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awd, the Homolog of Metastasis Suppressor Gene *Nm23*, Regulates *Drosophila* Epithelial Cell Invasion▽†

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Border cell migration during *Drosophila melanogaster* oogenesis is a highly pliable model for studying epithelial to mesenchymal transition and directional cell migration. The process involves delamination of a group of 6 to 10 follicle cells from the epithelium followed by guided migration and invasion through the nurse cell complex toward the oocyte. The guidance cue is mainly provided by the homolog of platelet-derived growth factor/vascular endothelial growth factor family of growth factor, or Pvf, emanating from the oocyte, although *Drosophila* epidermal growth factor receptor signaling also plays an auxiliary role. Earlier studies implicated a stringent control of the strength of Pvf-mediated signaling since both down-regulation of Pvf and overexpression of active Pvf receptor (Pvr) resulted in stalled border cell migration. Here we show that the metastasis suppressor gene homolog *Nm23/awd* is a negative regulator of border cell migration. Its down-regulation allows for optimal spatial signaling from two crucial pathways, Pvr and JAK/STAT. Its overexpression in the border cells results in stalled migration and can revert the phenotype of overexpressing constitutive Pvr or dominant-negative dynamin. This is a rare example demonstrating the relevance of a metastasis suppressor gene function utilized in a developmental process involving cell invasion.

Mobilization and migration of epithelial cells are important developmental processes that require stringent control. While epithelial to mesenchymal transitions (EMTs) are commonly observed during normal embryonic development, up-regulated or deregulated motility of epithelial cells is also a hallmark of metastatic tumor cells. Therefore, understanding factors that control epithelial cell motility under physiological conditions is of critical importance. In this context, migration of border cells during *Drosophila melanogaster* oogenesis is an accessible and quantifiable developmental system to study cellular processes such as invasion and directed migration.

Each *Drosophila* egg chamber contains a germ cell complex—one oocyte and 15 nurse cells—that originates from a germ line stem cell. A single layer of epithelial follicle cells envelops the germ cell complex. 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 (34, 42). At the beginning of stage 9 (about 48 h after the egg chamber is formed and about 24 h before egg laying), two anterior polar cells recruit four to eight 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 h later at stage 10, traversing a linear distance of ~100 to 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* platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) signaling pathway (Pvf ligand emanating from the oocyte and received by Pvr receptor on the border cells) (14, 32). Epidermal growth factor (EGF) signaling is also needed in the migration and plays a complementary role to Pvr signaling (5, 13, 31). It is, however, also required for a later, Pvr-independent movement along the oocyte surface (13). In addition to Pvr signaling, JAK/STAT (3, 17, 45, 53), steroid hormone (2), Notch (44, 52), and JNK (30) pathways contribute to various extents to the overall border cell motility. These multiple signaling pathways appear to control the cytoskeletal rearrangements and the complex interactions between the border cells and the substratum—the nurse cell surface—that propel the forward movement of the border cells (35). Previous studies provided evidence that chemotactic signal sensing via Pvr is central to the directional movement of border cells. Since both loss of function and gain of function of Pvr signaling can delay migration (14, 32), it was proposed that the signaling strengths from the guidance receptors are temporally and spatially refined to allow directional motility. Here we report a novel regulatory mechanism that utilizes the function of the *Drosophila* homolog of human metastasis suppressor gene *Nm23* to modulate signal strength from guidance receptors, providing the necessary fine-tuning and directionality of cell migration.

Human *Nm23* consists of a gene family of eight members, the H1 and H2 isoforms being the most closely related and most implicated in tumor progression. They are also evolution-

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arily conserved. *Drosophila* has one *Nm23* gene, *awd*, which shares 78% amino acid identity with either human H1 or H2 (6, 7, 43). *Nm23* was initially isolated as a cDNA clone down-regulated in highly metastatic variants of murine melanoma cell lines in a differential hybridization screen (47). Subsequent studies showed that re-expression of *Nm23* in metastatic cell lines can inhibit metastasis in the xenograft models and reduce cell motility in vitro without affecting initial proliferation (24, 27), thus consistent with the defined role of an antimetastasis gene as opposed to the tumor suppressor genes that control tumor growth. However, the exact cellular function of Nm23 has been a vexing issue since diverse activities, mostly demonstrated in cell culture, have been assigned to this protein (39, 46). *Nm23-M1* (the mouse H1 counterpart) knockout mice have been generated (1). The homozygous null animal is viable but showed delayed mammary gland development. The lack of severe developmental defects may be the result of compensation by other family members while the mild mammary phenotype seems correlative with the well-studied role of Nm23 in breast cancer metastasis. Also, the *Nm23-M1* knockout mice, when challenged to form hepatocellular carcinoma, showed higher incidence of metastasis to lung (8). Nonetheless, this animal model has not shed lights on the cellular function of Nm23. The reported functions of Nm23 include the following. (i) First, there is nucleoside diphosphate kinase (NDPK), which transfers the terminal phosphate from ATP to a nucleoside diphosphate (such as GDP), through the formation of an intermediate histidine-phosphate linkage at histidine 118 (39). (ii) Second, there are DNA binding and nuclease activities (28, 41), although their functional significance is in some dispute (33). (iii) Third, there is histidine-dependent protein kinase (15, 20, 51), the enzymatic activity of which is similar to that of NDPK but towards a protein substrate at aspartate or serine residues (4). None of these functions has been verified in the context of mammalian development. Consequently, the antimetastatic action of Nm23 and its developmental significance remain unclear.

To date, the most physiologically relevant function of *Nm23* was revealed by genetic studies in *Drosophila*. Initially, *awd* null alleles were shown to cause imaginal disc defects, hence its name: *a*bnormal *w*ing *d*iscs (11, 12). Subsequent studies showed that the *awd* transgene carrying the NDPK-dead mutation in the active site histidine residue (residue 119 in the *Drosophila* protein) failed to rescue the *awd* lethal phenotypes (54). In a more recent genetic screen for second-site mutations that exacerbate the neurological phenotype of a temperature-sensitive *shibire* (*shi*)/*dynamin* mutant, Krishnan et al. (25) isolated three lines of such *shi* enhancers; all three were alleles of *awd*. This suggests that the functional relationship between *awd* and *shi* is highly specific and almost exclusive in the endocytic pathway. The functional relationship between Nm23/Awd and dynamin prompted the suggestion that Nm23/Awd is a GTP supplier for dynamin, a GTPase. Nonetheless, the putative antimetastasis activity of Nm23/Awd has never been demonstrated in a physiologically relevant metastasis or EMT model.

In order to analyze the *awd* function in an analogous cell invasion and EMT model system, we examined its role in border cell migration in the *Drosophila* ovary. Although border cells retain a few epithelial characteristics, such as a high E-cadherin expression level, it is well established that cellular mechanisms activated during border cell migration closely resemble those observed in EMT (22, 37). Interestingly, consistent with its antimetastasis function, we encountered an intriguing finding that Awd expression is lost in border cells prior to initiation of cell migration. In this study, we present data related to the biological relevance of this observation.

