GTP exchange factor Vav regulates guided cell migration by coupling guidance receptor signalling to local Rac activation

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Summary

Guided cell migration is a key mechanism for cell positioning in morphogenesis. The current model suggests that the spatially controlled activation of receptor tyrosine kinases (RTKs) by guidance cues limits Rac activity at the leading edge, which is crucial for establishing and maintaining polarized cell protrusions at the front. However, little is known about the mechanisms by which RTKs control the local activation of Rac. Here, using a multidisciplinary approach, we identify the GTP exchange factor (GEF) Vav as a key regulator of Rac activity downstream of RTKs in a developmentally regulated cell migration event, that of the *Drosophila* border cells (BCs). We show that elimination of the *vav* gene impairs BC migration. Live imaging analysis reveals that *vav* is required for the stabilization and maintenance of protrusions at the front of the BC cluster. In addition, activation of the PDGF/VEGF-related receptor (PVR) by its ligand the PDGF/PVF1 factor brings about activation of Vav protein by direct interaction with the intracellular domain of PVR. Finally, FRET analyses demonstrate that Vav is required in BCs for the asymmetric distribution of Rac activity at the front. Our results unravel an important role for the Vav proteins as signal transducers that couple signalling downstream of RTKs with local Rac activation during morphogenetic movements.

Key words: Migration, Vav, Rac

Introduction

Directed cell migration plays a crucial role in many normal and pathological processes such as embryo development, immune response, wound healing and tumor metastasis (Friedl et al., 2004). During development, cells migrate to their final position in response to extracellular stimuli in the microenvironment. To migrate towards or away from a stimulus, individual cells or groups of cells must first achieve direction of migration through the establishment of cell polarity (Ridley et al., 2003). Guidance cues, such as growth factors, control cell polarization through the regulated recruitment and activation of receptor tyrosine kinases (RTKs) to the leading edge. A key event downstream of RTK signalling in cell migration is the localization of activated Rac at the leading edge (Ridley et al., 1992; Wang et al., 2010). However, little is known about the mechanisms by which external cues regulate Rac activity during cell migration. Rac is activated by GTP exchange factors (GEFs), which facilitate the transition of these GTPases from their inactive (GDP-bound) to their active (GTP-bound) states. Thus, GEFs appear as excellent candidates to regulate the cellular response to extracellular cues during cell migration.

Among the different Rac GEF families characterized so far, the Vav proteins are the only ones known to combine in the same molecule the canonical Dbl (DH) and pleckstrin homology (PH) domains of Rac GEFs and the structural hallmark of tyrosine phosphorylation pathways, the SH2 domain. In addition, Vav activity is regulated by tyrosine phosphorylation in response to stimulation by transmembrane receptors with intrinsic or associated tyrosine kinase activity (reviewed in Moores et al., 2000; Bustelo, 2000). These features make Vav proteins ideal candidates to act as signalling transducer molecules coupling growth factor receptors to Rac GTPase activation during cell migration. In fact, a number of cell culture experiments have suggested a role for the Vav proteins in cell migration downstream of growth factor signalling. Thus, the ubiquitously expressed mammalian Vav2 is tyrosine phosphorylated in response to different growth factors, including epidermal (EGF) and platelet-derived (PDGF) growth factors, and its phosphorylation correlates with enhanced Rac activity and migration in some cell types (Duan et al., 2011; Garrett et al., 2007; Liu and Burridge, 2000; Moores et al., 2000). However, the biological relevance for many of these interactions and the cellular mechanisms by which Vav regulates *in vivo* cell migration remains to be determined.

The Vav proteins are present in all animal metazoans but not in unicellular organisms. There is a single representative in

multicellular invertebrates and urochordata species (such as *C. elegans*, *Drosophila melanogaster* and *Ciona intestinalis*) and usually three representatives in vertebrates (Bustelo, 2001). The single *Drosophila vav* ortholog possesses the same catalytic and regulatory properties as its mammalian counterparts (Couceiro et al., 2005). In addition, the *Drosophila* Vav is tyrosine phosphorylated in response to EGF stimulation in *S2* cells (Hornstein et al., 2003; Margolis et al., 1992). Furthermore, a yeast two hybrid analysis has shown that the SH2-SH3 region of Vav can bind the epidermal growth factor receptor (EGFR) and the intracellular domain of PVR, PVRi, but not a kinase-dead version of PVRi, suggesting that Vav SH2-SH3-HA::PVRi interactions depend on PVR autophosphorylation (Bianco et al., 2007). Altogether, these results suggest that the role of mammalian Vavs as transducer proteins coupling signalling from growth factors to Rho GTPase activation has been conserved in *Drosophila*. Thus, we decided to take advantage of *Drosophila* to analyse *vav* contribution to growth factor-induced cell migration in the physiological setting of a multicellular organism.

The migration of the border cells (BCs) in the *Drosophila* egg chamber represents an excellent model system to study guided cell migration downstream of PVR/EGFR signalling *in vivo* (Montell, 2003; Rørth, 2002). Each egg chamber contains one oocyte and 15 nurse cells surrounded by a monolayer of follicle cells (FCs), known as follicular epithelium (FE). The BC cluster is determined at the anterior pole of the FE and it comprises 6–8 outer cells and two anterior polar cells in a central position (Montell et al., 1992). BCs delaminate from the anterior FE and migrate posteriorly between the nurse cells until they contact the anterior membrane of the oocyte (Fig. 1). BCs use the PVR and the EGFR to read guidance cues, the PDGF-related Pvf1 and the TGFα-related Gurken, secreted by the oocyte (Duchek and Rørth, 2001; Duchek et al., 2001; McDonald et al., 2006; McDonald et al., 2003). The Rho GTPase Rac is required for BC migration (Murphy and Montell, 1996). The current model proposes that higher levels of Rac activity present in the leading cell determine the direction of migration and that this asymmetric distribution of Rac activity requires guidance receptor input (Wang et al., 2010). The unconventional Rac GEF *myoblastcity*, *mbc*, is the only identified downstream signalling effector in this context. However, although genetic analysis have led to propose that the unconventional GEF for Rac, Mbc/DOCK 180, could activate Rac downstream of PVR during BC migration, this has not been formally proven (Duchek et al., 2001). In addition, *mbc* is unlikely to be the only Rac GEF actin downstream of guidance receptors in BCs as the migration phenotype due to complete removal of *mbc* is not as severe as the loss of both Pvr and Egfr. Thus, other effectors are likely to contribute to the complicated task of guiding BC migration. Many candidate molecules have been tested for their requirement in BC migration, MAPK pathway, PI3K, PLC-gamma, as well as RTK adaptors, such as DOCK, Trio, and Pak, but none of these is individually required.

Here, we use a multidisciplinary approach to identify the GEF *vav* as a key regulator of Rac activity downstream of guidance signals during BC migration. First, we show that *vav* is required for proper BC migration. Live imaging analysis reveals that *vav* is required for the stabilization and maintenance of protrusions at the front of the BC cluster. Second, stimulation of S2 cells with PVF increases tyrosine phosphorylation of Vav and its association with the phosphorylated form of PVR. Third, we show that Vav interacts genetically with both EGFR and PVR during BC migration. Finally, by FRET analyses, we demonstrate that Vav is required for the asymmetric distribution of Rac activity. Altogether, our findings uncover an essential role for Vav GEF as a signal transducer that couples signalling downstream of guidance receptors with local activation of the Rac GTPase in guided cell migration during development.

