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# Guidance of Cell Migration by the *Drosophila* PDGF/VEGF Receptor

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## Summary

**Directed cell migrations are important for development, but the signaling pathways and mechanisms responsible for guiding cell migration in vivo are poorly understood. Migration of border cells during *Drosophila* oogenesis is a simple and attractive model system in which to address these questions. We demonstrate that PVR, a receptor tyrosine kinase related to mammalian PDGF and VEGF receptors, acts in border cells to guide them to the oocyte. The oocyte is the source of a ligand for PVR, PDGF/VEGF factor 1 (PVF1). Intriguingly, the guidance function of PVR is largely redundant with that of EGFR. We present evidence implicating Rac and the Rac activator Mbc/DOCK180/CED-5 as mediators of the guidance signal.**

## Introduction

The directed migration of single cells or groups of cells takes place at multiple times during animal development. Guidance by external spatial cues is essential for the migrating cells to reach the correct target tissue. In the adult organism, cells involved in inflammatory responses are guided to sites of injury, and metastasizing tumor cells may be guided to target tissues (Müller et al., 2001). For cells to migrate in vivo, they must be motile, have adhesion to, and traction on, the relevant substrate, and often need to be actively invasive. To understand how cell migration is controlled, it is therefore necessary to understand how guidance signals control the cellular properties of motility, substrate adhesion, and deadhesion, as well as invasive behavior. Some aspects of the cell migrations that occur in multicellular organisms can be modeled in simpler systems, such as migration of mammalian tissue culture cells or *Dictyostelium* amoebae. Studies on these systems have led to an understanding of how cells can sense a gradient of attractant and crawl toward it (Lauffenburger and Horwitz, 1996; Firtel and Chung, 2000; Parent and Devreotes, 1999). Guidance of cell migration is also likely to show some mechanistic similarity to axon pathfinding, the guided movement of a cellular extension. Indeed, some guidance cues have been shown to be used for both axon pathfinding and cell migration (Wu et al., 1999; Holder and Klein, 1999; Wilkinson, 2001).

We have chosen to study guidance of cell migration directly in vivo. The migration of border cells in *Drosophila* is a relatively simple model for directional migration in a genetically tractable system. Border cells are a cluster of 6–10 specialized somatic follicle cells which perform a stereotypic migration during oogenesis (King, 1970). At the beginning of stage 9 of oogenesis, border cells delaminate from the anterior follicular epithelium and initiate their migration between the germline derived nurse cells, toward the oocyte. About 6 hr later, at stage 10, the border cells reach the oocyte, and then migrate a short distance dorsally toward the germinal vesicle (GV). Thus, the migration occurs in two steps: an initial posteriorly directed (oocyte directed) migration, and subsequently, a shorter, dorsally directed migration. The migration of border cells is essential for female fertility (Montell et al., 1992).

We have recently shown that EGF receptor (EGFR) signaling is responsible for guiding the second part of border cell migration, the dorsal migration (Duchek and Rørth, 2001). However, EGFR signaling is not essential for the first phase of migration of border cells toward the oocyte, indicating that an additional cue must direct this migration. Here we identify a *Drosophila* ligand of the PDGF/VEGF family (PVF1) and its receptor, PDGF/VEGF Receptor (PVR), and show that they are required for the first phase of border cell migration. We find that PVR and EGFR act in a partially redundant manner to guide border cells to the oocyte. We also analyze the signaling pathway downstream of PVR responsible for guiding cell migration.

## Results

### A *Drosophila* Protein Related to PDGF and VEGF, PVF1, and Its Receptor, PVR, Affect Border Cell Migration

Cell migration is guided by one or more spatial (guidance) cues. We reasoned that uniform expression of a key guidance cue, or a rate-limiting component in its production, throughout the target tissue could be expected to confuse the migrating cells and thus cause inefficient migration. To identify candidate guidance molecules for border cell migration, we made use of a gain-of-function genetic screen (Duchek and Rørth, 2001). Controlled ectopic expression of random genes in the genome can be obtained using the modular misexpression, or EP element, system (Rørth, 1996). In a screen of 8500 EP insertion lines, we identified two lines which caused inefficient border cell migration when overexpressed in the germline, consistent with the possibility that a guidance cue was being expressed. The first of these lines directed expression of the EGFR ligand Vein (Duchek and Rørth, 2001). For the second one, EPg11235, sequencing of flanking DNA showed that the EP element was positioned to drive expression of transcripts corresponding to the predicted gene CG7103 (Figure 1A). The gain-of-function phenotype was reproduced by expressing a corresponding cDNA. This cDNA was sequenced and encodes a protein with a signal sequence and a PDGF domain. It shows highest similarity to PDGF and VEGF ligands from vertebrates (Figure

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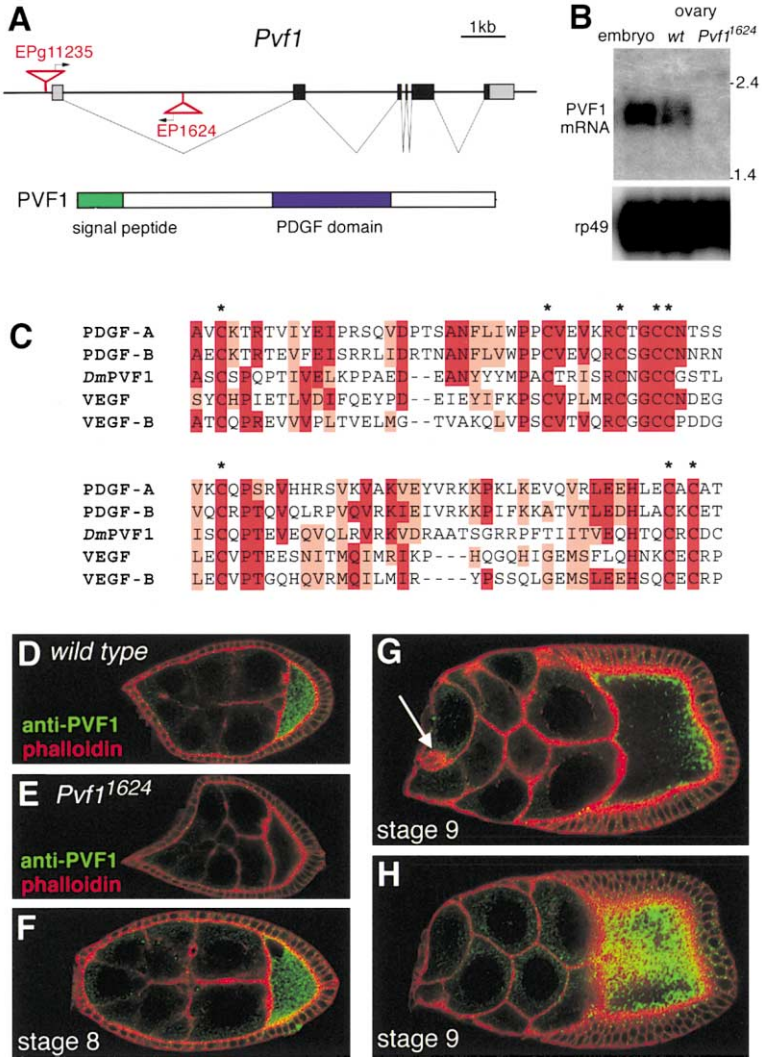


Figure 1. The PDGF-like Protein, PVF1, Is Found in the Oocyte at Mid-Oogenesis

(A) The *Pvf1* gene and encoded protein. The open reading frame (ORF) of the transcript is indicated as black boxes. The arrows on EP elements indicate direction of Gal4-induced transcription.

