RESEARCH ARTICLE

Pak3 regulates apical-basal polarity in migrating border cells during *Drosophila* oogenesis

Martina Felix, Mrinal Chayengia*, Ritabrata Ghosh, Aditi Sharma and Mohit Prasad‡

ABSTRACT

Group cell migration is a highly coordinated process that is involved in a number of physiological events such as morphogenesis, wound healing and tumor metastasis. Unlike single cells, collectively moving cells are physically attached to each other and retain some degree of apical-basal polarity during the migratory phase. Although much is known about direction sensing, how polarity is regulated in multicellular movement remains unclear. Here we report the role of the protein kinase Pak3 in maintaining apical-basal polarity in migrating border cell clusters during *Drosophila* oogenesis. Pak3 is enriched in border cells and downregulation of its function impedes border cell movement. Time-lapse imaging suggests that Pak3 affects protrusive behavior of the border cell cluster, specifically regulating the stability and directionality of protrusions. Pak3 functions downstream of guidance receptor signaling to regulate the level and distribution of F-actin in migrating border cells. We also provide evidence that Pak3 genetically interacts with the lateral polarity marker Scribble and that it regulates JNK signaling in the moving border cells. Since Pak3 depletion results in mislocalization of several apical-basal polarity markers and overexpression of Jra rescues the polarity of the Pak3-depleted cluster, we propose that Pak3 functions through JNK signaling to modulate apical-basal polarity of the migrating border cell cluster. We also observe loss of apical-basal polarity in Rac1-depleted border cell clusters, suggesting that guidance receptor signaling functions through Rac GTPase and Pak3 to regulate the overall polarity of the cluster and mediate efficient collective movement of the border cells to the oocyte boundary.

KEY WORDS: Collective cell migration, Apical-basal polarity, Border cell movement, *Drosophila* Pak3

INTRODUCTION

Collective cell migration or group cell movement plays an important role during diverse processes including development, morphogenesis, wound healing and tumor cell metastases (Christiansen and Rajasekaran, 2006; Friedl et al., 1995; Lecaudey and Gilmour, 2006; Vaughan and Trinkaus, 1966; Weijer, 2009). Unlike single cells, collectively moving cells are physically attached to one another and retain some degree of apical-basal polarity during migration (Pinheiro and Montell, 2004; Ewald et al., 2012). This observation is paradoxical, especially in the context of our current

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understanding of how cellular movement and multi-cellularity are linked. As per the classical model, retention of intercellular contact and maintenance of apical-basal polarity in a multicellular milieu hinders the ability of the cells to move. During development and tumor metastases, epithelial cells acquire the ability to move only when they lose multicellular organization and intercellular adhesion (Macara and McCaffrey, 2013; Chatterjee and McCaffrey, 2014). Given the inverse correlation between adhesion and mobility, how collective cell movement is favored in a multicellular context remains an enigma. Since this kind of movement is prevalent both in normal and pathological conditions, it is believed that cohesion between moving cells mediates efficient intercellular communication that helps in maintaining the integrity of the migrating cluster, potentiates efficient guidance and enables survival under unfavorable conditions (Pinheiro and Montell, 2004; Llense and Martin-Blanco, 2008). Although collective migration plays a widespread and physiologically relevant role in several processes, it is not clear how polarity is modulated during multicellular movement.

Movement of border cells in *Drosophila* oogenesis has emerged as an excellent model system for studying collective cell migration (Montell, 2001). The *Drosophila* ovary consists of a collection of oval structures called the egg chambers (Spradling, 1993). An egg chamber consists of 16 germline cells surrounded by a layer of epithelial follicle cells. The poles of the egg chamber are marked by the presence of a pair of specialized follicle cells called the polar cells (Ruohola et al., 1991). At stage 8, six to eight anterior follicle cells flanking the anterior polar cells round up, detach and migrate as a cluster towards the oocyte boundary (Montell et al., 1992; Silver and Montell, 2001; Pinheiro and Montell, 2004). This migrating group is the border cell cluster and they eventually aid in sperm entry during fertilization (Montell et al., 1992).