Results

Vav is required for BC migration

To examine the requirements for *vav* in BC migration, we analyzed mosaic BC clusters consisting of wild-type and *vav* mutant cells. We utilized two unrelated null alleles, vav^{3} and vav^{11837} (Malartre et al., 2010) that gave rise to indistinguishable phenotypes; however, in the text and in the figures we refer only

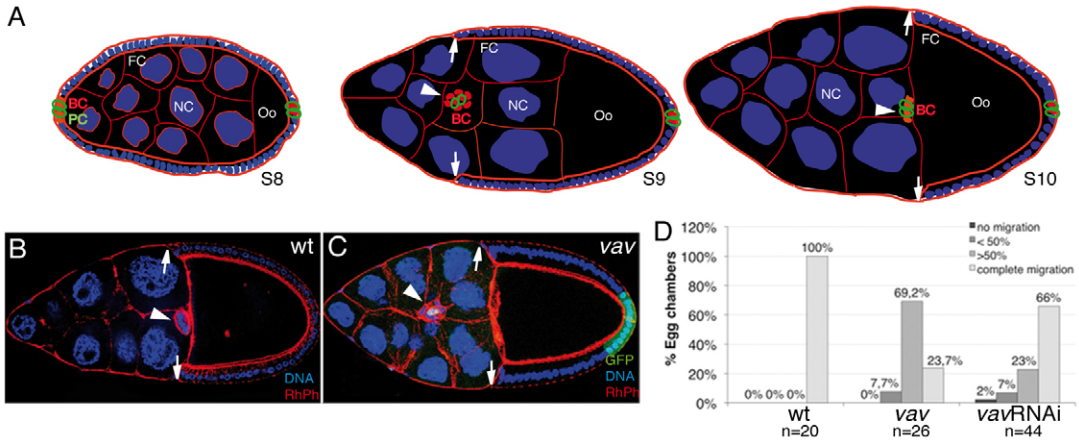

Fig. 1. Vav is required for BC migration. (**A**) Diagram of an ovariole showing stages 8, 9 and 10 egg chambers (S8–S10). NC, nurse cells; Oo, oocyte; FC, follicle cells; BC, border cells (red); PC, polar cells (green). White arrows mark the position of the main body FCs; arrowheads mark BCs. In all figures, egg chambers are labelled with anti-GFP (green), the nuclear marker TO-PRO-3 (blue) and rhodamine-phalloidin (Rh-Ph, red). (**B**) Wild-type and (**C**) *vav* mosaic S10 egg chambers. (B) Wild-type BCs complete their migration by S10 (arrowhead). (C) *vav* mutant BCs (labelled by absence of GFP, arrowhead) are delayed. (**D**) Quantification of BC migration phenotype of S10 egg chambers of wild-type (wt), *vav* mutant clusters and *vav*RNAi-expressing clusters.

to the results obtained with *vav*^{3}. The migration phenotype was quantified typically by scoring S10 egg chambers, which is when wild-type BCs reach the oocyte and migration has been completed (Fig. 1A). We used the following criteria: 0% refers to clusters that have not migrated at all and are found at the anterior pole of the egg chamber; <50% and >50% correspond to clusters that have migrated less or more than half the distance to the oocyte, respectively; and 100% indicates clusters with no defects in migration. Control flies showed no migration defects (n=26) (Fig. 1B,D), while only 23.7% of clusters containing a mixture of wild-type and *vav*$^{-/-}$ mutant BCs reached the oocyte at S10 (n=26) (Fig. 1C,D). The remaining 76% of mixed clusters showed delayed migration, with cells advancing only half the distance to the oocyte in 7.7% of the cases (Fig. 1C,D). Comparable results were found when a transgenic double-stranded RNA for *vav* (*UAS-vav*RNAi) was expressed in BCs (n=42) (Fig. 1D; Fig. 3B). These results show that *vav* is required for proper BC migration. These migration defects are similar to those found when the activities of either PVR or EGFR are reduced in BCs (Bianco et al., 2007; Prasad and Montell, 2007).

Vav regulates number, length and stabilization of cellular protrusions in the direction of movement

We next decided to investigate the dynamics of BC migration using time-lapse video recordings of living egg chambers and comparing the behaviour of control BCs to those with reduced *vav* function. In order to do this, we used *slbo*-GAL4 to drive the expression of UAS-GFP::Moesin in BCs to visualize actin filaments, and the vital dye FM4-64 to label all cell membranes (Prasad and Montell, 2007); (supplementary material Movies 1 and 2). In experimental ovaries, we also expressed a UAS-*vav*RNAi. We observed that although *vav*RNAi expressing BCs were individually active, the movement of the cluster was significantly much slower than the controls. We quantified this by measuring cell motility over a period of 2 hours using manual nuclear tracking (Fig. 2A–C, see Materials and Methods). We found that *vav*RNAi expressing BCs (n=12) move at a lower speed than controls (n=10) (Fig. 2C).

To further evaluate the dynamics of cell migration, we analyzed cellular protrusions of both experimental and control clusters at different time points (see Materials and Methods). Control BCs extend protrusions preferentially in the direction of migration (n=10) (Bianco et al., 2007; Fig. 2D; supplementary material Movie 1). In contrast, we found that although cells with compromised Vav function were able to extend protrusions, there was an increased in the number of protrusions formed in other directions (n=16) (Fig. 2D; supplementary material Movie 2). These results demonstrate that Vav is required for proper protrusion dynamics. Migration towards the oocyte has been divided in two phases, an early phase from detachment until halfway to the oocyte and a late one from this point until the