(B) Northern blot with PVF1 probe. Reprobing with a PVF1 ORF-only probe did not show the weak higher molecular weight band in the *Pvf1*$^{1624}$ lane, indicating that this transcript is truncated within the P element. rp49 serves as loading control.

(C) Alignment of PDGF domains; asterisks mark invariant cysteines. When the PDGF domains are individually aligned, PVF1 is 29% identical and 52% similar to human PDGF-A, and 25% identical and 50% similar to human VEGF.

(D–H) Egg chambers stained with anti-PVF1 antibody (green), counterstained with phalloidin to reveal the actin cytoskeleton (red). (E) is an egg chamber from a *Pvf1*$^{1624}$ homozygous mutant female, the rest are wild-type. (F) and (G) are two different confocal sections from the same stage 9 egg chamber. Migrating border cells are indicated by an arrow.

1C). We therefore call it PDGF/VEGF 1, PVF1. PVF1 affects border cell migration whether overexpressed uniformly in the germline (data not shown) or in border cells themselves, consistent with production of a secreted molecule. From an existing collection of P element insertions, one line, EP1624, was found to have an insertion in the first intron of the *Pvf1* gene (Figure 1A). This insertion is a loss-of-function mutant of *Pvf1 (Pvf1*$^{1624}$) with no detectable transcript remaining in the ovary (Figure 1B).

To investigate whether PVF1 could serve as a guidance cue for the migration of border cells to the oocyte, we first analyzed its expression. The *Pvf1* transcript was detected in the germline of the ovary at mid-oogenesis, more concentrated toward the oocyte (not shown). To look at the protein expression directly, we raised anti-PVF1 antisera. The anti-PVF1 sera showed specific staining in the ovary (Figures 1D and 1F–1H), which was absent from mutant egg chambers (Figure 1E). PVF1 was detected in the oocyte at stage 7 (Figure 1D) and at stage 8 (Figure 1F), filling the cytoplasm (the unstained GV is clearly seen in Figure 1F). At stage 9, when border cells have initiated migration, PVF1 is still enriched in the oocyte, but now only in the subcortical area of the large oocyte. Thus, the oocyte appears to be the major site of PVF1 protein production. PVF1 is expressed before and as the border cells migrate, consistent with the possibility that PVF1 serves as an attractant for border cells.

The *Pvf1*$^{1624}$ mutant was homozygous viable, and analysis of egg chambers from mutant females revealed minor delays in border cell migration. This phenotype will be described in more detail below. However, two additional PDGF, VEGF-like ligands appear to exist in *Drosophila* (see Experimental Procedures). To overcome the potential redundancy between PVF ligands, we therefore searched for a PVF receptor in order to directly investigate its role in border cells.

Gene predictions indicated that the *Drosophila* genome contained a single gene encoding a protein related in sequence and structure to mammalian PDGF and VEGF receptors (Figure 2A). We call the protein PVR for PDGF/VEGF receptor. It appears to be the only *Drosophila* member of this family of receptor tyrosine kinases, and thus could be the receptor for all three PVF ligands. PVR transcripts are detected in mRNA from ovaries and

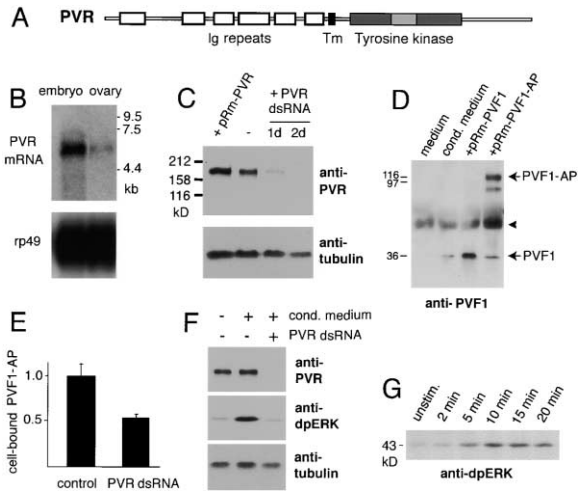


Figure 2. PVR Is a Receptor for PVF1

(A) Schematic drawing of the PVR protein. Tm indicates transmembrane segment. When the (split) tyrosine kinase domain of PVR is aligned individually with that of human PDGFR-α and VEGFR-1, the percent identity/similarity is 38/53 and 39/55, respectively.

(B) Northern blot of wild-type mRNA samples with a PVR probe.

(C) Western blot of total cell lysate from Schneider cells transfected with pRm-PVR, untreated (−) or treated with PVR dsRNA for 1 or 2 days. Anti-tubulin is used as loading control.

(D) Western blot of control medium (10% bovine serum) and conditioned medium from untreated Schneider cells or cells transfected with pRm-PVF1 or pRm-PVF1-AP expression vectors. The migration of the PVF1 band fits well with the predicted size of the secreted protein (32 kDa). Arrowhead points at a background band.

(E) Quantification of PVF1-AP binding to control cells or cells exposed to PVR dsRNA 3 days prior to testing. Cell-associated AP activity is indicated in arbitrary units. For both control and dsRNA samples, 6 independent samples were measured in duplicate. Similar numbers of cells were present as determined by protein content of total cell lysate.

(F) Western blots of total lysate from cells starved overnight in serum-free medium, then incubated for 10 min with medium (−) or Schneider cell conditioned medium (+). The PVR dsRNA sample was treated as in (E).

(G) Cells stimulated with conditioned medium for the indicated amount of time before harvest, as in (F).

from embryos (Figure 2B). *Pvr* mRNA is detected in embryonic hemocytes (not shown) and in the related tissue culture cells, Schneider cells. We raised antibodies directed against the C-terminal tail of PVR. Endogenous PVR protein was detected in Schneider cell extracts as an approximately 180 kDa protein (Figure 2C), corresponding well to the predicted molecular weight of 170 kDa. The protein band became undetectable after treatment of the cells with double-stranded RNA directed against *Pvr* (PVR dsRNA), confirming the specificity of the antibody (Figure 2C). As expected, a stronger signal was observed upon transfection with an expression construct for PVR (pRm-PVR).

