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Developmental Function of *Nm23/awd* - A Mediator of Endocytosis

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Abstract

The metastasis suppressor gene *Nm23* is highly conserved from yeast to human, implicating a critical developmental function. Studies in cultured mammalian cells have identified several potential functions, but many have not been directly verified *in vivo*. Here we summarize the studies on the *Drosophila* homologue of the *Nm23* gene, named *abnormal wing discs (awd)*, which shares 78% amino acid identity with the human *Nm23-H1* and *H2* isoforms. These studies confirmed that *awd* gene encodes a nucleoside diphosphate kinase, and provided strong evidence of a role for *awd* in regulating cell differentiation and motility via regulation of growth factor receptor signaling. The latter function is mainly mediated by control of endocytosis. This review provides a historical account of the discovery and subsequent analyses of the *awd* gene. We will also discuss the possible molecular function of the Awd protein that underlies the endocytic function.

Keywords

Nm23; awd; prune; Killer-of-prune; *Drosophila*

Nm23 was first isolated as a cDNA clone that was down-regulated in highly metastatic derivatives of murine melanoma cell line K-1735 (1). The gene is highly conserved in the eukaryotes from yeast to mammals. In vertebrates it consists of a gene family of eight.

Nm23 has been shown to inhibit metastasis, but not primary tumor growth, in xenografts of cells from human breast cancer, murine melanoma, rat colon cancer and human oral squamous cancer (2). However, in clinical cancer samples, this correlation is much weaker. While in initial breast cancer studies reduced *Nm23* expression levels were consistent with increased metastasis (3), in later studies no clear correlation could be discerned when comparing benign tumors and metastatic cancers (4–8). It has become clear that *Nm23* plays a critical role in normal tissue functions, and both up- and down-regulation of this gene can disrupt growth and differentiation.

The function of *Nm23*

Further complicating the issue of *Nm23*'s role in tumor progression is the multiple enzymatic functions assigned to *Nm23*: (a) Nucleoside diphosphate kinase (NDPK) that transfers the terminal phosphate group from ATP to a non-ATP nucleoside diphosphate (such as GDP), through the formation of an intermediate histidine-phosphate linkage at histidine 118 (residue 119 in *Drosophila*) (2). (b) DNA binding and DNA nuclease that is involved in transcriptional regulation (9,10). (c) DNase activity that is activated by granzyme A in caspase-independent apoptosis (11). (d) Histidine-dependent protein kinase (12–14). This enzymatic activity is similar to that of NDPK but toward protein substrates (15). Some tantalizing kinase targets

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have been identified such as aldolase C and kinase suppressor of Ras (Ksr), but the *in vivo* relevance of which requires further investigation (16). It is therefore critical that the apparently complex Nm23 functions be examined in physiologically relevant model systems. The *Drosophila* model to date has provided the most extensive set of studies, in part because *Drosophila* genome encodes only one major Nm23 homologue that accounts for >98% of the NDPK activity in the larval extracts (17). Interestingly many of these *Drosophila* studies originated from completely unrelated circumstances.