Border cells retain some epithelial characteristics as they move (Niewiadomska et al., 1999; Pinheiro and Montell, 2004). Apical determinants such as Par-6, Bazooka (Baz) and Crumbs (Crb) are maintained asymmetrically and disruption of their function (Par-6 and Baz) hinders cluster movement (Niewiadomska et al., 1999; Pinheiro and Montell, 2004). Another polarity regulator, the serine-threonine kinase Par-1, has been shown to facilitate border cell detachment from the follicle epithelia and help in sensing direction by favoring forward-directed protrusions (McDonald et al., 2008). Recently, c-Jun N-terminal kinase (JNK) signaling has been shown to modulate intercellular adhesion among the border cells and also to mediate Baz localization for efficient cluster movement (Llense and Martin-Blanco, 2008). Although we know that apical-basal polarity favors collective movement, a comprehensive understanding of how it is regulated is still not clear.

p21-activated kinases (Paks) have been shown to regulate polarity both in single and multicellular systems (Asano et al., 2009; Conder et al., 2007). The *Drosophila* genome encodes three Paks: Pak, Pak3 and Mushroom bodies tiny (Mbt; also known as *Drosophila* Pak2).

Based on their ability to self-modulate their activities, Paks have been divided into two categories; Pak and Pak3 belong to group I, while Mbt, which lacks the autoinhibitory domain, comes under group II (Arias-Romero and Chernoff, 2008). The group I Paks controls various processes, including polarity, whereas Mbt has been linked to cell proliferation and morphogenesis (Asano et al., 2009; Conder et al., 2007; Duan et al., 2012; Melzer et al., 2013; Schneeberger and Raabe, 2003). Our interest was to understand how polarity is regulated in collective cell movement so we focused on the group I Pak and Pak3. Pak regulates apical-basal polarity of the follicular epithelium of *Drosophila* egg chambers by affecting the distribution of basal actin (Conder et al., 2007), while in a *Drosophila* cell line Pak3 has been shown to regulate global polarity through modulation of actin distribution (Asano et al., 2009). We were curious to evaluate the role of both Pak and Pak3 in migrating border cells. Previously, *Pak* mutant border cells were shown to migrate normally and no anomaly was reported in their behavior (Duchek et al., 2001). We therefore decided to target Pak3, primarily because Pak and Pak3 exhibit a high degree of structural homology (35.4% identity and 47.9% similarity) and, most importantly, both have been reported to physically interact with the Scribble complex, an important modulator of apical-basal polarity in epithelial cells (Bahri et al., 2010; Mentzel and Raabe, 2005).

In this study, we evaluate the role of Pak3 in mediating the polarity of the collectively moving border cells during *Drosophila* oogenesis. Our results indicate that Pak3 protein is expressed in the border cells and that its functional depletion impedes cluster movement. Using live cell imaging analysis, we demonstrate that Pak3 affects the protrusive behavior of the migrating cluster, exhibiting short-lived and misdirected protrusions. Our analysis shows that Pak3 functions downstream of guidance receptor signaling to regulate F-actin in the border cells. Further, we provide evidence that Pak3 genetically interacts with the basolateral protein Scribble (Scrib) and functions upstream of JNK signaling to regulate apical-basal polarity of the migrating cluster. Pak3 depletion results in mislocalization of the apical proteins Pals1-associated tight junction protein (Patj), Stardust (Sdt) and Crb and the subapical marker E-cadherin. In addition, it also alters the distribution of the lateral markers Coracle (Cora) and Discs large 1 (Dlg1), and the basal marker βps-integrin (Myospheroid – FlyBase). Interestingly, we report that Rac1 depletion also disrupts apical-basal polarity of the migrating border cells, like Pak3. Since overexpression of Jra (D-jun) can rescue the polarity and migration efficiency of Pak3-depleted clusters, our results suggest that Pak3 functions through JNK signaling to regulate apical-basal polarity of the migrating cluster. Finally, we propose that guidance receptor signaling functioning through Rac GTPase and Pak3 modulates the overall polarity of the migrating cluster to favor efficient forward movement.