We used Schneider cells to determine whether PVF1 could bind to and activate PVR. The anti-PVF1 antibody detected a specific band of about 36 kDa in conditioned medium from Schneider cells (Figure 2D). This appeared to be secreted PVF1, as a stronger signal at the same position was observed in conditioned medium from Schneider cells transfected with an expression construct for PVF1 (pRm-PVF1). For binding studies, a PVF1-Alkaline Phosphatase (AP) fusion protein was produced in Schneider cells and harvested in conditioned medium (pRm-PVF1-AP in Figure 2D). The PVF1-AP fusion protein bound to Schneider cells in a PVR-dependent manner, as binding was significantly decreased by pretreatment of the cells with PVR dsRNA (Figure 2E). The remaining binding of PVF1-AP to cells may be nonspecific sticking or binding to other proteins. To look at consequences of PVF1 binding to PVR, we monitored MAP-kinase activation in recipient cells by anti-diphospho-ERK (anti-dpERK) (Gabay et al., 1997) staining (Figures 2F and 2G). MAP-kinase was activated by conditioned medium containing PVF1. This activation was abolished by prior treatment of the cells with PVR dsRNA (Figure 2F). Maximal activation was observed in 10 min (Figure 2G). Thus, PVF1 binds to PVR, and PVR activates the MAP-kinase pathway in Schneider cells.

We next asked whether PVR mediated the effect of PVF1 on border cell migration and whether the effect was direct. Immunofluorescence analysis of wild-type ovaries indicated that endogenous PVR protein was present in all follicle cells and thus might respond to PVF1 (Figure 3A). We identified PVF1 based on the ability of uniform expression to impede border cell migration. Direct uniform activation of the PVF1 receptor in border cells should give the same effect or a stronger effect. To test whether PVR would do this, we made an activated form of the receptor, λ-PVR. This was done by exchanging the normal extracellular ligand binding domain for a constitutive dimerization domain, as has been done for other receptor tyrosine kinases (Lee et al., 1996; Queenan et al., 1997). We used the Gal4-UAS system (Brand and Perrimon, 1993) and the *slboGal4* driver (Rørth et al., 1998) to drive expression of λ-PVR in border cells, centripetal cells, and a few other follicle cells (Figure 3B). λ-PVR was functional in vivo as it stimulated the MAP-kinase pathway (dpERK staining in Figure 3D). Expression of λ-PVR in border cells also completely blocked their migration (Figure 3B). In over 90% of control stage 10 egg chambers, border cells had reached the oocyte, and the rest were only slightly delayed (control in Figure 3E). In contrast, almost none of the border cell clusters expressing λ-PVR had moved at all (λ-PVR in Figure 3E). Thus, uniform activation of PVR in border cells blocks migration, as expected for a guidance receptor.

Ectopic expression of the ligand PVF1 had a detectable but modest effect on migration: All border cell clusters had moved by stage 10, and one-third had arrived at the oocyte (PVF1 in Figure 3E). Increased expression of the wild-type PVR receptor in border cells had, on its own, a negligible effect on migration (Figures 3C and 3E), but it sensitized the cells to ectopic expression of PVF1. Upon coexpression of PVR and PVF1, one-fourth of the stage 10 border cell clusters were at the oocyte, but another fourth had not moved at all (PVF1+PVR in Figure 3E). The effect was specific to PVF1, as border cells were not sensitized to ectopic expression of the EGFR-ligand Vein. In fact, PVR overexpression ameliorates the effect of ectopic Vein expression (compare Vein and Vein+PVR in Figure 3E), a point which will be addressed further below. The synergy between PVF1

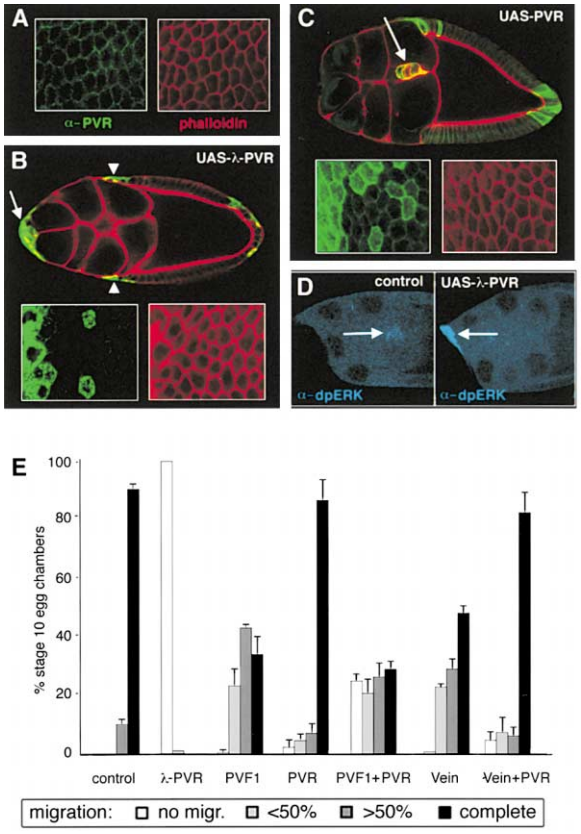


Figure 3. PVF1 and PVR Interactions in Border Cells

(A–C) Immunofluorescent staining with anti-PVR antibody (green) and phalloidin (red) as counterstain of wild-type (A), *slbo*[1],slboGal4/+; UAS-λ-PVR/+ (B), and *slbo*[1],*slboGal4*/+; *UAS-PVR*/+ (C) egg chambers. *slboGal4* drives expression in border cells (indicated by an arrow) and later in centripetal cells (indicated by arrowheads in B), plus variably in additional follicle cells. Border cells have migrated in (C), but not in (B). The boxed area is an enlargement of main body follicle cell staining with green and red channels shown separately. Endogenous (A) or overexpressed wild-type PVR protein (C) is predominantly at the cell cortex. λ-PVR appeared to be largely in intracellular vesicles (B).

(D) Control (*slbo*[1],*slboGal4*/+) and *slbo*[1],*slboGal4*/+; *UAS-λ-PVR*/+ (as in B) egg chambers stained with anti-dpERK antibody to visualize MAPK activation.