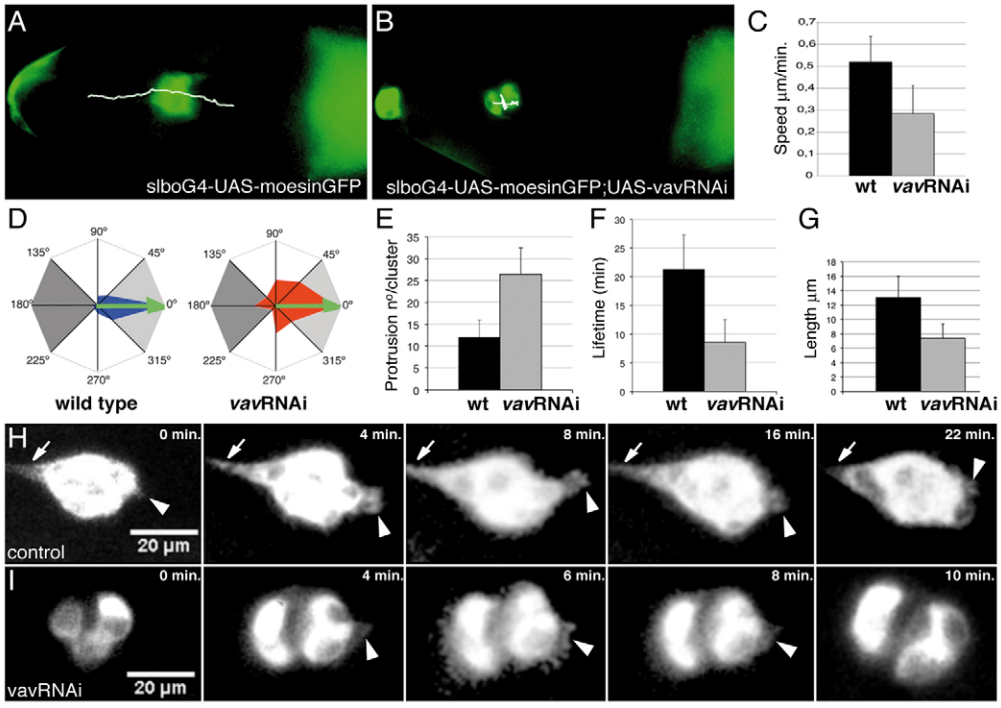

Fig. 2. Vav is required for protrusion formation at the leading edge. (**A**,**B**) Images from videos from controls (A; *slbo*Gal4/+; UAS-moesin-GFP) and from clusters expressing *vav*RNAi (B; *slbo*Gal4/+; UAS-moesin-GFP;UAS-*vav*RNAi) and the track of one nucleus. (**C**) Speed of a single tracked nuclei of wild-type and *vav*RNAi-expressing clusters. (**D**) Radial diagrams illustrating the distribution of protrusions in wild-type and *vav*RNAi-expressing clusters. The green arrow shows the direction of migration. (**E**–**G**) Histograms of protrusion number per cluster (E), protrusion lifetime (F) and length of front protrusions (G) in wild-type and *vav*RNAi-expressing clusters (values are means and s.d.). (**H**,**I**) Stills taken from live imaging of BCs expressing moesin-GFP undergoing the first phase of migration. Note that front protrusions (arrowheads) in control (H) are longer and more stable than in *vav*RNAi-expressing clusters (I). The arrow in H points to a lagging cell of the cluster that will delaminate at a later time point (supplementary material Movie 1). Differences in C, E, F and G are all statistically significant, P=0.0005, 0.02 and 0.0001, respectively, compared with wild type.

cluster contacts the oocyte (Poukkula et al., 2011). The bias in extending front protrusions over others is similar in both phases. In addition, front protrusions in both phases are on average more stable and longer than other protrusions, being this difference more notorious during the early phase (Poukkula et al., 2011). Thus, in order to analyse the role of Vav in protrusion dynamics, we focused our analysis on the early phase. We found that while depletion of *vav* induced more protrusions per cluster (Fig. 2E), the length of front protrusions was reduced compared to controls ($n=24$ and $n=29$, respectively) (Fig. 2G–I). In addition, the stability of front protrusions in *vav*RNAi expressing clusters ($n=38$) was reduced compared to controls ($n=27$), with an average persistence time over 10 and 22 minutes, respectively (Fig. 2F,H,I). Altogether, the results above show that the ability to grow, maintain and stabilize long front extensions is compromised when Vav function is reduced.

Vav regulates Rac activity in BCs

Vav proteins have been shown to act as GEFs for Rac GTPases in different cell culture models (reviewed in Bustelo, 2001). Here, we decided to test whether the *Drosophila* Vav could also act as a GEF for Rac in BCs. We used transgenic flies that express a Rac fluorescence resonance energy transfer (FRET) biosensor under the control of the GAL4 system (Wang et al., 2010). When expressed with Gal4 lines that drive expression in BCs, such as *slbo*-Gal4 or C306, a FRET signal is observed in BCs (Wang et al., 2010); (Fig. 3C). If Vav were required to activate Rac downstream of guidance signalling in BCs, we would expect Rac activation to be compromised in the absence of Vav function. To test this, we measured Rac activity in BC clusters expressing *vav*RNAi and compared it to wild type. We found that the average FRET efficiency was reduced in *vav*RNAi expressing clusters (Fig. 3C,D). A previous quantitative analysis of the FRET efficiency in different regions of the cluster showed that Rac activity is highest at the front and lowest at the back (Wang et al., 2010); (Fig. 3C,E). Furthermore, this asymmetry requires guidance receptor input (Wang et al., 2010). Thus, we next tested whether Vav was required for the asymmetric distribution of Rac activity by measuring FRET signal at the front and at the back in both control and *vav*RNAi expressing clusters. We found that the asymmetry in FRET signal between front and back was significantly reduced in *vav*RNAi expressing clusters (Fig. 3E). This analysis also revealed that the main difference in Rac activity between control and *vav*RNAi expressing clusters was found at the front of the cluster, the difference at the back was not statistically significant (Fig. 3E). These results strongly suggest that Vav is required for the asymmetric activation of Rac at the front of migration.

Expression in BCs of activated forms of Rac, PVR or EGFR blocked their migration and resulted in non-polarized massive F-actin accumulation (Duchek and Rørth, 2001; Duchek et al., 2001); (Fig. 4C–E). If Vav were able to activate Rac in BCs, one would expect expression of a constitutively active form of Vav to cause defects similar to those obtained upon ectopic Rac activation. We have previously shown that the total deletion of the CH region of *Drosophila* Vav (Vav^{act}) generates constitutively active mutant proteins with respect to its ability to activate Rac and to induce F-actin polymerization and morphological changes (Couceiro et al., 2005). We found that expression of Vav^{act} in BCs completely blocked their migration and resulted in a dramatic accumulation of F-actin, a phenotype that closely resembles that obtained upon expression of constitutively activated forms of PVR, EGFR or Rac (Fig. 4B–E).

Altogether these results strongly suggest that Vav could act as a signal transducer protein controlling Rac activity at the front of the cluster. Epistasis experiments quantifying BC migration could not be done because activated Vav and dominant negative Rac have the same effect. Thus, we decided to further test the ability of Vav to activate Rac in BCs by measuring Rac activity in BC clusters expressing the constitutively active form of Vav. We found that when Vav is activated equally in all cells of the