RESULTS
Pak3 regulates border cell movement
To test whether Pak3 mediates border cell movement, we first examined its expression in egg chambers. We observed a basal distribution of Pak3 protein in all cells of the egg chamber, including the migrating border cells (Fig. 1A-C). Since Pak3 protein is expressed in the border cells, we sought to investigate if it has any role in cluster movement. To downregulate Pak3 function effectively in border cells, we overexpressed a dominant-negative construct [Pak3V5(K322A)] in a *Pak3^{zyg}* heterozygous mutant background as reported by Duan et al. (2012); hereafter, this genetic combination is referred to as Pak3DN. Consistent with our

expectation, downregulation of Pak3 function impedes border cell movement in ~98% of stage 10 egg chambers (Fig. 1G-I). In addition, downregulation of Pak3 function using an RNA interference (RNAi) approach also hinders border cell movement in 29% of the egg chambers (Fig. S1A-C). We confirmed that the RNAi construct indeed targets *Pak3* by immunostaining, wherein flipout clones overexpressing the construct in random subsets of follicle cells and border cells were shown to exhibit lower levels of Pak3 protein compared with their wild-type counterparts (Fig. S1D-E′).

To corroborate the results above and to check the spatial requirement of Pak3 protein, we evaluated migration efficiency of the mosaic border cell clusters. The mutant clones were generated randomly using the MARCM (mosaic analysis with a repressible cell marker) technique and, consistent with the Pak3DN overexpression phenotype, we found that whole border cell mutant clusters exhibited significant migration defects, with the majority unable to travel even 50% of the total distance (Fig. 1L) (Lee and Luo, 1999). As expected in the mosaic analysis, we observed an inverse correlation between the size of the mutant clone and the migration efficiency of the cluster (Fig. 1L). For example, clusters with few mutant cells migrated normally and covered the same distance as wild-type clusters (Fig. 1J,J′). By contrast, clusters with larger numbers of mutant cells exhibited significant migration defects (Fig. 1K,K′). Significantly, we did not observe any migration defect in the corresponding wild-type clusters (n>180; data not shown). Overall, our data suggest that Pak3 probably functions in a cell-autonomous manner in the border cells to mediate efficient movement of the cluster.

Since the cluster consists of 6-10 cells, we examined whether Pak3 functions in a subset of the migrating border cells. We analyzed the migration efficiencies of mosaic clusters where the *Pak3* mutant clones were generated in a random manner. We reasoned that if Pak3 functions in specific cells of the cluster then we should observe a significant number of migration defects even when less than half the cluster was mutant. Contrary to our expectation, we observed very few instances in which border cell clusters with few mutant cells (less than half the cluster) exhibited migration defects, and the majority instead reached the oocyte boundary (Fig. 1L). Consistent with the MARCM results, overexpression of Pak3DN in the polar cells did not affect border cell movement, suggesting that Pak3 functions in the migrating border cells (Fig. S2D). From the data above we conclude that Pak3 functions in the majority of the border cells and its role is not restricted to a select few in the moving cluster.

Pak3 regulates protrusion dynamics in the migrating cluster
Since Paks function downstream of the small GTPases Cdc42 and Rac, and as the GTPases modulate front-rear polarity by affecting filipodia and lamellipodia polarity, we investigated whether Pak3 functions similarly in the migrating cluster. To test this, we evaluated the protrusive behavior of wild-type and Pak3-depleted border cell clusters using time-lapse live cell imaging.