MATERIALS AND METHODS

***Drosophila* strains.** *y w; UAS-awd* carrying the entire *awd* open-reading frame under *UAS* enhancer control has been described previously (9). For the RNA interference-mediated *awd* knockdown construct, tail-to-tail tandem copies of the *awd* open-reading frame were cloned in the *pCaSpe-hs* vector under heat shock promoter control. These expression vectors were used to transform *y w* flies. *w; UAS-λpvr* carries a modified *pvr* coding sequence containing the dimerization domain from λ repressor (14). It expresses a constitutively active Pvr receptor and is a generous gift from Pernille Rørth. The *UAS-shibire*^{K44A}, *slbo-GAL4*, and *C306-GAL4* lines were obtained from Bloomington Stock Center. The expression pattern of *slbo-GAL4* has been previously determined. It is identical to the β-galactosidase reporter gene inserted into the *slow border cell* (*slbo*) locus and correlates with the *slbo* expression pattern that is restricted to migrating border cells in addition to a small group of anterior follicle cells, centripetally migrating follicle cells and posterior follicle cells (36). *C306-GAL4* expression starts earlier in oogenesis than *slbo-GAL4* and spans a larger number of anterior follicle cells, including the future border cell cluster and a small group of posterior follicle cells (PFC) (26). The loss-of-function *awd* allele *awd*^{j2A4} is a P-element insertion as described previously (9, 25). The mutant allele was crossed into the *FRT*^{82B} chromosome, and the mitotic mutant clone was induced when combined with FLP recombinase directed by the follicle cell-specific *e22c-GAL4* driver. *w; hsp-awd*^{wt} and *w; hsp-awd*^{H119A} were gifts from A. Shearn of Johns Hopkins University.

Transgene and RNA duplex induction. For the *GAL4-UAS* expression system, the flies were conditioned in the presence of live baker's yeast at 25°C for 2 days and then incubated at 29°C for 3 days before dissection. For heat shock-induced expression, flies were conditioned in the presence of live yeast at 25°C for 2 days and then heat treated twice daily at 37°C for 30 min for a total of five heat treatments. The flies were dissected ~2 h after the final heat treatment.

Immunohistochemistry. Ovaries were dissected in 1× Ringers solution (182 mM KCl, 46 mM NaCl, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2) containing 1% bovine serum albumin. For immunostaining, the ovaries were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overlaid with heptane. All of the washes were done in PBST (1× PBS containing 0.2% Triton X-100). Anti-Pvr antibody incubations were in PBST containing 20% goat serum. For all other antibody staining, NP-40 (0.5%) was used instead of Triton X-100. Samples were analyzed using an Olympus IX70 microscope equipped with the Fluoview 300 confocal capability. Digital images were processed in Photoshop software without biased manipulations.

Antibodies. Protein A-purified polyclonal rabbit anti-Awd (3 mg/ml) was used at 1:1,000 dilution and has been described previously (9). Affinity-purified rabbit anti-STAT raised against a peptide corresponding to C terminus of STAT92E was used at a dilution of 1:1,000 (a generous gift from Stephen Hou). Mouse monoclonal antibody against a dually phosphorylated mitogen-activated protein kinase (MAPK) (Sigma) was used at 1:500. Rabbit anti-Dome was used at 1:200 (a generous gift from Stephane Noselli). Polyclonal rat anti-Pvr was used at 1:200 (a generous gift from Pernille Rørth). Mouse monoclonal anti-Arm and rat monoclonal anti-DE-cadherin were obtained from the Developmental Studies Hybridoma Bank. All of the primary antibodies were incubated overnight at 4°C. Alexa Fluor 488- or 546-conjugated secondary antibodies (Molecular Probes) were used at 1:200 dilutions for 2 h at room temperature. For Awd and Dome costaining, both using rabbit polyclonal antibodies, the anti-Awd antibody was preincubated with Alexa Fluor 647-conjugated anti-rabbit Fab fragment (Zenon Alexa Fluor 647 from Invitrogen) and used directly without separate secondary antibody detection.

RESULTS

Awd expression in border cells is down-regulated during migration. Fig. 1A illustrates the process of border cell migration. The border cell fate is induced by the two anterior polar