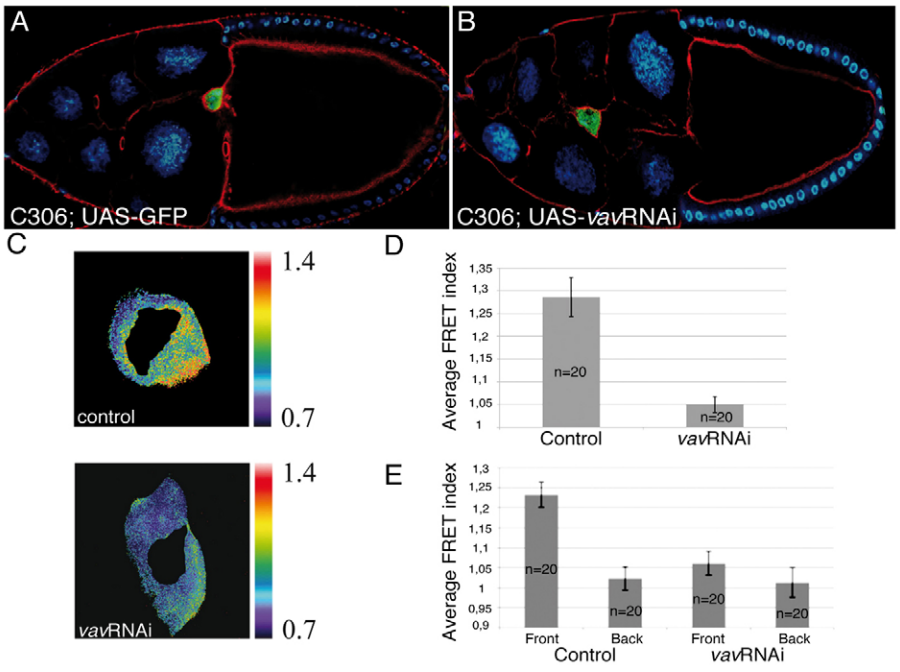

Fig. 3. Vav regulates Rac activity. (**A**) Wild-type egg chamber. (**B**) *vav*RNAi-expressing BCs. (**C**) Representative FRET patterns in control (*slbo*Gal4/+; UAS-RacFRET) and *vav*RNAi-expressing clusters (*slbo*Gal4/+; UAS-RacFRET; UAS-*vav*RNAi). (**D**) Average FRET efficiencies in wild-type and *vav*RNAi-expressing BCs, ($P=0.00021$). (**E**) Average FRET efficiencies at the front and at the back of wild-type and *vav*RNAi-expressing BCs. All differences are all statistically significant with P values between 0.0013 and 2×10^{-5}, compared with corresponding controls.

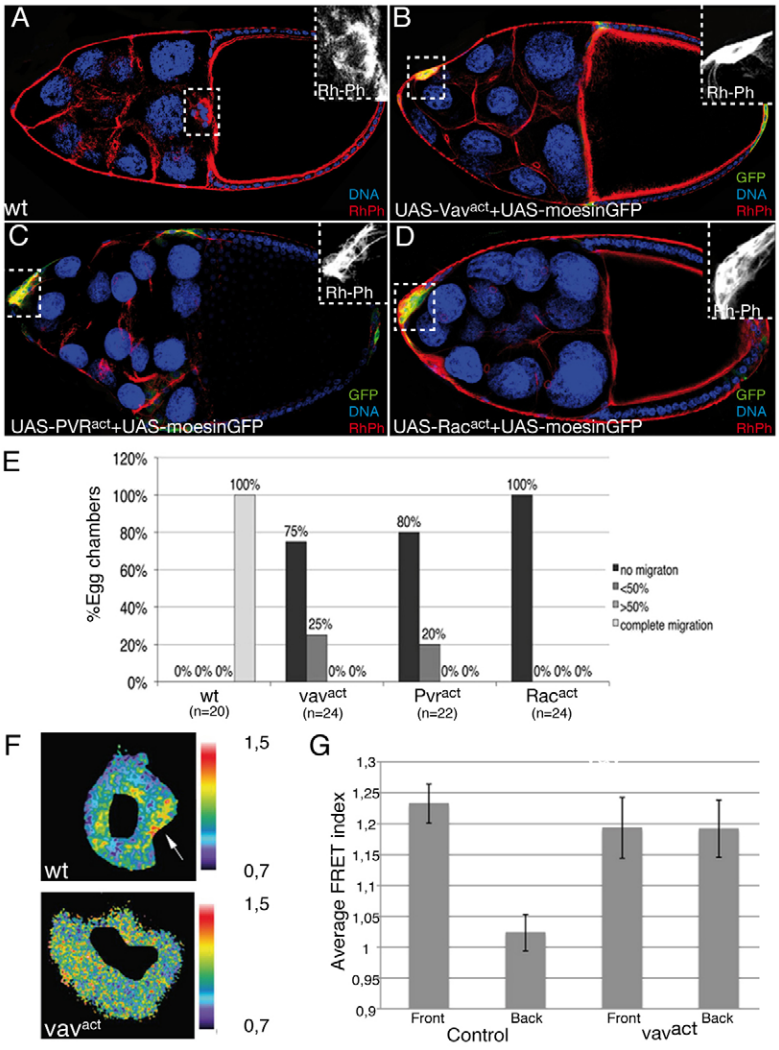

Fig. 4. Ectopic Vav activation inhibits BC migration. (**A**) Wild-type egg chamber. (**B**–**D**) Genotypes: *slbo*Gal4; UAS-moesinGFP and one copy of the indicated UAS-transgene. (B) Expression of an activated form of Vav (Vav^{act}) in BCs causes a strong migration phenotype, resembling that caused by activated PVR (PVR^{act}) (C) or Rac (Rac^{act}) (D). (**E**) Quantification of BC migration in S10 egg chambers of the indicated genotypes. (**F**) Representative FRET patterns in control (*slbo*Gal4/+; UAS-RacFRET) and Vav^{act}-expressing clusters (*slbo*Gal4/+; UAS-RacFRET; UAS-Vav^{act}). (**G**) Average FRET efficiencies at the front and at the back of wild-type and Vav^{act}-expressing BCs. Differences between front of wild type and front and back of Vav^{act}-expressing clusters are not statistically significant (P=0.517 and P=0.498, respectively).

cluster, Rac activity is distributed uniformly all over the cluster (Fig. 4F,G).

The *Drosophila* Vav is tyrosine phosphorylated in response to PVR stimulation and it associates with the stimulated receptor

The stimulation of cytokine, growth factor or antigen receptors by their ligands regulates the GEF activity of the mammalian Vav proteins via tyrosine phosphorylation. Interestingly, *Drosophila* Vav is also tyrosine phosphorylated in response to EGFR stimulation and it associates with the stimulated receptor in both mammalian and *Drosophila* S2 cells (Bustelo et al., 1992; Hornstein et al., 2003; Margolis et al., 1992). Here, we determined if PVF could also activate *Drosophila* Vav. In order to do this, we induced S2 cells with secreted PVF1 and followed the pattern of tyrosine phosphorylation of Vav (Fig. 5A). Comparable levels of Vav were found in stimulated and non-stimulated cells (Fig. 5A). Tyrosine phosphorylation of Vav was only observed when cells were stimulated with PVF1 (Fig. 5A, +PVF). In addition, immunoprecipitation of PVR from lysates of PVF1-stimulated S2 cells indicated that it associated with Vav (Fig. 5B). Similarly, immunoprecipitation of cell lysates with anti-Vav antibodies showed that it associated with tyrosine phosphorylated PVR (Fig. 5B). These experiments show that *Drosophila* Vav, as it is the case for its mammalian counterpart, can be tyrosine phosphorylated following the induction of signalling pathways by PVF1 and that it associates with the phosphorylated PVR.