As cellular movement is a dynamic process, we carried out live cell imaging to quantitate the migratory parameters of the wild-type and Pak3-depleted border cells. We overexpressed UAS-GFP:: Moesin (UAS-GMA) in the migrating cluster using the *c306*-GAL4 driver (Dutta et al., 2002; Geisbrecht and Montell, 2002) to label the protrusions, and from several time-lapse movies we calculated the average migration speed of the control and Pak3DN clusters (Fig. 2A,B). Compared with the control clusters, which moved at an average speed of 0.49 μm/min (n=11), the Pak3-depleted border cells were sluggish and migrated at an average of 0.19 μm/min (n=11) (Fig. 2C, Movies 1 and 2). These data suggest that Pak3
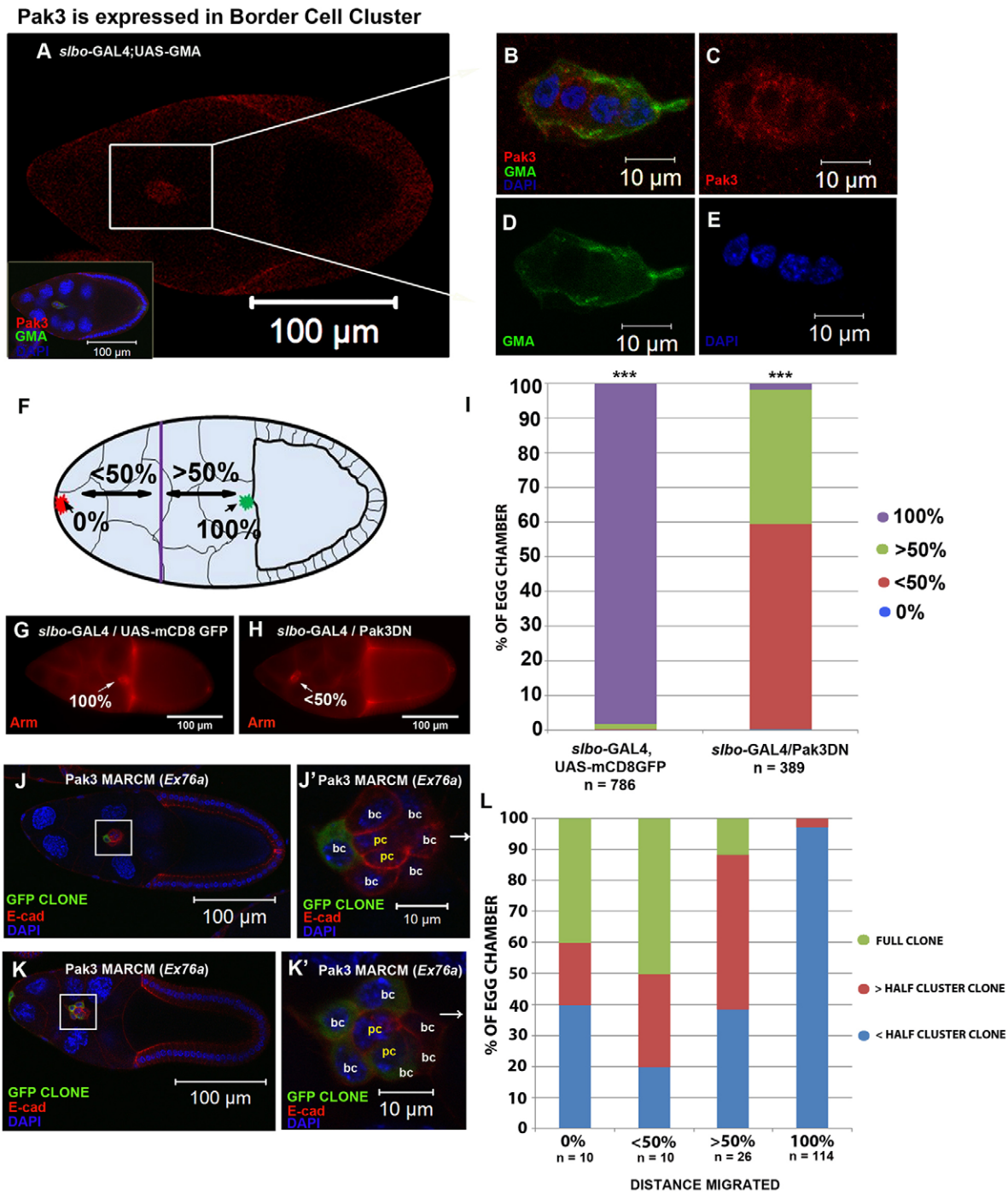
Fig. 1. *Drosophila* Pak3 regulates border cell movement. (A-E) Pak3 distribution in the genotype *slbo*-GAL4; UAS-GMA. (A) Stage 9 egg chamber. Inset is a merged image of the egg chamber shown in A. (B-E) Magnified image of border cells in A showing (B) merge, (C) Pak3 (red), (D) GMA (green) and (E) DAPI (blue). (F) Schematic of stage 10 egg chamber showing border cell in red (0% migration) and in green (100% migration). Double-sided arrows mark migration distance. (G,H) Pak3 depletion impedes border cell movement. Arrowheads mark border cells stained for Armadillo (Arm, red). (G) Wild-type stage 10 egg chamber showing complete migration. (H) Pak3DN stage 10 egg chamber showing <50% migration. (I) Quantification of migration efficiency. *n*, number of stage 10 egg chambers evaluated. ****P*<0.001. (J-K′) MARCM analysis. Confocal images of egg chambers stained for E-cadherin (red) and with DAPI (blue). GFP (green) marks homozygous *Pak3* mutant clones. Arrows indicate direction of migration. (L) The correlation between the size of *Pak3* mutant clones and the distance covered by the mosaic cluster. bc, border cell; pc, polar cell.

