDPK function depends on cellular context (single cells versus cell–cell aggregates). This is a recurring theme for this ancient and conserved protein family.

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