The ability of Vav to bind PVR was further confirmed by two-hybrid analysis. A yeast two-hybrid screen using the intracellular domain of PVR (PVRi) as bait identified three partial clones coding for the Vav protein (Fig. 5C). All three isolated clones encoded a complete SH2 domain, which is known to interact with phosphorylated tyrosines, plus the C-terminal Vav SH3 domain (Fig. 5C). These results confirmed previous studies demonstrating the ability of Vav to bind EGFR and the intracellular domain of PVR, PVRi (Bianco et al., 2007; Dekel et al., 2000). Next, we performed a preliminary analysis of Vav binding sites using a battery of modified PVRi baits (Jékely et al., 2005); (Fig. 5D). We found that while a deletion of PVRi at residue E1352 (ΔXho) did not have any effect in the ability of PVRi to bind Vav, a C-terminal truncation at residue F1261 (Δ313) resulted in a strong decrease in binding (Fig. 5D). This result maps the binding of Vav SH2-SH3-HA to PVRi to a region of 91 amino acids, including residues F1261 to E1352. In addition, we found that this interaction became gradually weaker

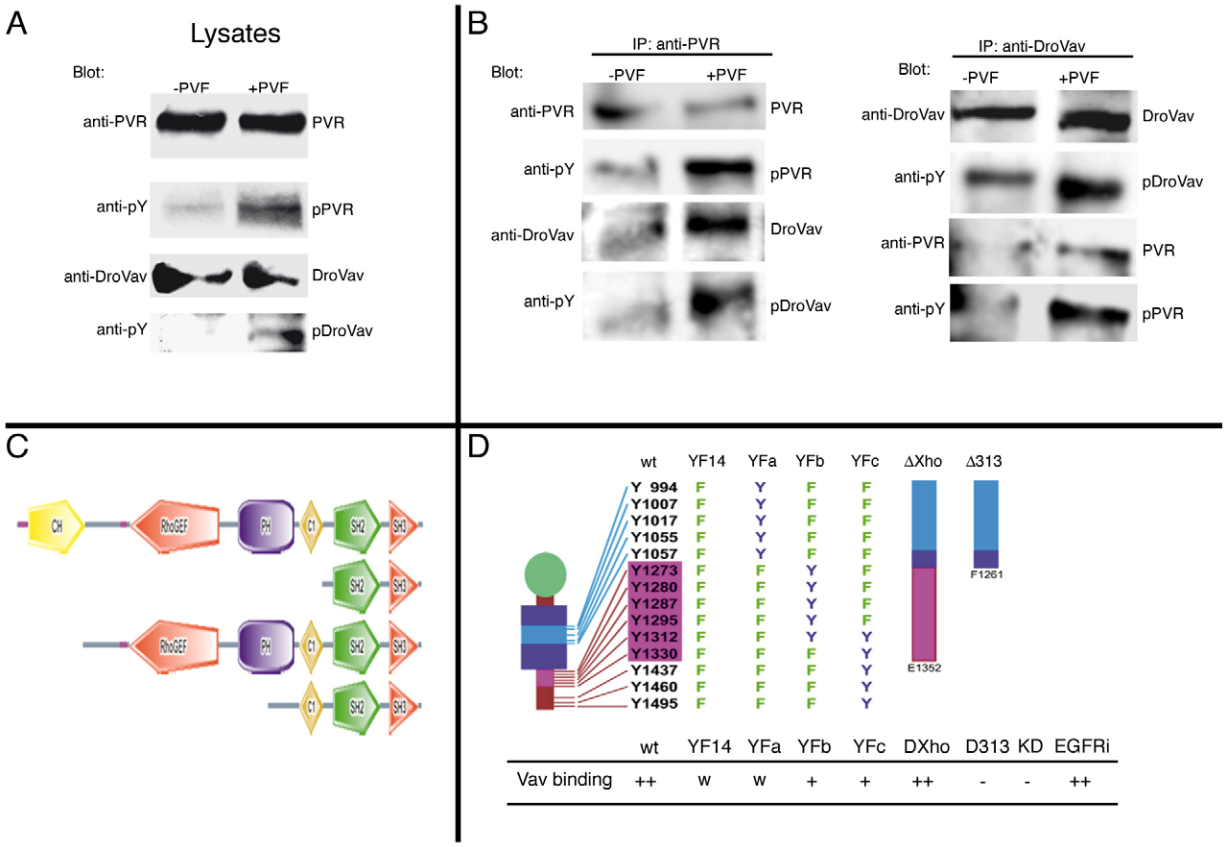

Fig. 5. Direct binding of PVR to Vav. (**A**,**B**) Extracts from control (−PVF) or PVF1-stimulated (+PVF) Schneider 2 cell extracts were subjected to western blot analysis (A) or were immunoprecipitated (B). (**C**) Scheme of three Vav partial clones isolated in the screen. (**D**) Scheme of the intracellular domain of PVR (PVRi) and mutations introduced in it. The truncation points are indicated by the last amino acid present in the construct, numbered below. Purple boxes highlight the PVR region required for interaction with Vav. Results from the yeast two-hybrid assay: ++, very strong interaction; +, strong interaction; w, weak interaction and −, no interaction.

as additional tyrosines were mutated (wild type >YFb=YFc >YF14; Fig. 5D).

Altogether, these results demonstrate that Vav can physically interact with the phosphorylated form of PVR and that this interaction could be modulated by tyrosine phosphorylation in different residues.

Vav interacts genetically with both EGFR and PVR

We next decided to explore the relationship between *vav* and the EGFR and PVR signalling pathways during BC migration. In order to do this, we manipulated signalling downstream of these receptors in *vav* mutant BCs. To attenuate signalling from either EGFR or PVR, we used dominant negative forms of the receptors, PVR^{DN} or $EGFR^{DN}$ (Duchek et al., 2001; O'Keefe et al., 1997). If Vav were required downstream of both PVR and EGFR pathways in BC migration, one would expect the phenotype of both PVR and EGFR mutant BCs to be enhanced when removing *vav*. Expression of any of these dominant negative forms in BCs has been shown to cause a delay in BC migration (Duchek and Rørth, 2001; Duchek et al., 2001); (Fig. 6C,E,G). We found that expression of either PVR^{DN} or $EGFR^{DN}$ in BCs mutant for *vav* enhanced the frequency and severity of the migration defects observed in the individual mutant conditions alone (Fig. 6D,F,G). This enhancement was stronger in the case of PVR. These interactions were confirmed by analyzing clones double mutant for *vav* and *egfr* and *vav* and *pvr* (supplementary material Fig. S1).

Thus, considering our results, the requirements for Vav in BC migration, the GEF activity of Vav, and the genetic and biochemical interactions between Vav and PVR, we propose that Vav acts downstream of both guidance receptors to regulate Rac activity during BC migration.

Discussion

Vav proteins were initially involved in lymphocyte ontology (Bustelo, 2000; Turner and Billadeau, 2002). Only recently, cell culture experiments have implicated these proteins in cell migration events downstream of guidance factors. Interestingly, Vav proteins can either promote or inhibit cell migration. In macrophages, Vav is required for macrophage colony-stimulating factor-induced chemotaxis (Vedham et al., 2005). In human peripheral blood lymphocytes, Vav is involved in the migratory response to the chemokine stromal cell-derived factor-1 (Vicente-Manzanares et al., 2005). Conversely, in Schwann cells, Vav2 is required to inhibit cell migration downstream of the brain-derived neurotrophic factor and ephrinA5 (Afshari et al., 2010; Yamauchi et al., 2004). In spite of the knowledge gained from cell culture experiments, the biological relevance for many