regulates the speed of the moving cluster and that downregulation of its function impedes the efficiency of cluster movement.

Since the speed of the moving cluster is affected by several factors, including protrusion dynamics, we analyzed cellular protrusions in detail for both experimental and control clusters for

the entire duration of migration. From several time-lapse movies, we mapped the number, length, direction and stability of the protrusions in order to identify how Pak3-deficient clusters differ from controls. Consistent with previous observations, control clusters extended protrusions preferentially in the direction of
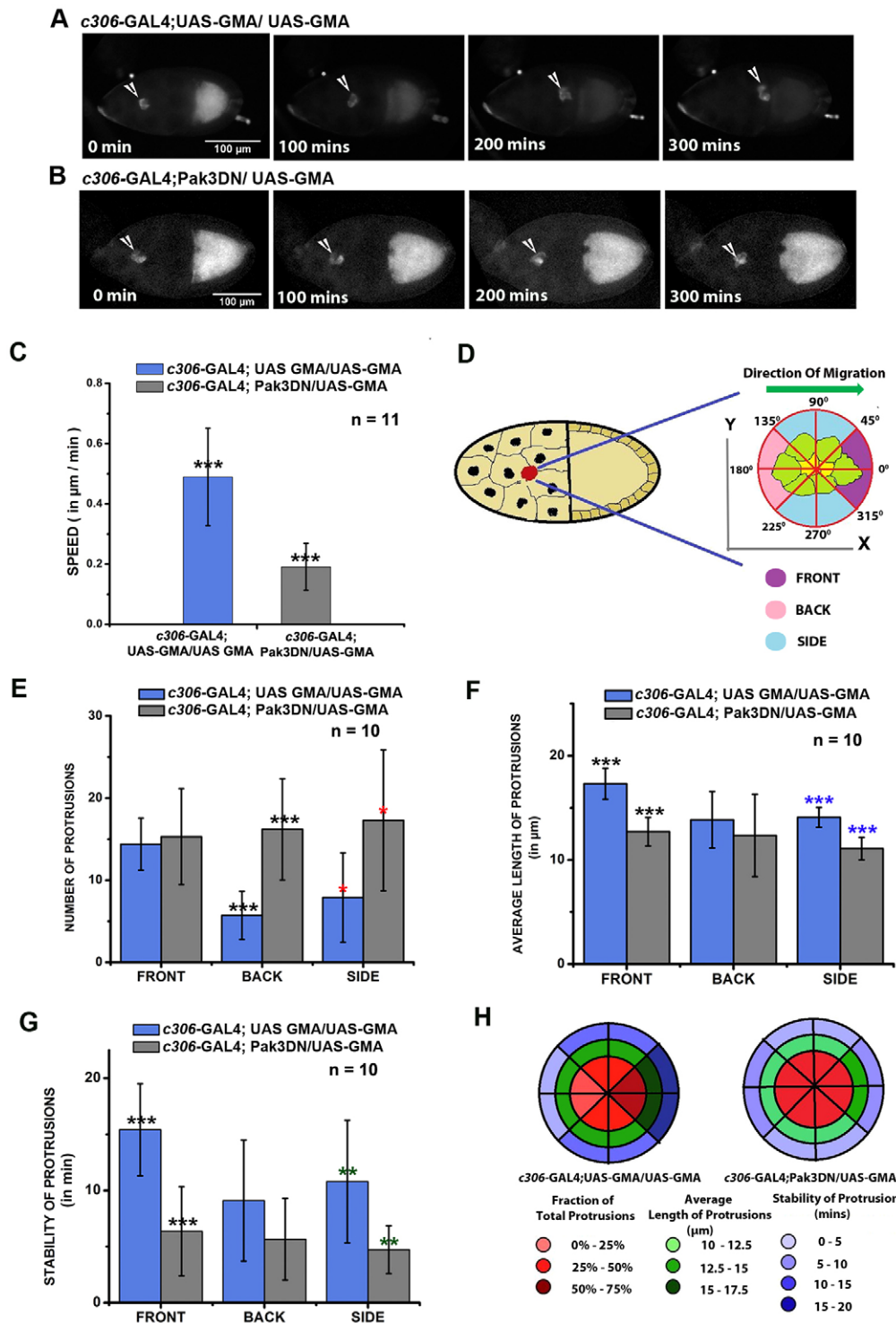
Fig. 2. Pak3 regulates protrusion dynamics in migrating clusters. (A,B) Time-lapse snapshot of stage 9 egg chambers of the indicated genotypes. Arrowheads mark border cell clusters. Note that Pak3DN clusters exhibit a migration delay. (C) Quantitative comparison of effect of Pak3DN on migration speed. (D) Radial diagram depicting classification of protrusions as front, back or side in the two-dimensional plane. (E-G) Effect of Pak3DN on the number (E), length (F) and stability (G) of protrusions. (H) Schematic summarizing the protrusion dynamics for wild-type and Pak3DN clusters. Loss of polarity is evident in Pak3-depleted clusters. Error bars represent s.d. *n*, number of movies analyzed. **$P < 0.01$, ***$P < 0.001$.

migration, with very few directed sideward or backwards ($n = 10$; Fig. 2E, Movie 1) (Bianco et al., 2007; Prasad and Montell, 2007; Fernández-Espartero et al., 2013). Interestingly, in Pak3-depleted clusters we observed an increase in the number of sideward and rearward protrusions ($n = 10$), while the preference for forward protrusions was compromised (Fig. 2E, Movie 2). This was primarily due to significant differences in the length and stability of frontal protrusions compared with the control. The average length of frontal protrusions for the control cluster was 17.3 µm, whereas for the Pak3-depleted cluster it averaged ~12.7 µm (Fig. 2F). In

addition, frontal protrusions in the control persisted on average for 15.4 min, whereas in Pak3-depleted clusters they were short lived and lasted on average for only 6.4 min (Fig. 2G). Altogether, the live imaging analysis suggests that Pak3 is required for polarizing the migrating cluster by regulating the direction, length and stability of forward-directed protrusions.