The case of *Killer-of-prune*

The story of the *Drosophila* Nm23 gene is figuratively and literally colorful. The first allele of the *Drosophila* Nm23 homologue was isolated, or more accurately, stumbled upon in the 1950s. In early 1954, Alfred Sturtevant, a key early pioneer in the establishment of the genetics discipline and the famed student of Thomas Hunt Morgan, was experimenting with the X-linked, recessive *prune* mutant (*pn*), which renders the eye brownish-purple color (like a prune) when homozygous or hemizygous (see Glossary, Supplementary Table 1). These *pn* mutants are otherwise viable. The three independently arisen *pn* mutants available to him at the time all showed the same characteristics and later turned out to be insertions leading to protein null lesions (18) (see more discussion below). To demonstrate the X linkage, he crossed homozygous *pn* females with what he thought was wild-type males. All the male progenies were expected to show *prune* phenotype since they would be *pn* hemizygotes, while all females would be *pn* heterozygotes and thus showing normal red eyes (Fig. 1a). Since no other mutations were known to be present in these flies, a male showing prune eye color would indicate a *pn* mutation on its lone X-chromosome. To his surprise, he could not recover any male offspring. Subsequent analysis led him to conclude that what he thought was the wild-type stock in fact carried a spontaneous dominant autosomal mutation that caused lethality in the *pn* homo- or hemizygous background (Fig. 1b). He mapped this allele to the tip of the chromosome 3R and named this curious mutation *Prune-killer* (19), which was later renamed *Killer-of-prune*. It is worth pondering that the only reason Sturtevant was unable to recover any male offspring in his cross was that the spontaneous *K-pn* stock turned out to be homozygous. The presence of any wild-type alleles would have given rise to male progenies from his cross, albeit at a lower rate. He conducted further analysis using more flies from the same stock, both males and females, and determined that the stock was indeed uniformly homozygous for *K-pn*. But why should a spontaneous mutation over time completely out-compete the wild-type and heterozygous siblings in his stock? This may suggest that *K-pn* can confer certain growth advantages. This fact may be worth keeping in mind when considering the normal function of Nm23/Awd proteins. The identity of this mutation would not be resolved for another 30 years and by another serendipity.

The discovery of *awd*

In a totally different line of work in the 1970s and 1980s, Allen Shearn was interested in the development of imaginal discs. Imaginal discs are small epithelial structures that are set-aside during embryogenesis for future development of adult tissues, including all appendages and the eye-antenna complex. The imaginal discs have been a favorite system of *Drosophila* geneticists for studying developmental control of cell growth and differentiation. The Shearn laboratory conducted a genetic screen for late larval/pupal lethality, in the belief that among these mutations would be those regulating imaginal disc development. Indeed 20 alleles, representing 12 genes, from this screen showed disc defects (20,21). One of these alleles encodes a single 0.8-kb transcript, mapped to the distal tip of the chromosome arm 3R, and was named *abnormal wing discs* (*awd*) (21). The mutant imaginal discs, including wing, leg and eye-antenna, could not differentiate properly, and showed extensive cell death. In a more complicated organ transplant experiment, they demonstrated the role of *awd* in ovary

development. In *Drosophila* as in mammals, gonad development begins with the specification of germ cells at the very early stage of embryogenesis (22). During gastrulation in both flies and mammals, the primordial germ cells move into the embryonic proper and coalesce with somatic gonadal mesoderm to form the primitive gonads. Dearolf et al. first transplanted the premature ovaries containing both somatic cells and germ cells from mutant larvae into wild-type surrogate females of corresponding age. None of the transplanted mutant ovaries developed fully. On the other hand, homozygous mutant germ cell precursors isolated from early *awd* mutant embryos transplanted into wild-type embryos (before gastrulation) gave rise to normal ovaries and fertile gametes (20). Since in the germ cell transplant, the surrogate wild-type females provided the somatic components of the future ovaries, the observation indicates that the ovarian phenotype in *awd* mutants is the result of defects in the somatic components (follicle cells) of the ovaries. This will become apparent later (see below).

In a case of accidental but beneficial scientific convergence, the isolation and cloning of *awd* happened at about the same time Patricia Steeg identified *Nm23* as the first metastasis suppressor gene (1), and it was quickly realized that *awd* is the *Drosophila* homologue of *Nm23*, which shares 78% amino acid identity with the *Nm23-H1* and *H2* isoforms (23).

Enzymatic activity of the Awd protein

The early studies from the Shearn laboratory showed that *awd* mutant larval brain exhibited mitotic defects correlated with defective microtubule polymerization (17). In a display of remarkable insight, the Shearn group noted that Nickerson and Wells had reported the purification of a 17-kDa protein, the same size as the Awd protein, from bovine brain microtubules that exhibited nucleoside diphosphate kinase activity (NDPK) (24). Remarkably, antibody raised against the bovine NDPK cross-reacted with Awd. They went on to show that larval extracts from *awd* mutant larvae contained dramatically reduced NDPK activity (17). In a concurrent but independent study, the NDPK enzyme from *Dictyostelium discoideum* was isolated and the amino acid sequence showed 60% identity with Awd (25). All these suggested that the Nm23/Awd family of proteins is a NDPK enzyme. This notion soon proved to be true (26).