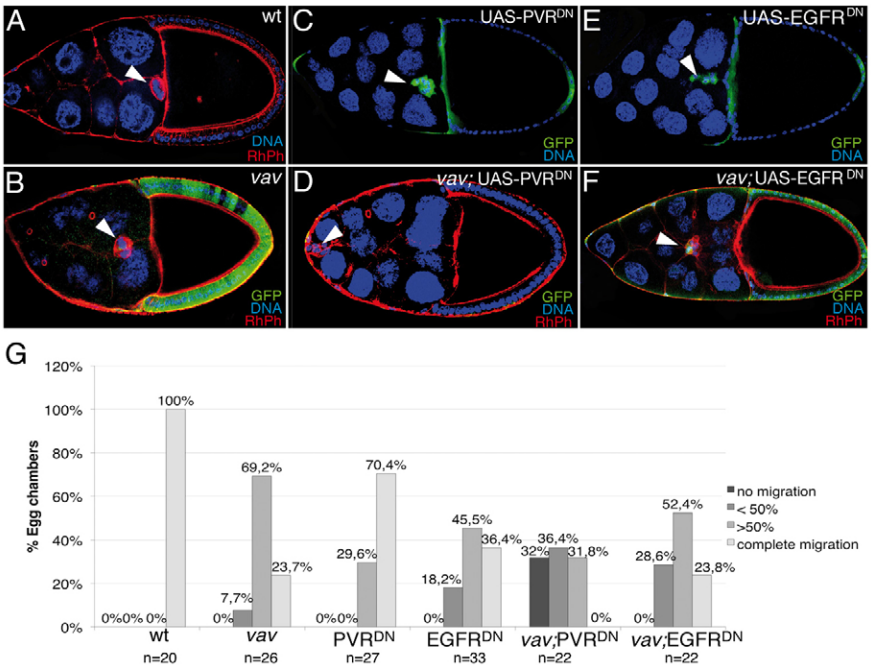

Fig. 6. Genetic interactions between Vav and PVR/EGFR. (**A**) Wild-type egg chamber. (**B**) BC cluster containing *vav* mutant cells labelled by the absence of GFP. (**D**,**F**) Reduction of *vav* function enhances the migration defects due to expression of dominant negative forms of PVR, PVR^{DN} (**C**) and EGFR, $EGFR^{DN}$ (**E**). (**G**) Quantification of BC migration in S10 egg chambers of the indicated genotypes. Arrowheads indicate BCs.

of the above interactions has remained elusive. In recent years, Vav proteins have started to emerge as critical Rho GEFs acting downstream of RTKs in diverse biological processes (Cowan et al., 2005; Hunter et al., 2006). Analysis of $Vav2^{-/-}Vav3^{-/-}$ mice revealed retinogeniculate axonal projection defects (Cowan et al., 2005) and impaired ephrin-A1-induced migration during angiogenesis (Hunter et al., 2006), suggesting a role for Vav in axonal targeting and angiogenesis downstream of Eph receptors *in vivo*. Here, we show that Vav can act downstream of growth factors receptors to promote BC migration in the developing *Drosophila* ovary, supporting a role of this family of GEFs in transducing signals from RTKs to regulate cell migration during development.

Analysis of the cellular mechanisms by which Vav regulates cell migration in vertebrates is hampered by the inaccessibility of the cells and the difficulty of visualizing them in their natural environment within the embryo. Thus, it is not yet clear how Vav proteins regulate cell migration downstream of RTKs during development. Here, by analysing cell movement in their physiological environment, we have been able to show that Vav is required to control the length, stabilization and life of front cellular protrusions. In addition, we found that disruption of Vav function *in vivo* results in a decrease in Rac activity at the leading edge. Defective signalling downstream of EGFR/PVR results in defects in the dynamics of cellular protrusion and Rac activation, which are very similar to those observed in $vav^{-/-}$ BCs (Poukkula et al., 2011; Wang et al., 2010). In addition, we have found that ectopic activation of Vav in BCs, as it is the case for PVR/EGFR and Rac, causes non-polarized massive F-actin accumulation. Thus, we suggest that one of the roles of Vav in directed cell migration downstream of EGF/PVF signals is to remodel the actin cytoskeleton via Rac activation, hence promoting the formation and stabilization of cellular protrusions in the direction of migration. Studies in cultured neurons, have shown that the main role for mouse Vav2 during axonal repulsion is to mediate a Rac-dependent endocytosis of ephrin-Eph (Cowan et al., 2005). Although endocytosis has been normally shown to be involved in attenuation of RTKs signalling, in BCs it has been proposed to ensure RTKs recycling to regions of higher signalling, thus promoting directed BC movement (Jékely et al., 2005). This is based on the fact that elimination in BCs of the ubiquitin ligase Cbl, which has been shown to regulate RTK endocytosis, leads to delocalized RTK signal and migration defects. In this context, another possible role for Vav downstream of EGFR/PVR could be to mediate RTK endocytosis, as it is the case during axonal repulsion. Further analysis will be needed to fully explore the molecular and cellular mechanisms by which Vav proteins regulate cell migration *in vivo* in other developmental contexts.

BC migration is a complex event and activation of EGF/PDGF receptors will most likely engage different GEFs to affect the distinct cytoskeletal changes necessary to accomplish it. In fact, the migration phenotype of BCs mutant for *vav* is less severe than that of BCs double mutant for both EGFR and PVR. In addition, although reducing Vav function decreases the asymmetry in Rac activity between front and back present in wild-type clusters, it does not eliminate it, as it happens when the function of both guidance receptors is compromised. All these results suggest that there are other GEFs besides Vav that could act downstream of EGFR and PVR to activate Rac. Previous analysis have implicated the Rac exchange factor Mbc/DOCK180 and its cofactor ELMO on BC migration (Bianco et al., 2007; Duchek et al., 2001). In this context, Vav and the Mbc/ELMO complex could act synergistically as GEFs to mediate Rac activation to a precise level and/or to a precise location. This awaits the validation of the Mbc/ELMO complex as a GEF for Rac in BCs. In the future, it will be important to determine how the different GEFs contribute to Rac activation, which specific downstream effectors of Rac they activate, and ultimately what cellular aspects of the migration process they control.

In summary, our work demonstrates that Vav functions downstream of RTKs to control directed cell migration during

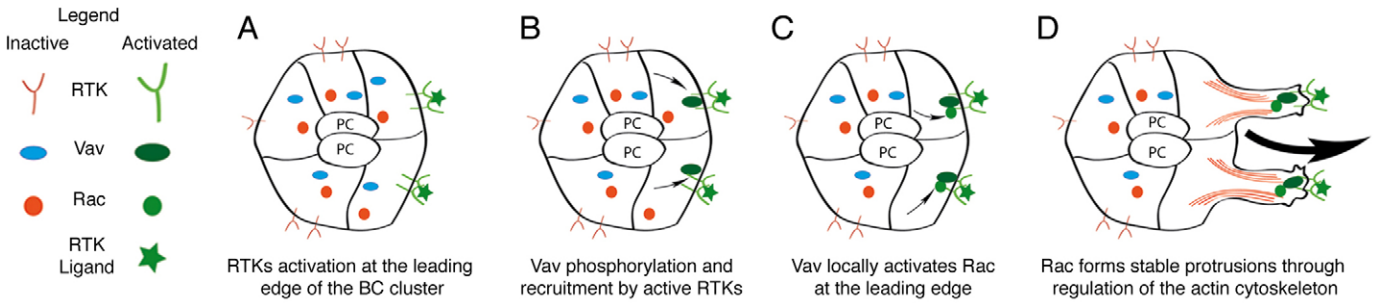

Fig. 7. Model for Vav function downstream of PVR/EGFR. Stimulation of EGFR and PVR upon ligand binding induces Vav recruitment and activation by tyrosine phosphorylation, resulting in Rac activation at the leading edge and subsequent forward movement. PC, polar cells.

development. Furthermore, we have unravelled the cellular and molecular mechanism by which Vav regulates cell migration in the developing *Drosophila* egg chamber: binding of PDGF/EGF to their receptors would induce Vav activation through tyrosine phosphorylation and its association with the activated receptors. This would lead to an increase in Rac activity at the leading edge of migrating cells, which promotes the stabilization and growth of the cellular front extensions, thus controlling directed cell migration (Fig. 7).