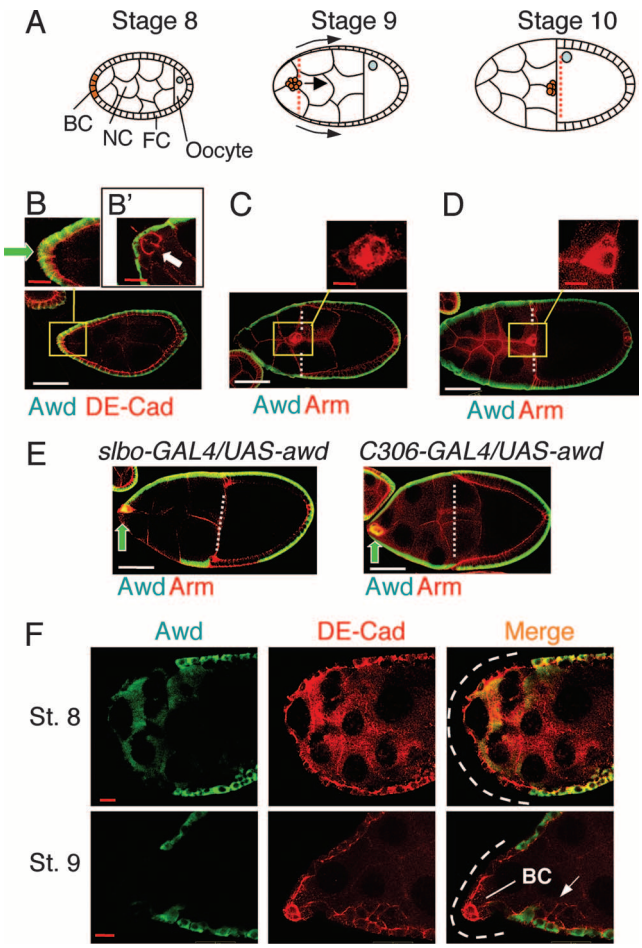

FIG. 1. Down-regulation of Awd expression correlates with border cell migration. (A) Illustration of border cell migration. At stage 8, a group of 6 to 10 cells at the anterior tip of the follicular epithelium begins to form the border cell (BC) complex (colored orange). Other cell types in the egg chamber include nurse cells (NC), follicle cells that form the main body of the epithelium (FC), and the oocyte. At stage 9, the BC complex delaminates from the epithelium and migrates toward the oocyte, moving through the midline of the NC complex (marked by the straight arrow). Concomitant with this process, the follicular epithelium begins moving posteriorly as a sheet (curved arrows). The posterior movements of the sheet and the BC complex are synchronized, since the anterior margin of the follicular sheet and the BC can be aligned (red vertical dashed line). The BC complex reaches the oocyte at stage 10 when the anterior margin of the follicular epithelium also aligns with the anterior of the oocyte (red dashed line). (B to F) Egg chambers were stained for Awd (green) and either β-catenin/Armadillo (Arm) or DE-cadherin (DE-Cad) as indicated (red). Arm and DE-cadherin are enriched in the BC and serve as BC markers. Anterior is to the left. (B) *y w* (representing wild type) stage 8 egg chamber showing Awd expression in the future BC complex (green arrow in inset). In an early stage 9 egg chamber (B′) when the border cell complex just delaminates from the epithelium, Awd is no longer expressed (white arrow). (C) *y w* stage 9 egg chamber. The border cell complex (inset) is moving along with the anterior margin of the follicle cell sheet (dashed line). No Awd is detected in the BC complex (inset). (D) *y w* stage 10 egg chamber. Both the BC complex and the anterior margin of the FC sheet have reached the anterior of the oocyte (dashed line). There is no expression of Awd in the BC complex (inset). (E) Stage 9 or 10 egg chambers carrying the *UAS-awd* transgene driven by *slbo-GAL4* or *C306-GAL4* were double stained for Awd (green) and Arm (red). Awd is detected in the BCs (green arrows) and the BCs are stalled at the tip of the egg chamber while the anterior margin of the FC sheet has moved posteriorly (dashed lines). (F) A stage 8 egg (upper panels) or a stage 9 (lower panels) chamber carrying a clone of the awd^{j2A4} mutant follicle cells at the anterior (dashed lines in the merged view), identifiable by the lack of Awd staining and positive for DE-cadherin staining. No delaminated follicle cells are observed in the NC proper at stage 8. At stage 9, a BC cluster within the *awd* mutant clone shows stalling at the tip. White scale bars are 50 μm; red scale bars are 10 μm.

cells at stage 7 (53), but the border cell complex remains a part of the anterior follicular epithelium until stage 8. At the beginning of stage 9, the border cell cluster containing four to eight border cells surrounding two polar cells delaminates from the epithelium and migrates posteriorly along the midline, invading through the nurse cell complex until it reaches the oocyte at stage 10. Concomitant with this process, the remaining follicle cells that form the main-body follicular epithelium move posteriorly as a single intact sheet. These two migratory events are not mechanically linked but occur at the same pace; therefore, the alignment of the border cells and the anterior margin of the follicular sheet provides a landmark for the normal progression of the border cells. At stage 10, when the border cells complete their posterior migration, the anterior margin of the follicular sheet also aligns with the anterior of the oocyte. Interestingly, during this developmental process, we observed that down-regulation of Awd protein expression is precisely correlated with border cell migration. That is, Awd is strongly expressed in border cells before migration begins (Fig. 1B), promptly disappears when delamination occurs at early stage 9 (Fig. 1B′), and stays undetectable during invasion through the nurse cell complex at stages 9 and 10 (Fig. 1C and D). On the other hand, Awd expression is maintained in the posteriorly moving follicular epithelium throughout stages 9 and 10. This border cell-specific dynamic expression pattern raised a distinct possibility that *awd* is developmentally down-regulated to allow optimal activation of cellular pathways utilized during border cell migration. If this is correct, re-expression of *awd* in the border cells should impede their migration. To test this, we specifically expressed Awd in the border cells using the *UAS-GAL4* binary expression system (*UAS-awd* driven by border cell-specific *GAL4* expression). Two *GAL4* drivers, *slbo-GAL4* and *C306-GAL4*, were utilized for this purpose. Re-expression of Awd by either of the two *GAL4* drivers specifically blocked border cell migration. In the most severe phenotype, border cells remained at the anterior tip of the germ cell complex at stages 9 and 10, instead of reaching the oocyte (compare Fig. 1E to D; also see Fig. 3 below). Identical results were observed using different lines of *UAS-awd* (data not shown). Also, the ectopically expressed Awd levels are comparable to the endogenous levels observed in the surrounding follicle cells.

It is noteworthy that ectopic expression of Awd does not interfere with determination of border cell fate or delamination as the border cell cluster is formed properly but fails to move (Fig. 1E), indicating that the function of Awd is specific to the cell migration phase. To confirm this assertion, we generated *awd* mutant clone of cells in the anterior follicle cell population. As shown in Fig. 1F (upper panels), these anterior *awd* mutant follicle cells at stage 8 did not show premature

delamination or migration into the nurse cell complex. We then asked what the cell behavior would be if these *awd* mutant anterior cells developed into the migratory phase. Interestingly, *awd*$^-$ border cells within the anterior *awd* mutant follicle clone showed stalling at the tip at stage 9 (lower panels). We also occasionally observed disorganized epithelial structure in the follicle cell clones of *awd* mutant (Fig. 1F). The significance and mechanism of this phenotype are unclear but may indicate additional function of *awd* in the follicular epithelium. Note that the *awd*$^-$ border cells observed here are part of the anterior *awd* mutant clone, recognizable because loss of Awd expression is observed in follicle cells surrounding the border cells, which normally are positive for Awd (compare with Fig. 1B and B′). It should also be noted that these *awd* mutant cells are generated by recombination between heterozygous chromatids during mitosis. Since follicle cells cease to proliferate at approximately stage 6, Awd in these border cells is lost at least 15 h prior to normal down-regulation at stage 9. Taken together, it appears that both loss of function and gain of function of *awd* resulted in delayed border cell migration. This is a phenomenon similar to that observed for the Pvr function. We next examined the functional relationship between *awd* and *pvr*.

Awd regulates the level of Pvr. The signaling strength of the *Drosophila* homologue of PDGF/VEGF receptor (Pvr) has been demonstrated to be critical for border cell migration. For example, both overexpression and loss of Pvr signaling impaired border cell motility (14, 32), suggesting that a measured dose of Pvr signaling is necessary to correctly guide border cell migration. We tested whether the role of Awd in border cell migration is to modulate the signaling receptor Pvr. In control border cells (carrying the *slbo-GAL4* transgene alone), endogenous Pvr is expressed in distinct puncta at or near the border cell membrane (Fig. 2A). These cells migrate to the anterior of the oocyte at stage 10 (Fig. 2A). Reexpression of Awd in the border cells reduced the endogenous level of Pvr and resulted in delayed migration (Fig. 2B). We next tested whether a more pronounced antagonist effect could be observed if Pvr is overexpressed and the resulting migration defects might be rescued by Awd re-expression. To this end, using *slbo-GAL4* we overexpressed a constitutively active form of Pvr (λPvr), which has been shown to result in severe stalling of border cells at the anterior of the egg chamber (14), concomitant with strong Pvr staining throughout border cell surface (Fig. 2C). Note that the *slbo-GAL4* driver is also active in centripetally migrating follicle cells (CMFC) and PFC, as evidenced by high anti-Pvr staining in these cells (Fig. 2C). Coexpression of Awd with λPvr completely ameliorated the overexpressed Pvr in border cells as well as in CMFC and PFC and restored normal border cell migration (Fig. 2D).