In a subsequent series of elegant studies, the Shearn laboratory showed that recombinant Awd protein contained NDPK activity *in vitro* (27); and that mutation at the presumptive active site histidine residue (H119A) abolished this NDPK activity and failed to rescue *awd* mutant phenotype when introduced as a transgene (28). Interestingly, they also found that human Nm23-H2, but not H1, could completely rescue the *awd* lethal phenotype when expressed from the *awd* gene promoter; and neither could rescue the female sterile phenotype (28). The implication is that, as suggested by the authors, the NDPK activity is necessary but not sufficient to account for all the *awd* functions. Although this notion could be explained as a failure of the human proteins to interact with all the native *Drosophila* targets, it may also suggest that activities other than NDPK are physiologically relevant.

The identity of *K-pn*

In another interesting twist, soon after they isolated the *awd* alleles, the Shearn laboratory set out to isolate deletion mutants of *awd* to serve as complete null alleles. That led them to revisit the *K-pn* mutant. Back in the 1960s, Lifschytz and Falk reasoned that the way to resolve the *K-pn* function was to isolate revertants (Supplementary Table 1) of the *K-pn* conditional lethal phenotype. They took advantage of Sturtevant's original demonstration: complete lethality of male offspring from *pn* mother and *K-pn* father. They mutagenized the *K-pn* males with X-ray, which produced chromosomal deletions, mated them with *pn* females, and recovered rare male progenies (29). These male escapers represented the *K-pn* revertants. It is important to note, from the hindsight, that this mutagenesis worked because *K-pn* is a dominant allele; that

is, one copy of (heterozygous) *K-pn* can cause lethality in *pn* mutant (Fig. 1). Therefore, loss-of-function mutation in one *K-pn* allele alone could result in viable male offspring. This also confirmed that *K-pn* is a gain-of-function or neomorphic mutant (Supplementary Table 1), since its phenotype could be ameliorated by loss-of-function mutations. However, surprisingly all attempts to generate homozygous stocks of these revertants failed, suggesting that all of the revertants were homozygous lethal (29). In addition, these revertants could not complement each other for their lethality (Supplementary Table 1). This is peculiar since non-complementation usually indicates that the alleles involved are different mutations of the same gene. Since loss-of-function *K-pn* mutation itself was expected in the revertants, could this mean all revertants they isolated by chromosomal deletion (X-ray irradiation) were new mutations in the *K-pn* gene? This seemed unlikely at the time so these authors suggested that the revertants generated by irradiation—usually large deletion mutants—uncover a few very closely linked genes including *K-pn* (so they could not complement each others; Supplementary Table 1), and that these genes are homozygous lethal. Shearn reasoned that since *K-pn* and *awd* are closely linked at the tip of the chromosome arm 3R—note that he had no reason to suspect that *awd* and *K-pn* are the same gene—he could generate at least a few new *awd* deletion alleles, in addition to the expected *K-pn* deletion alleles, by repeating the mutagenesis scheme of Lifschytz and Falk. To their surprise, all the *K-pn* revertants generated failed to complement the existing *awd* alleles (30). More importantly, *awd* cDNA cloned from the *K-pn* mutant could cause lethality in *pn* mutants when expressed as a transgene (30). Subsequent cloning confirmed that *K-pn* in fact is an allele of *awd*, which carries a proline to serine substitution at residue 97 (26, 27). This allele was renamed *awd^{K-pn}* (Supplementary Table 1). Interestingly, proline 97 is a highly conserved residue among NDPKs (26, 27, 31). The NDPK from *K-pn* is not altered in its catalytic activity, but the peptide chain is more flexible and exhibited reduced stability to heat treatment or denaturing agents (26). The increased flexibility is consistent with the neomorphic nature of the mutant, as it is possible that the mutant protein can interact with additional proteins. However, the Steeg laboratory showed that the *K-pn* mutation (P96S in Nm23-H1) retained the NDPK activity but lost the histidine-dependent protein kinase function (32), and that the mutant could not inhibit *in vitro* motility of cancer cells (33). The relative importance of NDPK and protein kinase functions of Nm23/Awd obviously requires further studies, and the enigmatic *K-pn* mutant may yet hold the key to this question.