(E) Quantification of border cell migration was done by scoring all stage 10 egg chambers ($n > 200$ per sample) in females of the genotype *slbo*[1],*slboGal4*/+ and one copy of the indicated UAS-transgene or EP insertion (for *Pvf1*, *EPg11235*; for *vein*, *EPg35521*). *EPg11235* and *EPg35521* give similar migration delays when expression is driven uniformly in the germline by *nanos-Gal4:VP16*.

and PVR expression supports a specific interaction between the two proteins on border cells.

### PVR and EGFR Together Guide Migration of Border Cells to the Oocyte

To investigate whether PVR is required for guiding border cell migration to the oocyte, we generated a dominant negative form of the receptor, DN-PVR. DN-PVR was made in the same way as the highly specific dominant negative EGFR (O'Keefe et al., 1997). DN-PVR contains only the extracellular and transmembrane domains of the receptor, allowing it to sequester ligand as well as to form inactive dimers with the endogenous receptor, and thus specifically attenuate signaling from this receptor. When expressed in border cells, DN-PVR caused some delay of posterior migration (compare Figures 4A and 4B). This result was confirmed by quantification of migration at stage 10. Upon expression of DN-PVR, less than 60% of border cell clusters had reached the oocyte (DN-PVR in Figure 4D). This phenotype was similar to that seen in *Pvf1* homozygous mutant females (*Pvf1*[1624] in Figure 4D), indicating that PVF1 is the major endogenous ligand for PVR in this context. Thus, PVR signaling, and lack thereof, affects the efficiency of border cell migration, but it is not essential for the process.

In addition to PVR, EGFR also has properties consistent with a role in guiding border cells to the oocyte: both receptor tyrosine kinases are expressed in border cells, and their ligands are found in key locations in the germline. Both give similar gain-of-function effects, and both dominant negative receptors give subtle effects with respect to migration to the oocyte (Figure 4D). One possible explanation for the subtle dominant negative effects is that the receptor/ligand pairs are partially redundant. We first addressed this possibility by coexpressing both dominant negative receptors in border cells. This gave a very dramatic effect. Border cells expressing both dominant negative receptors migrated very inefficiently (Figures 4C and 4D). When quantified at stage 10, 90% of border cell clusters expressing both dominant negative receptors had migrated less than halfway to the oocyte (DN-PVR+DN-DER in Figure 4D). In 5% of egg chambers, border cell clusters were found off the direct track to the oocyte (Figure 4C). This suggests that the cells were motile but poorly guided. We did not observe this "off track" phenotype in wild-type egg chambers or in egg chambers where border cell migration is impaired for another reason (*slbo* mutant).

We also tested the effect of expressing dominant negative receptors in *Pvf1*[1624] mutant egg chambers. As expected, the *Pvf1*[1624] mutant phenotype was not made worse by removing activity of its cognate receptor, PVR. However, reducing activity of the other pathway by expression of dominant negative EGFR had a strong effect (*Pvf1*[1624]+DN-DER in Figure 4D). Border cells were not able to reach the oocyte by stage 10, and they also showed a low level of "off track" migration. This confirms the redundancy of function for the two receptors, as well as their ligand specificity. Thus, if either EGFR or PVR (and corresponding ligand) are left intact, border cells can find the oocyte, but if both receptor functions are severely affected, they cannot. That EGFR is uniquely required for dorsal migration of border cells is explained by the ligand distribution. Only EGFR ligands are expressed differentially on the dorsal side. Gurken is expressed by the dorsally located GV, and the protein is found in a gradient originating from there (Neuman-Silberberg and Schüpbach, 1996). Spitz and Vein are expressed in dorsal follicle cells.

Our results indicate that PVR and EGFR are guidance receptors for border cell migration toward the oocyte. A guidance function implies that the critical parameter for proper migration is the differential distribution of signal (ligand) rather than absolute level of signaling. This is supported by the observation that increased expression of PVR in border cells suppressed the effect

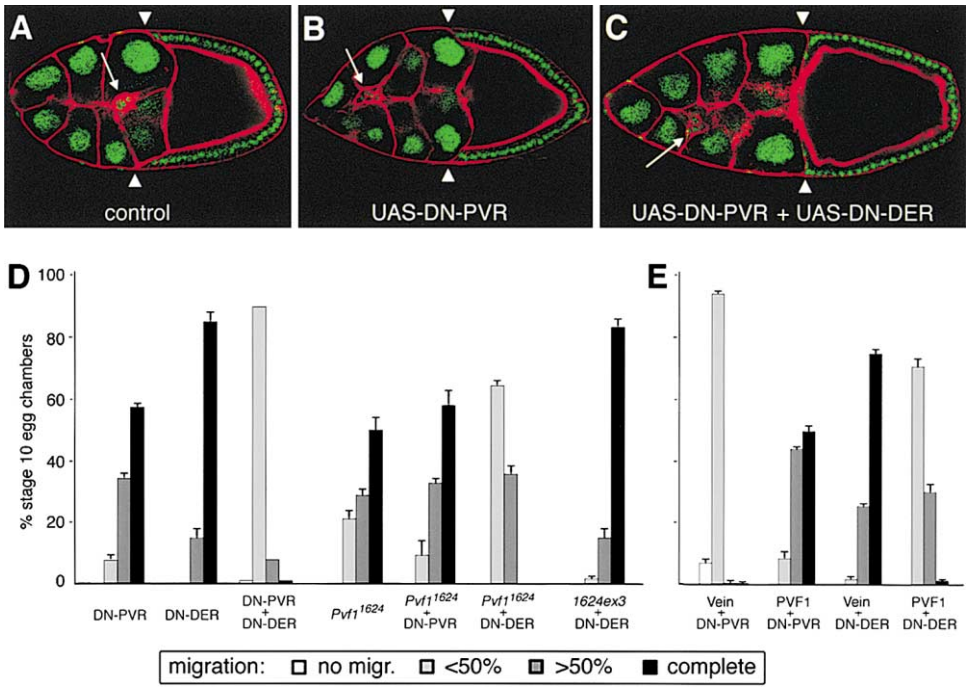


Figure 4. PVR and EGFR Are Required in a Redundant Manner for Border Cell Migration to the Oocyte

(A–C) Egg chambers from *slbo[1],slboGal4/+* (and indicated transgenes) females, stained with the DNA-dye DAPI (green) and phalloidin (red). Border cells are indicated with an arrow and, for reference, progression of general follicle cell movements with arrowheads. Typical stage 9 egg chambers are shown in (A) and (B), and a stage 10 egg chamber with the border cell cluster "off track" in (C). The F-actin (phalloidin) accumulation in border cells appears to be decreased upon expression of the dominant negative receptor(s).