Regulation of Vav signalling downstream of RTKs can participate not only in development or normal physiology but also in tumorigenesis (Billadeau, 2002; Lazer et al., 2009). Vav1 is miss-expressed in a high percentage of pancreatic ductular adenocarcinomas and lung cancer patients (Fernandez-Zapico et al., 2005; Lazer et al., 2009). Thus, understanding the mechanisms by which Vav controls cellular processes downstream of RTKs is likely to be relevant for both developmental and tumor biology.

Materials and Methods

Drosophila strains

The following fly stocks were used: yw; e22c gal4UAS-flp/CyO, w;*slbo*gal4/CyO, wc306gal4, Ubi-GFPnlshsFLP122FRT19A, yhsflp;Ubi-GFP FRT40A/(CyO), w;Ubi-GFPnlsFRT42D/CyO and w;UAS-Rac1^{v12} (Bloomington Stock Centre), UAS-EGFR^{DN}/TM6 (o'Keefe et al., 1997), UAS-PVR^{DN}/CyO, w;;UAS-λ-mycPVR/TM3 and *pvr*^{1}FRT40A/CyO (Duchek et al., 2001), UAS-RNAi for *vav* comes from VDRC, *vav*FRT19A/FM7 (Kyoto), *vav*^{3}FRT19A/FM7 (Malartre et al., 2010), UAS-Vav^{act}(Couceiro et al., 2005), UAS-Rac FRET (Wang et al., 2010). To analyse the genetic interactions between Vav and the EGFR/PVR pathways, the following stocks were generated: *vav*^{3}FRT19A/FM7; *pvr*^{1}FRT40A/CyO, *vav*^{3}FRT19A/FM7; EGFRFRT42D/CyO, *vav*^{3}FRT19A/FMZ; ; UASPVR^{DN}/TM6B, *vav*^{3}FRT19A/FMZ; ; UAS-EGFR^{DN}/TM6B, wUbi-GFPnlsFRT19Ahsflp; Ubi-GFPFRT40A/CyO, wUbi-GFPnlsFRT19Ahsflp; Ubi-GFPFRT42D/CyO, wUbi-GFPnlsFRT19Ahsflp; *slbo*gal4-UASmoesinGFP/CyO.

Immunohistochemistry

Drosophila ovaries were dissected and fixed following standard procedures and incubated with the DNA dye TO-PRO-3 (Molecular Probes, 1/1000) and rhodamine-phalloidin (Molecular Probes, 1/200). The following antibodies were used: rabbit anti-GFP (Molecular Probes, 1/10000), and anti-rabbit-FITC (Molecular Probes). Images were captured with a Leica TCS-SP2 confocal microscope.

Live imaging and processing

For live imaging of BC migration, stage 9 egg chambers were obtained from intact ovaries dissected from females of the following phenotypes: Control: *slbo*-Gal4-UAS-*moesin:GFP*/CyO; experimental: C306; *slbo*-Gal4-UAS-*moesin:GFP*/CyO; *UAS-vav*^{RNAi}.

Culture conditions and time-lapse microscopy were performed as described in (Prasad and Montell, 2007). Frames were taken every 2 minutes. Movies lasted on average 4 hours. Both movies were taken under the same conditions.

Measurements of cluster velocity, number, position, length and lifetime of protrusions were done manually as described in (Poukkula et al., 2011). In summary, once the body of the cluster is defined according to Poukkula et al., a line is drawn from the centre of the cluster body to the extension tip. The length of the extension is measured from the edge of the cluster body to the extension tip. The centre of the cluster body was used to measure cluster velocity by manual tracking. To analyse protrusion orientation, the angle of this line relative to the *x*-axis was calculated. Extensions were classified as front (0–45° and 315°–0°), side (255°–315° and 45°–135°) and back (135°–225°). To evaluate protrusion lifetime, each frame was compared with the next time point and extensions that overlapped by more than 5 pixels were considered the same extension. In all cases, an average of 150 protrusions was analyzed.

FRET images of fixed egg chambers were acquired with Zeiss LSM510 microscope equipped with a 40×/1.3 oil immersion objective as described previously (Wang et al., 2010). Final ratio image was generated with the ratio Image tool of Metamorph and represented using an eight-color scale code. Average FRET index measurements in whole border cell clusters were calculated on background-subtracted images that were thresholded and converted to binary mask with background set to zero. The mean cell fluorescence intensities (CFP and YFP) were then measured and the YFP/CFP ratios were calculated. To determine the front and back areas for FRET measurements a radar map was superposed on a cluster with the zero set to the direction of migration. For measurements in front and back areas CFP and YFP signals were measured only in the regions between 45 and 315 degree and between 135 and 225 degrees, respectively, as described in (Ramel et al., 2013).

Molecular biology

Two hybrid assays and screening of the library were done according to standard procedures, as previously described (Bianco et al., 2007; Cobreros et al., 2008; Jékely et al., 2005). Positive interactions were selected by plating the transformation on restrictive medium with double nutritional requirement; growing colonies were re-streaked and further tested for β-galactosidase activity, and DNA was recovered from the positive clones. We used a cDNA Library from Drosophila third instar larvae made on pSE1107 plasmid, gently provided by Dr Grace Gill (Harvard Medical School, Boston, MA), and a battery of modified baits including: the wild-type intracellular domain of PVR (PVRi), PVRi with specific mutations in specific tyrosines (YF14, YFa, YFb and YFc), a PVRi kinase-dead (KD) version (N1134 >A) unable to autophosphorylate and C-terminal truncated versions at residues E1352 (ΔXho) or F1261 (Δ313) (Fig. 3) (Jekely et al., 2005). In addition, an Epidermal Growth Factor Receptor derived bait consisting of the intracellular domain of the receptor (EGFRi; (Bianco et al., 2007) was used to identify PVR specific interactions. Binding of the mouse p53 protein to SV40 large T-antigen and to PVR-IP#112 were used as positive and negative controls, respectively (see Section 2 and Fig. 5C). Generation of baits and point mutants have been described elsewhere (Bianco et al., 2007; Cobreros et al., 2008; Jékely et al., 2005).

Cell culture, transfection and stimulation

S2 Schneider cells were grown at 25℃ in Schneider's medium (Beit-Haemek, Israel), supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 mg/ml streptomycin. For PVF stimulation, we used conditioned medium that was prepared as follows: S2 cells were transfected with pUASt-PVF1 and pA5C-ActinGal4 (kindly provided by B. Shilo) (Rosin et al., 2004) using ESCORT IV transfection reagent (SIGMA) according to manufacturer instructions. Six hours after transfection, the media of the cells was changed with serum-free media and after 72 hours conditioned media containing secreted PVF1 was collected. For stimulation, starved S2 cells were incubated for 15 minutes at 25℃ with either conditioned media containing PVF1 or control media without serum.