The function of Pn

Unfortunately the functional basis of the *K-pn* phenotype has remained a mystery. What does a fruit fly eye color mutant have to do with a metastasis suppressor gene? The *pn* gene was cloned and sequenced soon after the identity of *K-pn* was revealed (34,35). Since the NDPK function is to generate non-ATP nucleoside triphosphates, notably GTP, the authors' suggestion that Pn protein may be a GTPase-activating protein (GAP) generated plenty of excitement, as this function would link Nm23/Awd to the all-important GTPases such as the Ras family of proteins or G-proteins. However, subsequent analysis did not support a GAP function for Pn. Further sequence analyses suggested that Pn belongs to a large family of phosphodiesterase (36) and that human Nm23-H1 physically interacts with human Pn (37). It was also shown that human Pn protein contains a cyclic nucleotide phosphodiesterase activity that promotes cancer cell motility (38). At the time of Sturtevant, there were only three independent alleles of *pn* (alleles 1, 2 and 3), all of which showed lethal interaction with *K-pn*. All are insertions that resulted in peptide truncations of varying lengths (18). These mutants do not produce proteins detectable by a polyclonal antibody raised against full-length protein. Most, if not all, of the *pn* mutants isolated later show lethality when combined with *K-pn*. Whether they show residual enzymatic activities is not known. Curiously, some of these mutants resulted in short peptide truncation at the C-terminus. These observations indicate that the C-terminus is critical for function or protein stability.

Another important link among Nm23/Awd, Pn, and the nucleoside diphosphate-triphosphate conversion function is the original Pn function. *pn* mutants affect *Drosophila* eye color. The *Drosophila* red eye pigment is a derivative of pteridines (drospterin). The first and rate-limiting step in pteridine synthesis is catalyzed by the enzyme GTP cyclohydrolase (GTPCH), which converts substrate GTP to dihydroneopterin triphosphate (39). It has been shown that in *pn* mutants the pteridine level is reduced and the activity levels of GTPCH becomes variable (40). Since Nm23/Awd as a NDPK is presumed to supply nucleoside triphosphate, thus driving the forward reaction catalyzed by GTPCH, the defects in *pn* mutants are consistent with the genetic data that implicate Pn as an antagonist of Nm23/Awd. The antagonistic relationship between Nm23 and Pn was later demonstrated in human cells (37,38).

Sturtevant himself offered a hypothesis in explaining the *K-pn/pn* interaction (19): "One possible hypothesis is that *pn* leads to the production (or accumulation) of some substance that is absent, or present only in small amounts, in *pn+* flies, and that K-*pn* converts this substance to a different and toxic substance. Such a hypothesis at least suggests experiments to be carried out, and offers some hope of relating the phenomena to the reactions concerned in the synthesis of pigments." This insight suggested a link to GTP metabolism and may still serve as a guide for Nm23 researchers today. No such substance has been detected so far. However, indirect evidence may hint at the validity of the hypothesis. In a series of protein analyses, Timmons and Shearn showed that the phosphorylation (activation) pattern of K-*pn* protein is not altered compared with the wild-type Awd protein (27). This observation, combined with the finding that *K-pn* mutant changed the substrate-binding cleft of the NDPK enzyme, suggested to the authors that K-*pn* may catalyze an ectopic substrate (27,41). More recently, the Shearn laboratory repeated the genetic screen for *K-pn* revertants and isolated a rare non-*awd* allele (42,43). Interestingly, this allele encodes a protein exhibiting glutathion S-transferase (GST) activity. GST is usually a detoxification enzyme but in some cases, addition of glutathion to certain substances can lead to cytotoxicity (44). Although direct biochemical evidence is lacking, the authors suggested that the GST activity is needed in the *pn-K-pn* flies to convert the hypothetical ectopic substrate into the toxic substance. Mutation of the GST function therefore rescued the *pn-K-pn* lethality.