To quantify the above observations (Fig. 2E), the area traversed by the border cells was divided into four zones. Zone 1, at the tip of the stage 10 egg, represents little or no migration, while zone 4, immediately adjacent to the anterior of the oocyte, represents completed migration. Zones 2 and 3 represent significant but not complete defects in migration. In control flies carrying only the *slbo-GAL4* transgene, ~90% of the stage 10 egg chambers showed border cells reaching the oocyte. The remaining ~10% of the border cells show partially delayed migration, which represents the background level of defects

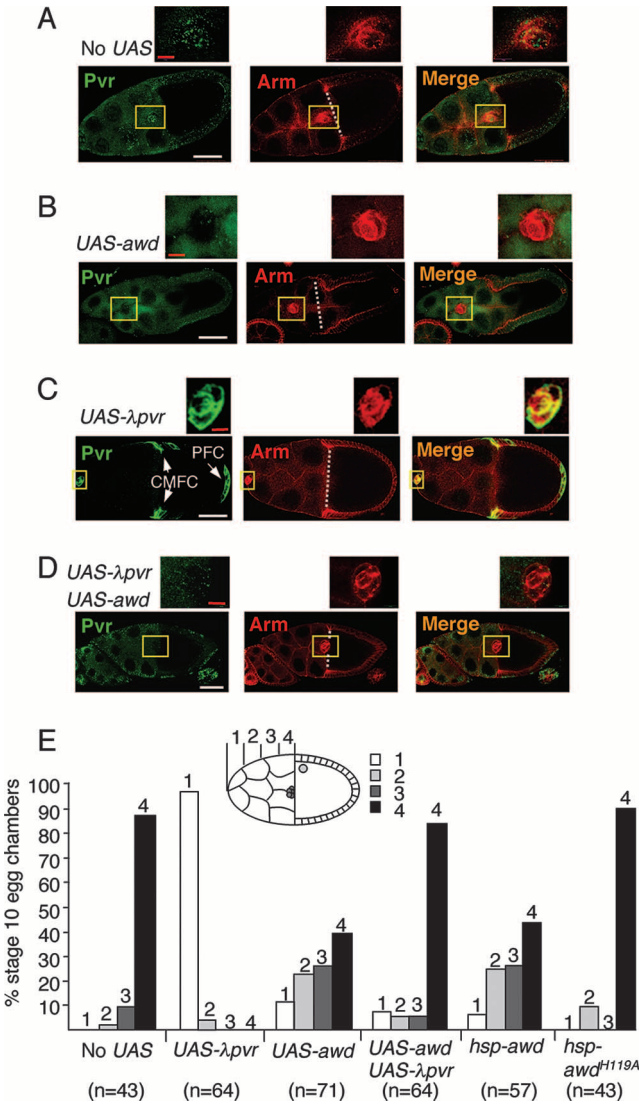

FIG. 2. Awd down-regulates Pvr. *slbo-GAL4* flies carrying no *UAS* transgene (A), one copy of *UAS-awd* (B), one copy of *UAS-λpvr* (C), and one copy each of *UAS*-λpvr and *UAS-awd* (D) were incubated at 29°C for 3 days before ovaries were dissected and stained for Pvr (green) and Arm (red). Stage 10 eggs are shown. Anterior is to the left. (A) In control eggs, endogenous Pvr is expressed in puncta in the border cells (insets). The border cells have reached the anterior of the oocyte (vertical dashed line). (B) Overexpression of Awd reduced the endogenous level of Pvr (insets), accompanied by the delayed border cell migration (anterior to the anterior margin of the follicle cell [FC] sheet; dashed line). (C) Overexpressed λPvr is detected at very high levels on the surface of all border cells (insets). The border cells remain at the anterior of the egg chamber. The vertical dashed line marks the anterior of the oocyte, where the border cells should have been. (D) When λPvr and Awd are coexpressed, the Pvr levels are reduced to equal to or lower than the control levels (insets). The border cell migration is normal. (E) Quantification of the border cell migration defects of the fly strains described in panels A to D). In addition, *hsp-awd*^{wt} and *hsp-awd*^{H119A} were heat treated at 37°C for 30 min daily for 3 days before ovaries were dissected. For quantifying the migration defects, the space traversed by the border cells are divided into four zones as depicted in the diagram on the top. The numbers of border cells within each zone at stage 10 were counted and expressed as per cent total. The numbers (*n*) of egg chambers counted for each genotype are indicated. White scale bars are 50 μm; red scale bars are 10 μm.

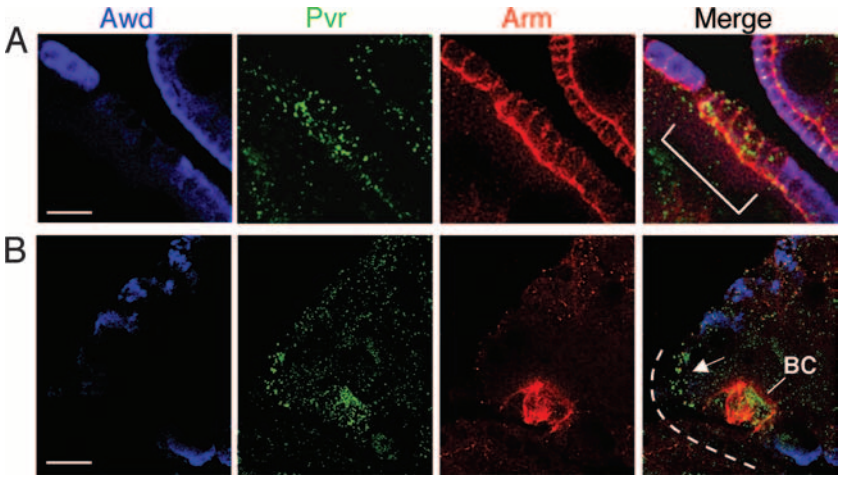

FIG. 3. Awd is a negative regulator of endogenous Pvr. (A) Flies carrying two copies of the *awd* duplex constructs under heat shock promoter control were heat treated as described in Materials and Methods. The ovaries were dissected and stained for Awd (blue), Pvr (green), and Arm (red). The *awd* knockdown cells are marked by a bracket. These cells express higher levels of Pvr than their awd^{+} neighbors. (B) Anterior of a stage 9 egg chamber carrying a clone of the awd^{j2A4} mutant, stained for Awd (blue), Pvr (green), and Arm (red). The *awd* mutant cells are marked by a dashed line. Pvr expression is elevated in the border cells and in the neighboring anterior follicle cells (arrow). Scale bars are 20 μm.

under our assay conditions. Overexpression of λPvr resulted in 97% of stage 10 egg chambers showing border cells stalling near the anterior tip (zone 1), and none completed the migration. Reexpression of Awd alone caused milder but significant migration defects: 11% showed total lack of migration (at zone 1), and ~50% showed delayed migration (at zones 2 and 3). Only 39% completed migration. The stalled-migration phenotype resulting from hyperactive Pvr signaling is at first counterintuitive. However, as suggested previously (5, 14), the delayed migration is the net outcome of "roundabout" movement of the border cells as a consequence of lack of directional cues due to symmetrical signal activation of the receptor.