Western blotting and immunoprecipitation

Extracts from control or PVF1 stimulated S2 cells were subjected to western blot analyses using various antibodies (Abs) as described below. Alternatively, extracts from stimulated S2 cells were immunoprecipitated with anti PVR Abs (kindly provided by B. Shilo) (Rosin et al., 2004) or anti DroVAV Abs (Hornstein et al., 2003). The resulting immuno-complexes were harvested with protein G or A-sepharose beads respectively

(Pharmacia, Peapack, NJ) and were subjected to western blot analysis. Anti-phosphotyrosine monoclonal antibody (4G10) (Upstate Biotechnology, Lake Placid, NY) was used to determine the phosphorylation state of DroVav and PVR.

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Author contributions

C.H.F.-E, performed all the genetic and *in vivo* experiments and D.R. and G.E. carried out the FRET analysis; they all contributed to the writing of the paper. M.M. contributed to the initial analysis of Vav requirements in BC migration; C.M.L. performed the two-hybrid analysis and M.F., S.L. and S.K. contributed with the S2 cells experiments; these authors provided useful comments on the manuscript. M.D.M.-B. conceived, designed and wrote the paper.

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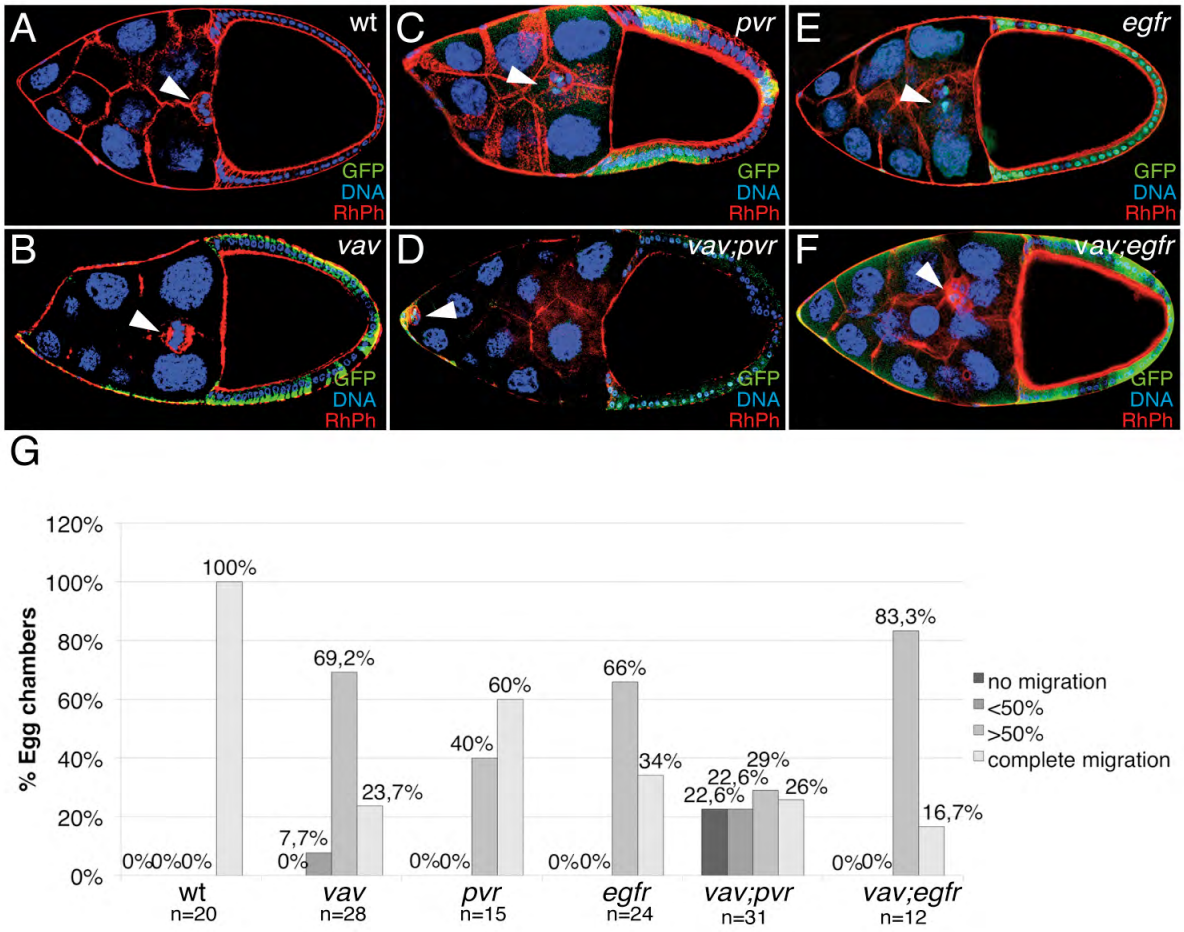
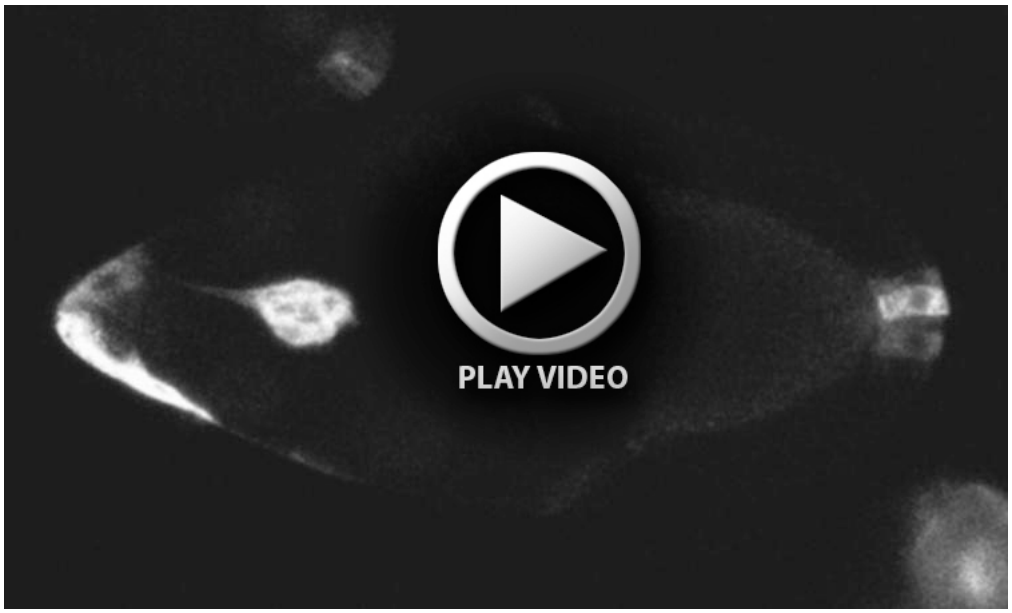


Fig. S1 Vav interacts genetically with PVR and EGFR during BC migration. Wild type (**A**), *vav* (**B**), *pvr* (**C**), *vav*; *pvr* (**D**), *egfr* (**E**) and vav; *egfr* (**F**) mutant BC clusters (arrowhead). (**G**) Quantification of the BC migration phenotype of S10 egg chambers of the indicated genotypes.



Movie 1. Movie showing dynamics of the migration of wild type BCs.

Movie 2. Movie showing dynamics of the migration of *vav* mutant BCs supporting Fig. 2. It shows "*in vivo*" that the ability to grow, maintain and stabilize long front extensions is compromised when Vav function is reduced.