***awd* in neurotransmission**

In another case of serendipity, a team of neurobiologists in Arizona and India were interested in using a genetic approach to solve the endocytic pathway that regulates neurotransmission at the synaptic junctions. One ingenious idea they came up with was to use a temperature-sensitive allele of the *Drosophila* dynamin gene, *shibire* (*shi*). At non-permissive temperature (29°C) the flies become paralyzed (*shibire* is the Japanese term for numbness) due to defective uptake of neurotransmitters at the neuromuscular junctions (38,45). In order to identify other players in the vesicle recycling pathway, the authors conducted a genetic screen for enhancers of the *shi* phenotypes. That is, they looked for second site mutations that would make *shi* flies pass out at a lower temperature (25°C). They isolated three such alleles and to their (and everybody else's) surprise, all three alleles were mutations of the *awd* gene (46). The authors went on to show that the existing *awd* mutations could indeed cause the same *enhancer-of-shi* phenotype, and the His119 mutant did not confer such activity. This important finding suggested a highly specific and almost exclusive functional interaction between *dynamin* and *awd*.

***awd* and epithelial tubule morphogenesis**

Our laboratory has been interested in the developmental function of the von Hippel-Lindau tumor suppressor gene (*VHL*), the human germline mutations of which predispose carriers to kidney cancer, a blood vessel tumor called hemanigioblastoma, and a number of other, less

frequent tumors (47). We had shown that *VHL* loss-of-function in *Drosophila* caused disruption of an epithelial tubule organ termed trachea (48). We subsequently found that *Drosophila* VHL protein physically interacts with Awd in a yeast two-hybrid screen (unpublished data). This prompted us to examine the possible trachea phenotype in *awd* mutants. This was an attractive idea since the trachea is an epithelial tubule organ that has been used to model the mammalian vascular system as well as other epithelial ductal tissues such as mammary gland and kidney tubules. The implications for human epithelial tumors and perhaps angiogenesis would be highly interesting.

We quickly observed that in *awd* mutants, the tubule cell migration became ectopic, and in severe cases, the epithelial cells became detached from the epithelium in a phenotype resembling epithelial-to-mesenchymal transition (EMT) (Fig. 2a) (49). Tubule migration in *Drosophila* trachea is mainly guided by the fibroblast growth factor (FGF) signaling system (50,51). The ligand FGF is produced in the target tissues distal to the migrating tips while the receptor (FGFR, encoded by the *Drosophila* *breathless* gene) is expressed in the tracheal cells. We reasoned that the ectopic migration phenotype observed in *awd* mutants might result from hyperactive chemotactic FGF signaling. And the underlying defect, taking note of the *awd-shi* functional relationship, might be over-accumulation of FGFR on the tracheal cells due to defective internalization of the receptor. This is indeed the case (49). We demonstrated a massive accumulation of FGFR both in the *awd* mutant trachea (see Fig. 2b for an example) and in cultured insect cells treated with *awd*-specific siRNA. In addition, *awd-shi* double mutants greatly exacerbated the tracheal phenotypes exhibited by the individual mutants. Conversely, when *awd* mutants were crossed into the *breathless/FGFR* loss-of-function genetic background, which reduced the dosage of FGFR, the ectopic migration phenotype was reduced.