(D–E) Quantification of border cell migration in stage 10 egg chambers ($n > 200$ per sample). Females were of the genotype *slbo[1], slboGal4/+* and one copy of the indicated UAS-transgene or EP insertion (PVF1 and Vein) and, where indicated, homozygous for *Pvf1[1624]* or the EP1624 excision allele (revertant) *EP1624ex3*.

of ectopically expressed EGFR ligand, Vein (Figure 3E). The level of PVR+EGFR signaling in border cells was likely higher upon coexpression, but the signal distribution might be more normal due to increased sensitivity to the spatially graded PVR ligand relative to the ectopically expressed EGFR ligand. To test the importance of signal distribution versus level more directly, we reduced signaling from one receptor by expression of its dominant negative form and asked whether the deleterious effect of ectopic ligand for the other receptor would be enhanced or suppressed. For guidance signaling, the expectation is that cells which can only respond to one type of ligand will require this ligand to be properly distributed and thus be very sensitive to its misexpression. If just the correct level of signal is required, then simultaneously increasing and decreasing signaling should give a less severe phenotype than either alone. The experiment was done for both receptors, and in both cases, we saw a strong enhancement of the migration defect (Vein+DN-PVR and PVF1+DN-DER in Figure 4E). Ectopic expression of one ligand and the dominant negative form of the other receptor caused a phenotype similar to one expressing both dominant negative receptors: border cells did not reach the oocyte at stage 10. They usually had migrated less than halfway, and sometimes were found off track. As expected, coexpression of a ligand with a dominant negative version of its cognate receptor had little or no additional effect. These results indicate that both receptors receive directional information which guides cell migration. Migration can proceed to some extent if only one receptor receives nonuniform (directional) signaling, consistent with a partially redundant guidance function.

## Signaling Downstream of PVR in Guiding Cell Migration

Similar to the *Drosophila* EGFR and to the mammalian PDGFR family, stimulation of PVR activates the MAP-kinase pathway in Schneider cells (Figure 2F) as well as in border cells (Figure 3D). However, we have previously shown by loss-of-function and gain-of-function experiments that MAP-kinase signaling does not affect border cell migration (Duchek and Rørth, 2001). In addition, we found no effect of phospholipase C-γ (PLC-γ) or phosphatidylinositol 3′ kinase (PI3K) on this migration, using loss-of-function mutants (PLC-γ) or border cell expression of dominant negative and dominant activated forms (PI3K) (Duchek and Rørth, 2001). This was somewhat unexpected, as PLC-γ and PI3K have been implicated in motility and guidance effects of RTKs (in particular PDGFR) in tissue culture cells (Wennstrom et al., 1994; Chen et al., 1994; Kundra et al., 1994). To address how PVR signaling might be affecting cell migration in vivo, we decided to test the effect of PVR signaling on cell morphology and cytoskeleton. In border cells as well as in other follicle cells, expression of λ-PVR had a dramatic effect on the actin cytoskeleton (phalloidin staining (red) in Figure 5A). Massive F-actin

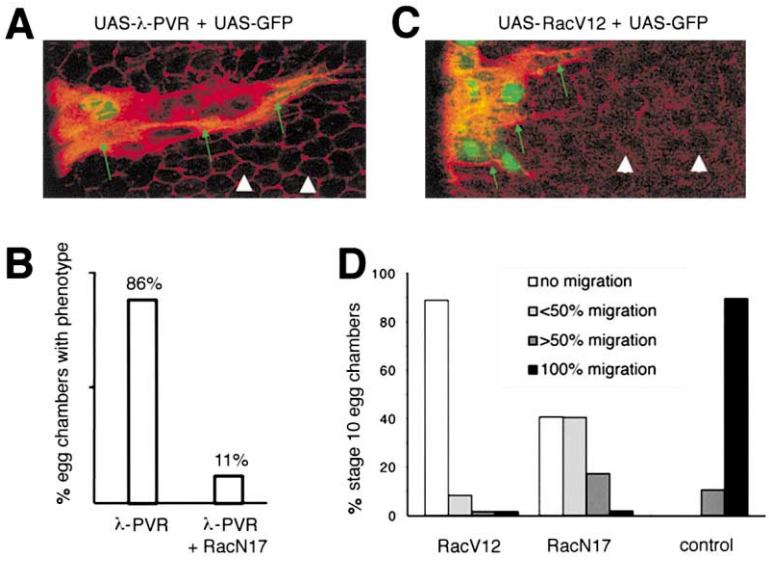


Figure 5. PVR Affects the Actin Cytoskeleton in a Rac-Dependent Manner

(A) A clone of follicle cells expressing activated PVR (λ-PVR) and the clone marker GFP (green). Phalloidin staining of F-actin is in red. Green arrows mark expressing cells and their F-actin rich extensions; white arrowheads indicate control cells. Stage 8 egg chamber with actin-Gal4 "flip-out" clone, genotype *hsFLP/actin>>Gal4;;UAS-λ-PVR/UAS-GFP*.

(B) Suppression of activated PVR-induced phenotype by dominant negative Rac (RacN17). Absence or presence of the actin phenotype shown in A was quantified in stage 7–8 egg chambers of the genotype *hsFLP/actin>>Gal4;;UAS-λ-PVR/UAS-GFP* and *hsFLP/actin>>Gal4;;UAS-λ-PVR/UAS-RacN17.* The same suppression of the phenotype was seen with *hsFLP/actin>>Gal4;UAS-λ-PVR/+; UAS-RacN17/UAS-GFP.*

(C) A clone of follicle cells expressing activated Rac (RacV12) and the clone marker GFP (green). Staining as in (A). Stage 8 egg chamber, genotype *hsFLP/actin>>Gal4; UAS-RacV12/+;UAS-GFP/+*.

(D) Both activated and dominant negative Rac arrest border cell migration. Quantification of border cell migration in stage 10 egg chambers. Genotypes: *slbo[1],slboGal4/+* and one copy of the indicated UAS-transgene.

accumulation, actin-rich extensions, and changes in cell shape were produced in λ-PVR expressing follicle cells (green arrows). The normal cells have modest cortical F-actin accumulation (white arrowheads). This result was likely to be relevant to the guidance function of PVR, as direct control of F-actin accumulation would allow receptor activation to control cell migration.

The actin cytoskeleton has been shown to be affected by small GTPases of the Rho superfamily in many systems (Hall, 1998), with the exact effects depending on the cellular context. In this system, Rac was an attractive candidate for mediating the effect of activated PVR, as dominant negative Rac (RacN17) had previously been shown to inhibit border cell migration (Murphy and Montell, 1996). Epistasis experiments could not be done by quantifying border cell migration because activated PVR and dominant negative Rac have the same effect. Instead, we tested whether Rac was required for the effect of PVR on the actin cytoskeleton in follicle cells. Coexpression of dominant negative Rac suppressed the effect of activated PVR on the actin cytoskeleton (Figure 5B). In addition, we found that follicle cells expressing activated Rac (RacV12) have dramatic accumulation of F-actin, resembling that caused by activated PVR (Figure 5C). Finally, if Rac were directly downstream of PVR, we would expect activated Rac to inhibit border cell migration, as observed for the activated receptor. Although a previous study reported that activated Rac did not affect border cell migration (Murphy and Montell, 1996), we reexamined this using the *slboGal4* driver and found that activated Rac completely blocked border cell migration (Figure 5D). These results are consistent with a role of Rac in the guidance pathway downstream of PVR.