This observation is interesting as both guidance receptor signaling and the apical-basal polarity regulator Par-1 have been shown to regulate the polarity of protrusions from the migrating cluster (McDonald et al., 2008; Prasad and Montell, 2007). Since Pak3

regulates the polarity, growth and stability of frontal protrusions, we examined whether Pak3 interacts with guidance receptor signaling to modulate the apical-basal polarity of the moving border cells.

Pak3 functions downstream of Rac GTPase in guidance receptor signaling

The guidance receptor PDGF- and VEGF-receptor related (Pvr) is known to regulate front-rear polarity in migrating border cells (Fernández-Espartero et al., 2013; Zhang et al., 2011). Since Pak3DN border cells exhibit misdirected protrusions, we investigated the relationship between Pak3 and Pvr in migrating clusters. We overexpressed a dominant-negative form of the Pvr receptor (PvrDN) (Duchek et al., 2001) in border cells in the wild-type and Pak3 heterozygous (*Ex76aPak3/+*) genetic backgrounds. Overexpression of PvrDN in the wild-type border cells impeded cluster movement in 22% of the egg chambers, whereas in the *Ex76aPak3/+* background we observed an enhanced migration defect of 46.2% (Fig. 3A-D), suggesting that Pak3 genetically interacts with Pvr in migrating border cells.

To further validate this result, we examined the F-actin distribution in Pak3-depleted clusters. Since Pvr is known to generate actin-rich protrusions in border cells (Duchek et al., 2001), we investigated whether Pak3 affects the F-actin distribution in the migrating cluster, as in *Pvr* mutants. We analyzed F-actin in the border cells by overexpressing UAS-GMA. In the wild type, moesin-GFP accumulates around the outer rim of the migrating cluster, with higher levels at the front (leading edge) and lower levels at the rear (Fig. 3E′). In addition, moesin-GFP labels F-actin and one can observe distinct long fibers distributed in the migrating clusters (*n*=15 clusters). Consistent with our expectation, Pak3 depletion impeded the formation of these long F-actin fibers and, in 53% of the clusters (*n*=7), only actin puncta were observed, whereas in the remaining 47% there was mix of actin puncta and small fibers (Fig. 3F′,G′). We obtained comparable results with the F-actin probe Rhodamine-phalloidin (Fig. S3E-G′). A similar effect on actin distribution has been reported during myoblast fusion in *Drosophila* embryogenesis when Pak3 function was downregulated (Duan et al., 2012).

Next, we examined whether Pak3 functions downstream of guidance receptor signaling in the migrating border cells. Guidance receptors are known to stimulate Rac GTPase activity in the border cells to modulate actin polymerization and cellular protrusions (Duchek et al., 2001; Wang et al., 2010). We compared actin fiber length in border cells overexpressing Rac GTPase in the wild-type and *Ex76aPak3/+* background. If Pak3 functions downstream of Rac1 in the moving border cells, one would expect the phenotype associated with Rac1 overexpression to be suppressed in the *Ex76aPak3/+* background. This was indeed the case, as the average length of F-actin upon Rac1 overexpression in wild type was 7.3 µm, whereas in the *Ex76aPak3/+* background it was 3.8 µm (Fig. 3H-J), suggesting that Pak3 functions downstream of Rac1 in guidance receptor signaling to regulate F-actin in the border cells. This conclusion is supported by our live imaging data, where we observed smaller, short-lived cellular protrusions from the cluster when Pak3 function was downregulated.