Interestingly, in collaboration with Janis O'Donnell, we showed that the neurotransmitter dopamine could promote endocytosis in the tracheal cells (52). Dopamine synthetic pathway enzyme tyrosine hydroxylase (TH) requires the cofactor tetrahydrobiopterin, which also relies on the activity of GTPCH for biosynthesis. Therefore the cellular level of dopamine is also dependent on the function of GTPCH (encoded by the *Punch* gene in *Drosophila*) (53). We demonstrated that *Punch* mutant exhibited the same tracheal phenotype as in *awd* and *shi*, with over-accumulation of FGFR on the tracheal cell surface. Furthermore, *awd* and *Punch* double mutants resulted in exacerbated phenotype (52). Thus, *awd* and *Punch* may function in parallel to promote endocytosis of FGFR, or they can work in the same GTP-GTPCH enzymatic pathway that leads to elevated endocytosis. The latter, indirect role for *awd* is not favored in light of the strong and specific genetic interaction between *awd* and *shi/dynamin* (46). In addition, we showed that in *Punch* mutants dynamin is down-regulated and diffused in the tracheal cells (52) while we did not observe such change in dynamin expression pattern in *awd* mutants (unpublished observation).

Thus, these studies reaffirmed the endocytic function of Awd and suggested a plausible mechanism for the Nm23 antimetastasis function. That is, Nm23/Awd may be involved in internalization and turnover of growth factor receptors. In the absence of Nm23/Awd, the cells are over-sensitized to chemotactic signals, resulting in elevated metastatic potential. It would be interesting to examine whether the *pn-awd* functional relationship can be manifested in this epithelial model system.

***awd* and epithelial cell invasion**

In the normal course of examining the developmental expression pattern of Awd in *Drosophila* using our Awd antibody, we noticed very early on that Awd is expressed in all the somatic follicle cells but conspicuously lost in a group of migrating epithelial cells called border

cells in the developing egg chambers. This is highly interesting since border cell migration has been considered an *in vivo* model for epithelial cell invasion and epithelial-to-mesenchymal transition (54,55). Each *Drosophila* egg chamber contains a germ cell complex—one oocyte and 15 nurse cells—that originates from a germline stem cell. A single layer of somatic epithelial follicle cells envelops the germ cell complex (Fig. 3a). The follicular epithelium mechanically supports the integrity of the egg chamber and communicates with the germ cells to provide positional cues for the developing oocyte. One key morphogenic event involving the follicular epithelium is the migration of border cells (55,56). At the beginning of stage 9 (about 48 hrs after the egg chamber is formed and about 24 hr before egg laying), two anterior polar cells recruit 4–8 neighboring cells to form a border cell cluster. The border cell cluster delaminates from the epithelium and invades through the nurse cell complex until it reaches the anterior of the oocyte about 6 hr later at stage 10, traversing a linear distance of ~100–150 μm . In its final location, the border cell cluster is critical for providing the opening in the micropyle (an eggshell structure for sperm entry) and providing anterior spatial cues for the future embryo. The precise movement of the border cells is guided by the *Drosophila* PDGF/VEGF signaling pathway (Pvf ligand emanating from the oocyte and received by Pvr receptor on the border cells) (57,58).

Given our finding that Awd expression is lost in border cells prior to initiation of cell migration (59), we tested if forced expression of Awd in the border cells could dominantly interfere with developmentally programmed invasion of these cells. Indeed, re-expression of Awd in border cells reduced the level of Pvr and ameliorated downstream signaling and blocked migration (59) (see Fig. 3b for an example). Over-expression of a dominant-negative *shi/dynamin* transgene could result in over-accumulation of Pvr and spinning (without forward moving) of border cells, and this phenotype could be rescued by co-expression of *awd* (59). Interestingly, Awd also regulates the turnover of Domeless, the *Drosophila* cytokine receptor that is also required for border cell migration, but has no effect on the level of an ectopically expressed human transmembrane protein CD8 (59). Therefore the presumed endocytic function of Awd is not promiscuous. The exact specificity of this function is currently unknown. It is worth noting that Awd protein is detected only in the somatic follicle cells and not in the germ cells (59), which is consistent with the early Shearn finding that the ovarian function of *awd* is exerted in the somatic component of the ovary, not in the germ cells (20).