In mammalian tissue culture cells, PDGF stimulation can cause Rac-dependent F-actin accumulation (Ridley et al., 1992) suggesting that the effect we observe in follicle cells may reflect a conserved pathway. PI3K has been implicated as a mediator of the effect of PDGFR on Rac in Swiss 3T3 cells (Nobes et al., 1995). However, PI3K does not appear to play a key role in guidance of border cell migration as discussed above. To investigate how PVR might lead to activation of Rac, we tested two groups of *Drosophila* mutants for their effect on border cell migration: mutants in genes shown to be downstream of receptor tyrosine kinases in other contexts, and mutants linked to Rac activation (Table 1). Most mutations were homozygous lethal, so their effect in border cells was tested by generating mutant clones in a heterozygous animal (mosaic analysis). Of the 8 genes tested, only *myoblast city (mbc)* had a detectable effect on border cell migration. Mbc is homologous to mammalian DOCK180 and *C. elegans* CED-5. Mbc/DOCK180/CED-5 acts as an activator of Rac (Kiyokawa et al., 1998; Nolan et al., 1998; Reddien and Horvitz, 2000).

We had independently identified *mbc* in a screen for gain-of-function suppressors of the *slbo* mutant phenotype (details in Experimental Procedures). *slbo* mutant border cells migrate poorly. Increased expression of *mbc* in *slbo* mutant border cells improved their migration, suggesting that *mbc* has a positive role in promoting border cell migration. Mbc protein was detected in follicle cells, including border cells (Figure 6A), and was overexpressed upon induction of the EP element EPg36390 located upstream of *mbc* (Figure 6B). Removing *mbc* function from border cells by generating mutant clones caused severe delays in their migration (Figures 6C and 6D). At stage 10, when 100% of control (GFP) clones have reached the oocyte, only 10% of *mbc* mutant border cell clusters had done so, and these were the oldest egg chambers. Thus, *mbc* is not absolutely required for border cell migration, but, contrary to the other genes implicated in RTK and Rac signaling (Table 1), loss of *mbc* function severely impairs this cell migration.

To test whether Mbc could act downstream of PVR,

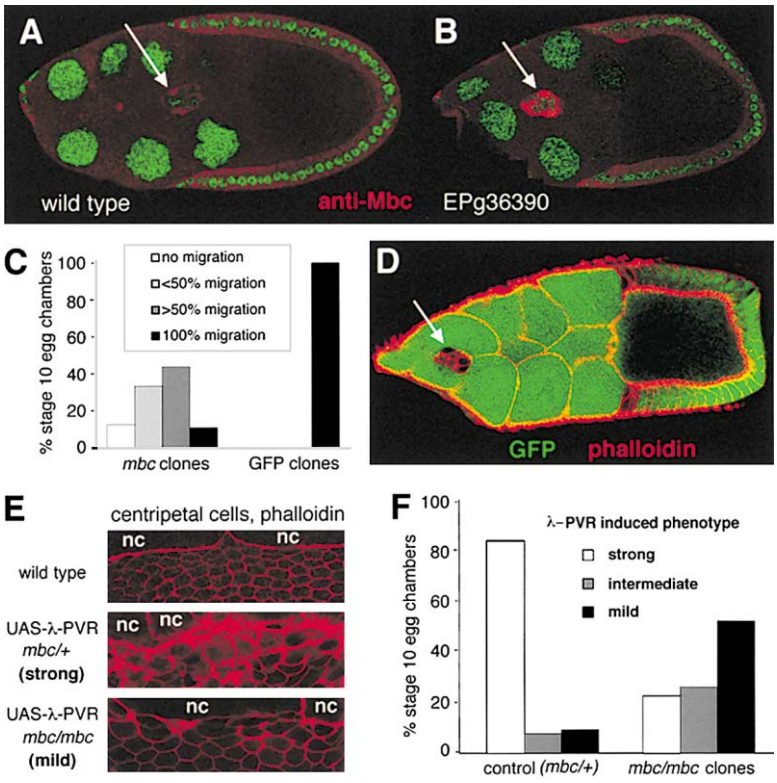


Figure 6. Mbc, an Activator of Rac, Is Required for Normal Border Cell Migration and Acts Downstream of PVR

(A and B) Egg chambers from wild-type or *slbo*[1]*,slboGal4/+; EPg36390/+* females stained with the DNA-dye DAPI (green) and anti-Mbc (red). EPg36390 is an insertion immediately upstream of the *mbc* gene.

(C) Migration of *mbc*$^{D11.2}$ border cell mutant clones (n = 57) and GFP control clones (n = 24) at stage 10. Similar results were obtained with *mbc*$^{C2}$ (strong allele).

(D) Stage 10 egg chamber with border cells (arrow) mutant for *mbc* (lack of GFP). Genotype: *hsFLP/+;;FRT82,mbc*$^{D11.2}$*/FRT82,ubiGFP.*

(E) Phalloidin staining of centripetal cells (adjacent nurse cells are labeled nc) in wild-type stage 10 egg chamber (top panel) or egg chambers expressing λ-PVR in centripetal cells (*slboGal4, UAS-λ-PVR*). In the bottom panel the cells are mutant for *mbc*.

(F) Quantification of the actin phenotype as shown in (E) in females of the genotype *hsFLP/+; slboGal4/UAS-λ-PVR;FRT82, mbc*$^{D11.2}$*/FRT82,ubiGFP* with somatic clones. *mbc/mbc* means all centripetal cells were mutant (n = 27); control means none were mutant (n = 525). Strong and mild phenotypes are illustrated in (E).

we focused on the effect of λ-PVR on F-actin accumulation in follicle cells (as outlined for Rac). In order to obtain *mbc* mutant clones in egg chambers which express λ-PVR, the experiment was done slightly differently than for dominant negative Rac. Expression of λ-PVR under control of *slboGal4* (shown in Figure 3B) caused disruption of centripetal cell morphology and abnormal actin accumulation (Figure 6E). When follicle cells were mutant for *mbc*, this effect was strongly attenuated (Figures 6E and 6F), indicating that Mbc acts downstream of PVR. Taken together, our results suggest that PVR affects guidance of border cell migration, at least in part, by signaling through Mbc to Rac, which then controls F-actin accumulation.