The observations that Awd can reduce both endogenous and overexpressed Pvr suggested that *awd* antagonizes *pvr* gene function. Since ectopic expression of either Awd or λPvr resulted in stalled border cell migration, we sought genetic evidence for the inferred antagonist relationship between Awd and Pvr. In phenotypic rescue analysis, indeed, coexpression of Awd and λPvr restored normal migration to 83% of the egg chambers (Fig. 2E). The rescue effect of coexpressing Awd and λPvr is not due to dilution of the available GAL4, as compared to expressing single transgenes, since inclusion of a *UAS-GFP* reporter transgene with either *UAS-awd* or *UAS-λpvr* alone did not alter the phenotypic outcome (see Fig. S1 in the supplemental material).

To ensure that Awd activity is required for the phenotypic outcome of Awd overexpression, we compared overexpression of wild-type *awd* and a mutant that is defective in the histidine-mediated phosphate transfer activity (H119A mutation) from the *hsp70* promoter. As shown in Fig. 2E, $hsp70\text{-}awd^{wt}$ induced a significant border cell migration defect while $hsp70\text{-}awd^{H119A}$ had little effect.

To further confirm the regulatory role of Awd on Pvr expression, we examined the Pvr levels in an *awd* loss-of-function genetic background. Pvr is normally expressed at low but detectable levels in the follicle cells that comprise the main body of the epithelium (14), coincidental with high expression levels of Awd (see Fig. 1). We therefore tested whether reduced Awd expression could lead to increased expression of Pvr in these cells. For this purpose, *awd* RNA duplex-mediated knockdown was preferred to avoid indirect developmental defects that may be associated with genomic *awd* mutant clones. As shown in Fig. 3A, when *awd*-specific RNA duplex is induced in the follicle cells and endogenous Awd is reduced, Pvr is clearly overexpressed and forms large aggregates in these *awd* knockdown cells as compared to the neighboring normal cells. Thus, Awd is a general suppressor of Pvr expression in a wider population of follicle cells. However, these *awd* knockdown follicle cells, although overexpressing Pvr, did not delaminate or migrate ectopically. This could be because the RNA interference effect was more immediate or because these mature follicle cells have lost their intrinsic capability to mobilize. We next examined whether the stalled border cells observed in the anterior *awd* mutant clone (Fig. 1F) could be correlated with elevated Pvr expression. As shown in Fig. 3B, the stalled border cells within an anterior *awd* mutant clone exhibit large patches of aggregated Pvr. This is consistent with the stalling phenotype observed with increased levels of Pvr signaling (14). It should be noted that under our assay conditions, overexpression of wild-type Pvr also resulted in significant levels of border cell migration defects (data not shown), in agreement with the *awd* loss-of-function phenotype observed here. We conclude that at least part of the normal *awd* function is involved in down-regulating chemotactic receptor in nonmigrating follicle cells and in premigratory border cells.

Signaling events downstream of Pvr are affected by Awd expression in border cells. With respect to downstream signaling events, two major characteristics define border cell migration. (i) MAPK activation is a major signaling event downstream of receptor tyrosine kinases and migrating border cells exhibit strong activation of MAPK (5, 10, 14). (ii) Asymmetrical activation of guidance receptors (detected by phosphotyrosine [p-Tyr]) creates differences in input signal levels