The molecular function of Awd protein

The genetic studies in *Drosophila* clearly implicated a role of Awd in endocytosis. The most straightforward explanation for this function is that Nm23/Awd is a GTP supplier for dynamin GTPase. However, a guanine nucleotide exchange factor (GEF)-like function is not likely since physical interaction between Nm23/Awd and dynamin has not been demonstrated in physiological conditions. It is therefore possible that Nm23/Awd affects dynamin-mediated endocytosis through an indirect mechanism. Further genetic experiments should help resolve this issue.

There are indeed other ways Awd can regulate endocytosis. For example, David Deitcher (60) proposed that the NDPK activity of Nm23 can provide CTP to the enzyme cytidine diphosphate diacylglycerol synthase (CDS) that catalyzes a rate-limiting step in the phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)P₂] synthetic pathway (61). PtdIns(4,5)P₂ has been implicated as a second messenger that regulates clathrin-mediated endocytosis of receptors (62,63). Dynamin is known to bind PtdIns(4,5)P₂ through its pleckstrin homology domain (64) and PtdIns(4,5)P₂ is also required in subsequent steps in endocytosis (65). On the other hand, since other small GTPases such as Rab5 and Arf6 are also regulators of endocytosis, a GTP supplier function of Nm23/Awd may also apply in such capacity. It is interesting to note that among the myriad of interacting proteins of Nm23 in mammalian cells, many are

related directly or indirectly with the GTPases, such as Arf6 (66), TIAM1 (a guanine exchange factor for Rac) (67), Lbc (a guanine exchange factor for Rho) (68), and Rad (69). Whether these GTPase-related functions hold true requires further *in vivo* investigation.

The developmental studies showed that substitution of the active-site histidine residue that is critical for the NDPK activity could not stall border cell migration (59). This is consistent with previous finding that this residue is required for rescuing the *enhancer of shi* phenotype (46). Curiously, this residue is not required for suppressing the *in vitro* motility (assayed by Boyden chamber) of the metastatic breast cancer cells (32,33). However, the histidine substitutions employed in the two systems are different (phenylalanine in human vs. alanine in fly). It is therefore difficult at this time to draw direct comparison. On the other hand, human mutants that affect the histidine-dependent protein kinase activity failed to suppress motility of the cancer cells (32,33). So far very few Nm23 protein kinase targets have been identified (16) but it would be highly interesting to investigate whether any of the endocytic components would be a phosphorylation target of Nm23/Awd.

Perspective

The story of *Drosophila awd* illustrated the value of an accessible genetic system that can bring seemingly unrelated interests together in solving a complex biological problem. Much of the progress in *awd* research appears serendipitous, but the underlying theme is that the careful use of the genetic system can lead to unexpected and valuable new insights, which may be otherwise difficult to attain using biased approaches. We have shown an important endocytic function for *awd*. This should lead to further investigation, in both mammalian and fly systems, into possible Nm23/Awd functions in regulating pathways that are known to involve endocytosis and vesicle recycling, such as epithelial cell adhesion, integrin turnover, etc. More recent results, both published and unpublished, have also indicated an interaction between Nm23/Awd and the tumor suppressor protein VHL. For example, *Drosophila awd* and *vhl* mutants show similar tracheal phenotypes (49). Human VHL can stabilize Nnm23-H1 in the endocytic pathway that regulates FGFR signaling strength (70). We also found that Awd and *Drosophila* VHL protein can physically interact (Dammai and Hsu, unpublished observations). Such interaction is likely independent of the known E3 ubiquitin ligase activity of VHL, and thus independent of the hypoxia-inducible-factor function (71), because VHL stabilizes Nm23, not destabilizing it. Nonetheless, such novel interactions warrant further investigations. Although so far in *Drosophila* the main Awd activity appears to be an involvement in endocytosis, which can manifest into various physiological functions, Nm23 homologues in other developmental systems have suggested other functions such as regulation of cyclin dependent kinase inhibitors (reviewed in Bilitou et al., this issue). Also, mouse Nm23 has been shown to localize to primary cilia (72). This is highly interesting considering the early observations that Nm23/Awd is associated with microtubules (17,24). Such activity may link Nm23/Awd function to cilia-dependent signaling pathways such as sonic hedgehog, Wnt and TGF β (73). The established *Drosophila* assay system—imaginal discs, trachea and the border cells—should continue provide a valuable tool for testing the Nm23/Awd functions *in vivo*, including those of the various disease-related Nm23 mutants.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a