## Discussion

In this study, we have identified PVR as a guidance receptor for border cell migration and its ligand, PVF1, as the localized guidance cue. PVR appears to be the only member of the PDGFR/VEGFR family of receptor tyrosine kinases in *Drosophila*. Analysis of PVR may shed light on the basic conserved properties of this receptor family.

We find that PVR affects actin accumulation in follicle cells through Mbc and Rac. In addition, both Mbc and Rac are required for normal border cell migration. Together, these observations suggest that PVR signaling controls actin accumulation via Mbc and Rac in migrating border cells. Other receptor tyrosine kinases may also use this signaling module to guide cell migration in vivo. *mbc* was first identified in *Drosophila* based on its requirement in myoblast fusion (Rushton et al., 1995). Mbc has since been implicated in multiple processes requiring cytoskeletal reorganization, and is intriguingly expressed in early germ cells of the embryo, which undergo a guided migration (Erickson et al., 1997). Genetic data indicate that *mbc*, as well as the *C. elegans* homolog *ced-5*, acts as an upstream activator of Rac (Erickson et al., 1997; Nolan et al., 1998; Reddien and Horvitz,

Table 1. Clonal Analysis of Genes Implicated in RTK and Rac signaling

| Gene | Function | Homolog | Allele Used | Reference | B.C. Clones |
|---|---|---|---|---|---|
| Drk | adaptor | Grb2 | Drk$^{J03}$ | Raabe et al., 1995 | no effect |
| Dos | adaptor | (Gab2) | Dos$^{R31}$ | Raabe et al., 1996 | no effect |
| Shc | adaptor | Shc | Shc$^{111/40}$ | Luschnig et al., 2000 | no effect |
| Sty | RTK inhibitor | Sprouty | Sty$^{\Delta 5}$ | Reich et al., 1999 | no effect |
| Dock | adaptor | Nck | Dock$^{04723}$ | Garrity et al., 1996 | no effect |
| Pak | kinase | Pak | Pak[1] | Newsome et al., 2000 | no effect |
| Trio | adaptor-GEF | Trio | Trio[1] | Newsome et al., 2000 | no effect |
| mbc | Rac activator | DOCK180 | mbc$^{C2}$ | Rushton et al., 1995 | strong delay |
| | | | mbc$^{D11.2}$ | Erickson et al., 1997 | strong delay |

2000). No GTP-GDP exchange activity has been shown for Mbc/DOCK180/CED-5, but Mbc/DOCK180/CED-5 interacts with nucleotide-free Rac, indicating that it plays a role in activation or localization of Rac (Kiyokawa et al., 1998; Nolan et al., 1998). The small adaptor protein Crk interacts specifically with Mbc/DOCK180/CED-5 in all three systems (Hasegawa et al., 1996; Galletta et al., 1999; Reddien and Horvitz, 2000). In mammalian cells, Crk and another adaptor protein, p130-CAS, have been shown to regulate cell migration in a Rac-dependent manner (Klemke et al., 1998). Crk, CAS, and DOCK180 regulate membrane ruffling in a Rac-dependent manner (Cheresh et al., 1999). In *C. elegans*, CED-5, CED-2 (Crk), and CED-10 (Rac) are required for normal distal tip cell migration as well as cell engulfment, but the receptors regulating this behavior are not known (Reddien and Horvitz, 2000; Wu and Horvitz, 1998). Cell engulfment (phagocytosis) by mammalian 293T cells involves the $\alpha_v\beta_5$ integrin receptor which, in an unknown manner, can stimulate the formation of a p130-CAS-Csk-Dock180 complex and also activation of Rac1 (Albert et al., 2000). Thus, Mbc/DOCK180/CED-5 and Rac are linked in a well conserved signaling module which affects cell behavior, including migration. With the PVR receptor identified, it should now be possible to determine how this guidance receptor affects Mbc and Rac.

Signaling through Mbc and Rac is unlikely to be the only effect of the guidance receptors in border cells. *mbc* null clones give a phenotype which is stronger than loss of signaling from either receptor alone, but not as severe as loss of both PVR and EGFR activities. EGFR acts partially redundant with PVR in guiding border cells, but preliminary evidence suggests that EGFR may act differently than PVR (P.R., unpublished data). PVR may also have additional effects, given that the dominant effect of activated PVR on the actin cytoskeleton is strongly attenuated but not abolished in *mbc* null clones. Thus, the receptor pathways may be only partially overlapping, and other effectors are likely to contribute to the complicated task of guiding cell migration in vivo. We have tested many candidate signaling molecules for their requirement in border cell migration: MAPK pathway, PI3K, PLC-$\gamma$ (Duchek and Rørth, 2001), as well as RTK adaptors, DOCK, Trio, and Pak (this study, Table 1). Neither of these was (individually) required; thus, Mbc and Rac remain the only identified downstream signaling effectors in this context. A number of other genes have been shown to be important for border cell migration, but these are either transcription factors and modulators thereof which are likely to affect cell fate (Montell et al., 1992; Bai et al., 2000; Liu and Montell, 2001), or components of the basic cellular machinery for movement/adhesion (Chen et al., 2001; Edwards and Kiehart, 1996; Niewiadomska et al., 1999).

Receptor tyrosine kinases serve multiple roles during development. The ability of PVR to activate the MAP-kinase pathway may be important for control of cell growth and differentiation in other tissues, as is the case for EGFR (Schweitzer and Shilo, 1997). Both EGFR and PVR retain the ability to activate the MAP-kinase pathway when serving the guidance receptor function in border cells, indicating that they can simultaneously display multiple signaling properties. The mammalian PDGF receptors and VEGF receptors also have multiple functions during development and in tissue culture cells, including effects on proliferation and on cell migration. Which pathways downstream of these receptors are critical for which function, and how cell type specific responses are generated, remains an important question. Studies of mice with targeted mutations in specific tyrosines of PDGFR-$\beta$ indicate that the requirements for specific docking sites in vivo are not easily predicted from the effect of the same mutations in tissue culture cells (Tallquist et al., 2000). There may be more compensation and redundancy in vivo, or the importance of different pathways may simply differ in vivo and in tissue culture. In either case, such findings underscore the importance of in vivo analysis. We have analyzed guidance signaling in a simple and well defined cell migration process in vivo. Based on analyses of mutations in signaling pathway components, we can rule out essential involvement of some pathways and implicate another putative pathway (Mbc-Rac) downstream of PVR (and EGFR). There may be some redundancy in downstream pathways leading to guided border cell migration. In the case of MAP-kinase pathway and PI3K, we investigated both loss-of-function and gain-of-function (constitutive activation) mutations. If a signaling molecule was instructive but redundant, then ubiquitous activation would probably have some effect. However, border cell migration was unaffected, arguing that these signaling molecules do not play instructive roles.