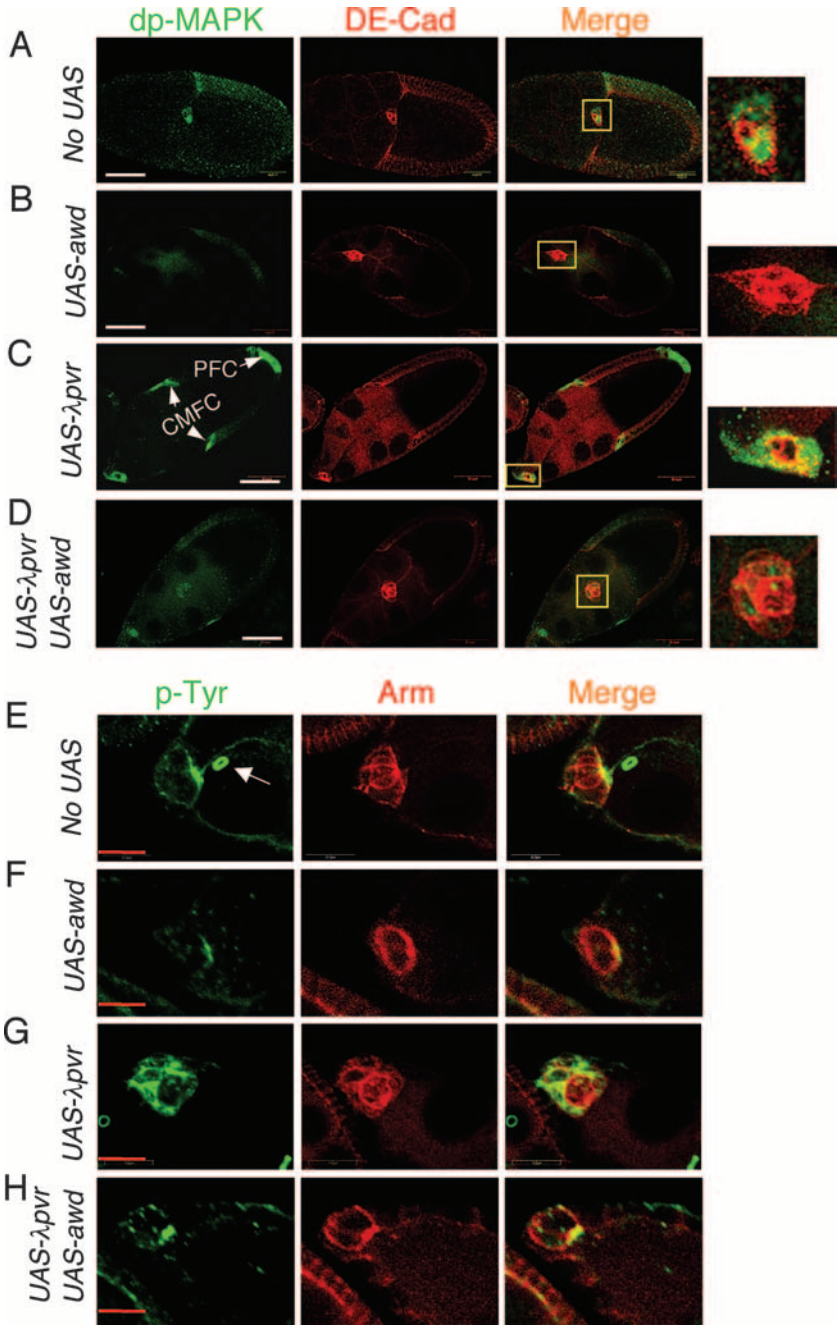

FIG. 4. Awd down-regulates MAPK and p-Tyr levels. The four groups of flies are the same as in Fig. 2. Anterior is to the left. (A to D) Stage 9 and 10 eggs were stained for dually phosphorylated MAPK (dp-MAPK; green) and DE-cadherin (red). Enlarged views of the border cells in the merged fields are shown on the right. Reexpression of Awd down-regulates both the endogenous dp-MAPK in border cells (compare B with A) and the dp-MAPK induced by λPvr in border cells, CMFC and PFC (compare D with C). (E to H) Stage 9 and 10 eggs were stained for p-Tyr (green) and Arm (red). The prominent ring-like structure in (E) is one of the ring canals that provide cytoplasmic linkages between nurse cells. They express p-Tyr but have no structural or functional relationship with border cells. Reexpression of Awd can down-regulate both the endogenous p-Tyr (compare F with E) and the p-Tyr induced by λPvr (compare H with G). White scale bars are 50 μm. Red scale bars are 10 μm.

within the cell cluster (for example, leading cells have high p-Tyr activity compared to the cells behind them) to maintain directionality of migration. It has been shown that ectopic induction of MAPK by expressing activated fibroblast growth factor receptor (FGFR) or Raf did not produce gross border cell migration defects, nor did expression of a dominant-negative Raf (13). However, MAPK is needed in the border cells for down-regulation of the transcription factor Yan, which promotes DE-cadherin turnover, which in turn results in asymmetrical distribution of DE-cadherin (44). Thus, constructive activation of MAPK in border cells may require spatially restricted signaling components either within the individual bor-

der cells or within the migrating border cell cluster or may depend on the context of specific receptor kinase pathway(s) (5). While the intricate role of MAPK requires further investigation, its activation serves as an accessible readout for the Pvr activity (44). Dually phosphorylated MAPK (indicative of activation) is readily detected in wild-type border cells (Fig. 4A). The activity is severely reduced upon Awd expression (Fig. 4B) and greatly enhanced by λPvr expression (Fig. 4C), indicating that both Awd and Pvr influence MAPK activation. Significantly, coexpression of Awd with λPvr reduced dramatically the up-regulated MAPK activity levels (Fig. 4D), again demonstrating the antagonist relationship between Awd and Pvr signaling. An important observation from previous studies is that propagation of signal within the border cell cluster is asymmetrical. For example, Pvr is activated at the migrating front, presumably because the Pvf ligand emanates from the oocyte. This asymmetric activation has been observed by staining for p-Tyr (23). We reasoned that if the action of Awd is to down-regulate the Pvr level, a prediction would be that in border cells overexpressing Awd, the level of p-Tyr should be reduced at the migrating front, but still the receptor activity should be restricted to the migrating front. We find that this is indeed the case. p-Tyr is enriched in the migrating front of the control border cell complex (Fig. 4E), which is significantly reduced when Awd is overexpressed (Fig. 4F). Consistent with the MAPK results, this indicates that Awd action potentially involves reduction in signaling through the receptors and does not cause mislocalized receptor activation or enhanced ectopic activation of unknown pathways. On the other hand, λPvr expression resulted in very high levels of p-Tyr in all border cells (Fig. 4G), consistent with the overwhelming ligand-independent Pvr signaling activity. As expected, coexpression of Awd and λPvr restored the level and spatial pattern of p-Tyr to that of the control (Fig. 4H).

***awd* functionally interacts with *shi/dynamin*.** We next asked what might be the mechanism of Awd action. In *Drosophila* and human systems, Nm23/Awd has been demonstrated to participate in endocytosis via enhancing dynamin activity (9, 19). However, it is also known that in the absence of Nm23/Awd, dynamin activity in these pathways is significantly reduced but not entirely abolished. Therefore, Nm23/Awd enhances the endocytic activity of dynamin, but is not an essential component of the endocytic pathway. This is consistent with a role in fine-tuning the signaling activity. If this is correct, overexpression of Awd should be able to rescue a *shi/dynamin* mutant with residual wild-type *shi* function.

To first test if indeed Pvr activity could be regulated by dynamin-mediated endocytosis, we utilized a dominant-negative mutant of *shi/dynamin* (*shi*^{K44A}). When Shi^{K44A} was overexpressed in the border cells, the endogenous Pvr formed large aggregates (Fig. 5A). This pattern is reminiscent of the Pvr expression pattern seen in the clone of *awd* mutant border cells (Fig. 3B). Consistent with increased accumulation of Pvr, overexpression of Shi^{K44A} also resulted in delayed migration (Fig. 5C), demonstrating that Pvr signaling is regulated by dynamin-mediated endocytosis. To test the model that Awd negatively regulates border cell migration by promoting internalization and/or turnover of surface receptors, we examined the functional relationship between *awd* and *shi/dynamin*. Incredibly,