heterozygous *pn* female
normal red eyes, viable

hemizygous *pn* male
prune eye color, viable

b

heterozygous *pn* and
K-pn female
normal red eyes, viable

hemizygous *pn*,
heterozygous *K-pn* male
lethal

Fig. 1. Genetic cross that uncovered *K-pn*

The diagram follows the conventional designation of *Drosophila* genotype: X genes/X genes (or X/Y); 2nd chromosome genes/2nd chromosome genes; 3rd chromosome genes/3rd chromosome genes. Note that the 4th chromosome contains very few genes and is commonly omitted in genotyping. (a) In the original experiment conducted by Sturtevant to show the X-linkage of *pn* mutant, *pn* homozygous females were crossed with presumed wild-type males. The expected progenies would be that all females were phenotypically normal (red eyes) and all males had *pn* eyes. (b) Since the presumably normal fathers were actually *K-pn* homozygous mutant, all female progenies were *pn* and *K-pn* double heterozygotes while all male progenies were *pn* hemizygous and *K-pn* heterozygous. Since all female progenies were normal and

viable, but no male progenies were recovered, the conclusion was that *K-pn* is a dominant, gain-of-function (or neomorphic) conditional lethal mutation in the *pn* genetic background.

Fig. 2. *awd* mutation results in ectopic tracheal tubule migration and accumulation of FGFR
 (a) Wild-type and *awd* mutants embryos carrying a trachea-specific *lacZ* transgene were stained for LacZ to visualize tracheal cells. Wild-type embryos show organized array of tracheal tubules. In severe *awd* mutants, the tubule structure was completely disrupted and randomly motile tracheal cells can be seen (arrowheads point to some examples). Bars are 100 μ m. (b) Wild-type and *awd* mutant embryos carrying the trachea-specific *lacZ* transgene were transformed with another transgene expressing FGFR-GFP fusion protein. The embryos were double stained for LacZ (red) and GFP (green). Zoom-in view of the migrating tips is shown. The tips migrate toward the top in this view. FGFR-GFP in wild-type embryo shows some membrane localization (arrow) but mostly is internalized. In *awd* mutants, FGFR-GFP is seen accumulated on the cell surface. Bars are 5 μ m.

Fig. 3. *awd* expression inhibits border cell migration

(a) Schematic presentation of stage 8 and stage 9 *Drosophila* egg chambers. Anterior of the eggs are on the left. At stage 8, the germ cell complex consisting of nurse cells (NC) and the oocyte was enveloped by a single layer of somatic follicle cells (FC). At stage 9, a group of 6–10 follicle cells named border cells (BC, colored orange) originally located at the anterior end of the follicular epithelium delaminate from the epithelium and invade through the nurse cell complex. Arrow indicates the direction of border cell movement. (b) Stage 8 and stage 9 egg chambers from wild-type (left and middle panels, *wt*) and transgenics re-expressing *awd* in the border cells (right panel, +*awd*) were stained for β -catenin (red) and Awd (green). β -catenin stains follicle cell apical-lateral membrane and marks the border cells (arrows). In wild-type egg chambers, border cells delaminate from the follicle cell layer and move into the nurse cell proper (middle panel). These invading border cells lost Awd expression as compared to stage 8 when they remain in the follicle cell layer at the anterior (left panel). When Awd is re-expressed in the border cells (right panel), the border cells fail to move from the anterior tip.