It is intriguing that, even in this simple cell migration system, there is substantial redundancy between the guidance cues (and between the guidance receptors). It is not a priori obvious that these two different types of receptor tyrosine kinases should show such overlap in function. Redundancy in biological functions of receptor tyrosine kinases is likely to be even more prominent in mammalian systems, which have multiple receptors of each type. Subtle effects of individual factors and genetic redundancy are more the rule than the exception in analysis of axon guidance. It is interesting to speculate that partial reliance on multiple signals is biologically advantageous for continuously and subtly modulated processes such as guidance, as compared to all-or-none cell fate determination switches.

## Experimental Procedures

### Analysis of *Pvf1* and *Pvr*

The *Pvf1* gene is located at 17E. Sequencing of plasmid-rescued DNA showed that EPg11235 was positioned about 100 nt upstream of the LD30334 cDNA, which was sequenced. Two additional predicted genes encoding proteins with PDGF motifs, *Pvf2* and *Pvf3*, are located at 27D–E. Transcripts encoding PVF2 and PVF3 were detected by RT-PCR on ovary mRNA. The cDNA sequences for *Pvf1*, as well as for *Pvf2*, *Pvf3*, and *Pvr*, have recently been submitted independently to GenBank. For Northern blot, a 1.8 kb EcoRI-XhoI or a 1.1 kb PvuI-XhoI (ORF only) fragment from LD30334 was used. EP1624 was mapped by the Berkeley *Drosophila* Genome project (BDGP). EP1624 excision alleles were generated and two of four tested showed reversion of $Pvf1^{1624}$ phenotypes (see also Figure 4D).

We used the PVR cDNA SD04172 for all of our studies. SD04172 retains two small introns (position 893 and 1068), but produces functional protein of the expected size. A 1.5 kb EcoRI-XhoI fragment was used as a probe in Northern blot. To make UAS-PVR, full-length cDNA was cloned into UAST (Brand and Perrimon, 1993). To make $\lambda$-PVR, the adaptor GATCGCTAGCAGATCTACGAGCAGAAG CTGATCTCCGAGGAGGACCTG (encodes a Myc tag) was inserted

into the BglII site at amino acid 785 (N-terminal of the transmembrane domain). The BglII fragment encoding λ-repressor dimerization domain from λ-*btl* (Lee et al., 1996) was added and the resulting fusion cloned into pUASp (Rørth, 1998) as NotI-XhoI. DN-PVR was constructed by PCR with CGGAATTCAGATGGCGATGCTTCCGCG and GCTCTAGATTATCCGGCTGCCTTAAGGGC (from 5′ UTR to downstream of the region encoding the transmembrane domain) and cloned as EcoRI-XbaI into UASp2 (UASp (Rørth, 1998) with altered polylinker). Transgenic flies were made by coinjection with helper plasmid (Δ2-3) into $w^{1118}$.

### Fly Screens and Phenotypic Assays

The *slbo* suppression screen in which EPg36390 (*mbc*) was identified was performed as described in Rørth et al. (1998), but with a new set of EPg lines generated as described in Mata et al. (2000). Sequencing of plasmid-rescued DNA showed that EPg36390 was inserted 350 bases upstream of *mbc*. The *slboGal4* driver is described in Rørth et al. (1998), and quantification was done as described by scoring of Xgal-stained egg chambers. Quantification of migration in genetic interaction tests was done blindly.

For clonal analysis in border cells, the mutants listed in Table 1 were either obtained as, or recombined onto, the appropriate FRT chromosomes (Xu and Rubin, 1993) and their identity checked with deficiencies or other alleles. Recombination was induced by applying heat shock to animals of the genotype *hsFLP/+; FRT, mut/FRT, ubiquitinGFP* as larvae or adults, at least 4 days prior to analysis. For each genotype, we recovered at least 3 stage 10 egg chambers where all outer border cells were mutant.

For actin-flipout-Gal4 (actin>>Gal4) experiments, females were heat-shocked at 37°C for 20 min, incubated at 18°C for 4–5 days then shifted to 25°C for 16 hr before analysis. Stage 7–8 egg chambers were analyzed, as expression of either λ-PVR, RacV12, or RacN17 induced degeneration from stage 9 on. Scoring of the actin accumulation phenotypes in Figure 5B (λ-PVR +/− RacN17) and Figure 6F (λ-PVR +/− *mbc*) was done blindly, by scoring the egg chamber genotype (GFP expression) last.

### Antibodies, Staining, and Cell Culture

Fragments encoding the C-terminal 240 amino acids of PVR (SD04172) and amino acids 31 to 315 from PVF1 (LD30334), respectively, were PCR amplified and cloned into pGEX4 (Promega). The GST fusion proteins were expressed in *E. coli*, purified on glutathione beads, and used as antigens for polyclonal antibody productions in rats (PVR) or mice (PVF1).

Standard procedures were used for Northern blot and Western blots. For antibody and phalloidin staining of ovaries, fixation was done with 4% paraformaldehyde in PBS and all washes in PBS +0.1% triton X-100 (+5% normal goat serum in antibody incubations). Additional primary antibodies were: anti-Mbc (kind gift from Susan Abmayr), mouse anti-α-tubulin (Sigma), and rabbit anti-diphosphoERK (Sigma). Fluorescent secondary antibodies were from Jackson ImmunoResearch and rhodamine-phalloidin from Molecular Probes. All images were captured using confocal microscopy (Leica).

Schneider S2 cells were maintained in Schneider's medium +10% FCS and antibiotics. For stimulation experiments, cells were washed, incubated in serum-free medium overnight, washed, and incubated with conditioned medium or control medium. DNA transfections were performed with lipofectin (Gibco/BRL) according to instructions. Double-stranded RNA was also added with lipofectin (6 μg per 1 ml of lipofection mix). For *Pvr*, two nonoverlapping fragments of SD04172 (830 bp PstI-BglII and 900 bp BamHI-XhoI) were independently used (each transcribed in sense and anti-sense orientation). Both PVR dsRNA gave effects shown in Figure 2, which were not seen with dsRNA directed against other genes. Over 3 days incubation of cells with PVR dsRNA appeared to inhibit growth. *Pvr* and *Pvf1* cDNAs were cloned into pRmHa-3 (Bunch et al., 1988) and expression induced with 0.7 mM $CuSO_4$. The PVF1-AP fusion construct was made by replacing the coding region of Delta in pRm-AP-delta (Bruckner et al., 2000) with that of PVF1. The binding assays were performed as in Bruckner et al. (2000).

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