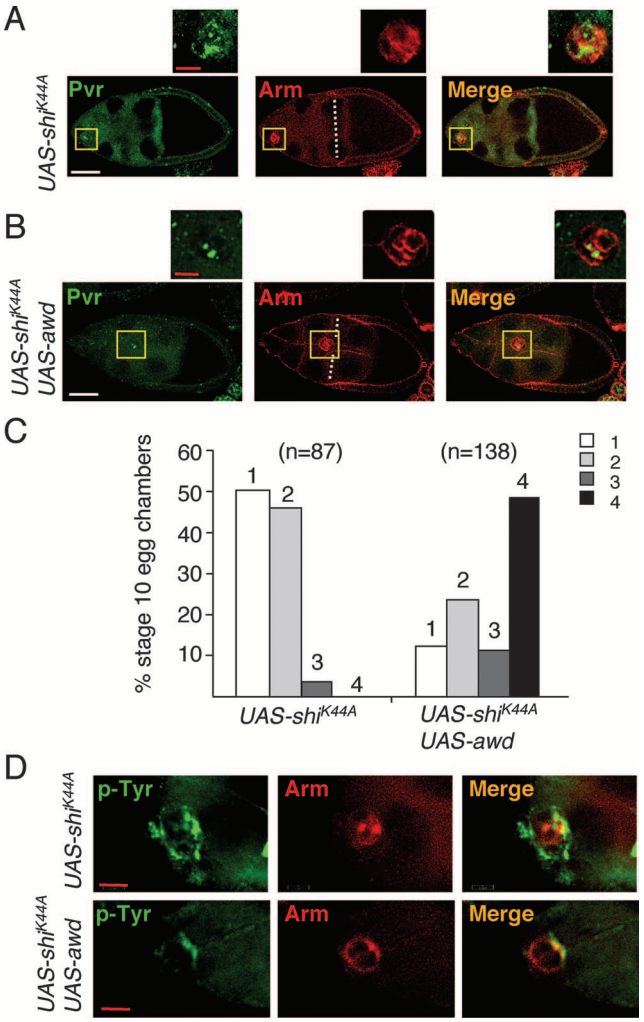

FIG. 5. *awd* functionally interacts with *shi/dynamin*. *slbo-GAL4* flies carrying one copy of *UAS-shi*^{K44A} or one copy each of *UAS-shi*^{K44A} and *UAS-awd* were incubated at 29°C for 3 days before ovaries were dissected and stained for Pvr (green) and Arm (red) (A and B) or p-Tyr (green) and Arm (red) (D). Anterior is to the left. (A) Endogenous Pvr form large aggregates in the border cells overexpressing dominant-negative *Shi*^{K44A} (insets). (B) Coexpression of Awd with Shi^{K44A} reduced the level and aggregation of Pvr in the border cells (insets) and restored normal migration. (C) Quantification of the border cell migration defects in *UAS-shi*^{K44A} and *UAS-shi*^{K44A}/*UAS-awd* flies, as in Fig. 2. Expression of *awd* rescued the severe migration defects induced by overexpressing Shi^{K44A}. (D) In *UAS-shi*^{K44A} flies, p-Tyr is overexpressed in the border cells and the asymmetrical distribution is lost (compare to Fig. 4E). Coexpression of Awd rescued the p-Tyr expression back to the wild-type pattern. White scale bars are 50 μm; red scale bars are 10 μm.

coexpression of Awd with Shi^{K44A} reduced Pvr aggregation (Fig. 5B) and rescued the migration defects (Fig. 5C). The effect of overexpressing Shi^{K44A} on Pvr signaling is also evident in the overall accumulation of p-Tyr throughout the border cell cluster (Fig. 5D), as anticipated. This ectopic p-Tyr activity was rescued by coexpression of Awd (Fig. 5D), demonstrating that Pvr signaling in border cells is optimized by dynamin-dependent endocytosis that can be dominantly interfered with by Awd.

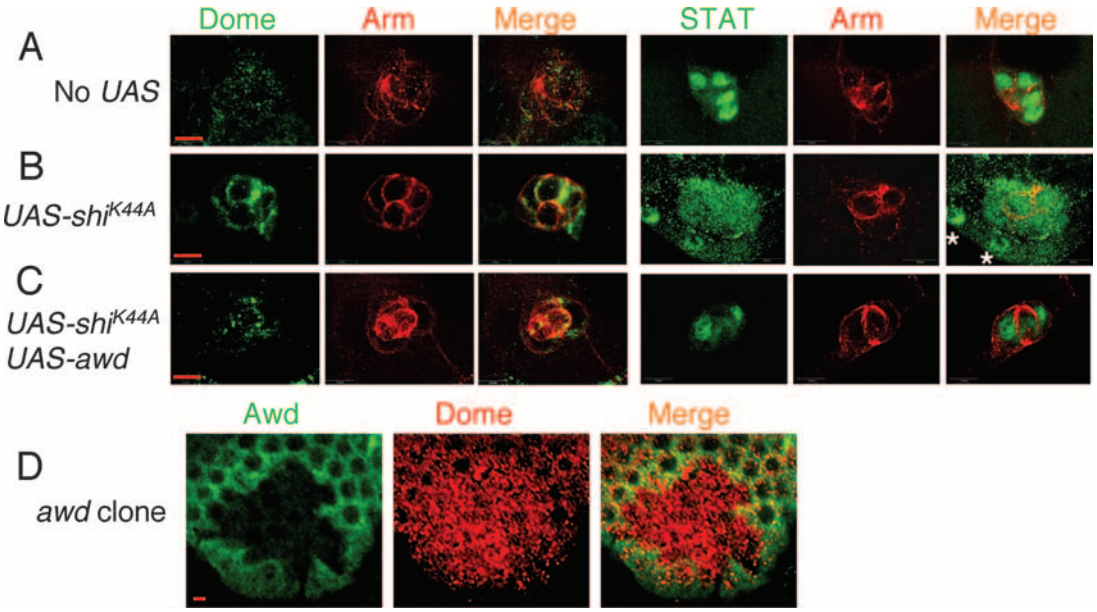

FIG. 6. *awd* regulates Dome signaling. (A to C) The three groups of flies are the same as in Fig. 5. Egg chambers were stained for Dome (green) and Arm (red) or STAT92E (green) and Arm (red), as indicated. Close-up views of border cells in stage 9 eggs are shown. Anterior is to the left. (A) In control flies (No *UAS*), Dome is expressed in small puncta at or near the border cell membrane and STAT92E is mostly nuclear. (B) In Shi^{K44A}-overexpressing flies, Dome overaccumulates throughout the border cell surface and STAT92E becomes overexpressed in the cytoplasm. In this view, a couple of nearby follicle cells with nuclear STAT92E (*) provide a convenient control for proper STAT92E staining. (C) Coexpression of *awd* reduces Dome accumulation, and cytoplasmic STAT92E is reduced (that is, nuclear localization becomes evident). (D) *awd* mutant clone is generated in the main-body follicle cells of a stage 7 egg chamber. Dome is overexpressed throughout the cytoplasm in the clone, as compared to the neighboring cells that show Dome expression in the cell periphery. Bars are 20 μm.

Receptor Domeless is also regulated by Awd in border cells. Besides receptor tyrosine kinase signaling, the JAK/STAT pathway is also needed for border cell migration (3, 17, 45, 53). This signaling is initiated from the surface receptor Domeless (Dome) and leads to nuclear translocation of STAT (45), which is required as a transcription factor for Yan expression (45). In contrast to vertebrates, in which multiple surface receptors activate JAK/STAT pathway, Dome is essential for all JAK/STAT functions in *Drosophila*. Also, similar to Pvr, border cell-specific overexpression of Dome or Dome-green fluorescent protein (GFP) fusion halts cell migration (17). Apart from this, it is known that Dome is continually endocytosed throughout the border cell migration phase and this is crucial for normal migration and signaling through Dome (45). Dome signaling strength can be verified by transcription factor STAT92E activation. When Dome is active, STAT92E is predominantly nuclearly localized and this has been used as a reliable and sensitive marker for Dome signaling strength in border cells. We therefore tested the effects of Awd expression on Dome and on the subcellular localization of STAT92E by inducing Shi^{K44A}-mediated accumulation of Dome and subsequent rescue by Awd. In control border cells, Dome is present in punctate structures, accompanied by predominantly nuclearly localized STAT92E (Fig. 6A). In border cells overexpressing Shi^{K44A}, Dome accumulates in large aggregates at or near the cell membrane, as anticipated, and STAT92E distribution is now shifted to roughly even nuclear and cytoplasmic staining (Fig. 6B), indicating high levels of Dome signaling that overwhelms the nuclear transport mechanism for STAT92E. However, when Awd is coexpressed with Shi^{K44A} (Fig. 6C), surface accumulation of Dome is reduced and the predominant nuclear localization of STAT92E is restored (highlighted by reduced cytoplasmic STAT). We also showed that *awd* mutant clones in the main-body follicle cells exhibited significant overaccumulation of Dome, confirming that Awd is a negative regulator of Dome expression (Fig. 6D).

Our results indicate that Pvr and quite possibly a wider spectrum of membrane proteins are targets for the action of Awd and, therefore, removal of Awd prior to and during border cell migration is an important developmental event to ensure the activity and adequate signal strength of these multiple pathways. Importantly, we observed that Awd did not alter the expression of a nonreceptor mammalian membrane protein CD8 exogenously expressed in the border cells (see Fig. S2 in the supplemental material). Thus, the activity of Awd may not be promiscuous and may be limited to active signaling receptors.

DISCUSSION

In this report, we described a novel role of a negative regulator of directional migration in border cells. Specifically, we studied the significance of developmentally regulated loss of Awd expression in border cells during their active migratory phase. We show that ectopic expression of Awd effectively blocks border cell movement, suggesting that Awd is involved in modulating the directional movement of the border cell complex. Conversely, we observed a high level of Awd expression in the nonmigrating follicle cells, possibly promoting rapid turnover of surface receptors to prevent ectopic cell migration.

Indeed, loss of *awd* in these cells and in premigratory border cells resulted in up-regulation of Pvr and stalling of border cells, consistent with the phenotype of overactive Pvr signaling reported previously (14) and our unpublished observation of overexpression of wild-type Pvr. Our results show that the function of Awd is to promote down-regulation of the surface receptors such as Pvr and Dome, in collaboration with Shi/dynamin, thereby regulating the chemotactic signal strength. Although the function of Awd has been linked to dynamin (9, 25), this report is the first on the relevance of the Nm23/Awd antimetastasis function in an analogous developmental model. We have demonstrated that border cell migration is stalled by both ectopic expression of Awd in the migrating cells and knockdown of Awd in premigrating cells, although through opposite consequences of reducing and increasing Pvr expression, respectively. This is consistent with the published finding that both loss of function and gain of function in Pvr signaling can disrupt border cell migration, due to loss of chemotactic signal or overwhelming signal, respectively. It was proposed and subsequently demonstrated by time-lapse microscopy that border cells that are oversaturated with Pvf signaling spin around without moving forward, consistent with the overborne, nondirectional chemotactic signaling response; while *pvr* loss-of-function border cells do not move (5). That is, although the phenotypic outcomes are the same, the cellular behaviors of the two genetic conditions are just the opposite.

At this time, the cellular events that precisely down-regulate Awd expression in migrating border cells remain unknown. However, our observations suggest that the regulatory mechanism, besides potential transcriptional regulation, could at least in part be posttranscriptional. For example, the *slbo-GAL4* driver can usually induce very high levels of ectopic expression, as evidenced by the expression of *UAS-λpvr* in our study. However, with *UAS-awd* (without the endogenous 3′ untranslated region), we could at best achieve a level equal to the endogenous one in nearby follicle cells and very often much lower.

As mentioned earlier, the histidine-dependent phosphotransferase activity of Nm23/Awd has functional correlation with the production and usage of GTP and the Awd-GTP link is worth noting since dynamin is a GTPase. In a classic study to identify components of eye color pathway, one peculiar, otherwise healthy mutant caused dominant lethality in the viable eye color *prune* null mutant background (49). This dominant conditional lethal allele was named *Killer-of-prune* (*K-pn*) and turned out to be a missense mutant allele of *awd* (7). This is highly interesting because the *Drosophila* eye pigmentation is determined by pteridines that is also a precursor of essential enzyme cofactors. The rate-limiting enzyme in pteridine biosynthesis is GTP cyclohydrolase, which uses substrate GTP to generate dihydroneopterin triphosphate. It was suggested that the Prune protein, which contains pyrophosphatase activity, stabilizes or promotes Nm23/Awd multimeric protein activity by channeling the phosphate. It is possible that Awd and Prune proteins together form a relay system for generating GTP. Therefore, the *K-pn* mutation of *awd* in the *prune* mutant background renders the phosphate transfer function of the Prune-Awd protein complex even less stable. Indeed, among the myriad of interacting proteins of Nm23 in mammalian cells, many are related directly or indirectly to the GTPases, such as Arf6 (40), TIAM1 (a guanine exchange factor for Rac) (38), Lbc (a guanine exchange factor for Rho) (21), and Rad (50). Whether or not these GTPase-related functions hold true requires further in vivo investigation. Recently, the lysophosphatidic acid receptor EDG2 was found to be overexpressed in Nm23-H1 mutant metastatic breast cancer cells, which can account for the metastatic activity of this cell line (18). However, whether the up-regulation is a direct or downstream effect of Nm23 loss of function is not clear. It therefore remained to be determined whether the similar receptor down-regulation mechanism by Awd observed in this report is applicable to EDG2 regulation.

It should be noted, however, that although the observed genetic interaction between *awd* and *shi* suggests that Awd may promote the endocytic activity of Shi/dynamin, it is formally possible that Awd may promote protein turnover that is downstream of the initial endocytic event. On this note, it is also worth considering other activities of Nm23/Awd. Our results showed that substitution of the active-site histidine residue that is critical for the nucleoside diphosphate kinase activity could not stall border cell migration. This is consistent with previous finding that this residue is required for rescuing the *enhancer of shi* phenotype (25). Curiously, this residue is not required for suppressing the in vitro motility (assayed by Boyden chamber) of the metastatic breast cancer cells (16, 29). However, the histidine substitutions employed in the two systems are different (phenylalanine in human versus alanine in fly). It is therefore difficult at this time to draw a direct comparison. On the other hand, human mutants that affect the histidine-dependent protein kinase activity failed to suppress the in vitro motility of the cancer cells (16, 29). So far, very few Nm23 protein kinase targets have been identified and none verified in physiological settings (48). Nonetheless, the protein kinase activity may be of specific functional significance since the range of targets is likely limited (48), so that specific pathways that contribute to metastasis may be identified more readily. The border cell migration model describe here should be used in future studies to test the functions of Nm23/Awd based on the above-mentioned human mutations.

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