Interestingly, polarized distribution of actin is also linked to the maintenance of apical-basal polarity in various cellular milieu, including follicle cells in *Drosophila* egg chambers (Conder et al., 2007). Since we observed actin downregulation in Pak3-depleted clusters, we investigated whether Pak3 affects apical-basal polarity in moving border cells.

Pak3 genetically interacts with Scrib and regulates JNK signaling in border cells

Actin and myosin are known to play an important role in regulating the polarity of migrating cells (Li and Gundersen, 2008). Since Pak3 co-immunoprecipitates with Scrib (Bahri et al., 2010) and its downregulation affects F-actin in the border cells, we hypothesized that Pak3 regulates the apical-basal polarity of the migrating border cell cluster. To test this, we first checked the genetic interaction of Scrib and Pak3 in moving border cells. Overexpression of *scrib* RNAi using the *c306*-GAL4 driver impeded border cell movement in 1.9% of stage 10 egg chambers. Consistent with the immunoprecipitation results, this was enhanced to 27.1% in the *Ex76aPak3/+* background, suggesting that Pak3 and Scrib genetically interact in migrating border cells (Fig. 4A-C).

In addition, we observed several instances in which the border cell clusters were elongated, a phenotype mimicking loss of JNK signaling (Llense and Martin-Blanco, 2008). Since JNK signaling has been implicated in the proper localization of the apical marker Baz, this prompted us to examine the status of JNK signaling in Pak3-depleted clusters (Llense and Martin-Blanco, 2008). Upon activation by JNK, the transcription factor Jra mediates the expression of JNK target genes (Martín-Blanco, 1997; Martin-Blanco et al., 1998). The levels of phospho-Jun have been routinely used to evaluate the status of JNK signaling in various developmental contexts (Llense and Martin-Blanco, 2008). In wild-type clusters, phospho-Jun is localized to the nucleus and the average intensity observed per nucleus is 73.7 a.u. (arbitrary units; *n*=58), whereas in Pak3-depleted clusters this value was significantly lower at 55.6 a.u. (*n*=35) (Fig. 4D-F). This suggests that Pak3 probably functions upstream of JNK signaling to regulate border cell movement.

Since Pak3 genetically interacts with the apical-basal polarity regulator Scrib and affects JNK signaling, Pak3 appears to be a strong candidate to regulate apical-basal polarity in the moving border cells.

Pak3 affects the apical-basal polarity of migrating border cells

JNK signaling is known to regulate the apical polarity marker Baz in the moving border cell cluster (Llense and Martin-Blanco, 2008). Since Pak3 depletion affects phospho-Jun levels, we proposed that Pak3 might affect apical-basal polarity in the moving border cell cluster. To test this, we examined the distribution of various polarity markers in wild-type and Pak3-depleted border cell clusters.

Proteins such as Crb, Sdt, Patj and Baz localize to the apical domain. A complex consisting of Dlg1, Lethal (2) giant larvae (Lgl) and Scrib proteins marks the lateral domain, while integrin is used to identify the basal domain. To get a complete overview of the distribution of these polarity proteins, we examined three-dimensional (3D) reconstructions of these markers in the border cells. In wild-type clusters (*n*≥15), the apical proteins Sdt and Patj exhibit a characteristic rosette pattern in 90% of the clusters, with distinct enrichment at the polar cell-border cell (pc-bc) and border cell-border cell (bc-bc) interface. This enrichment is on one side of the border cell cluster, mostly perpendicular to the direction of migration, consistent with previous observations for Crb, Par-6 and Baz (Pinheiro and Montell, 2004). We observed a similar distribution for Crb in wild-type clusters (*n*=11). We do observe some degree of enrichment for Sdt, Patj and Crb proteins at the border cell-nurse cell (bc-nc) interface in less than 20% of wild-type clusters (Fig. 5A,A′,C,C′, Fig. S4A,A′, Movie 3A,B). In Pak3-depleted clusters the distribution of these apical markers is affected,
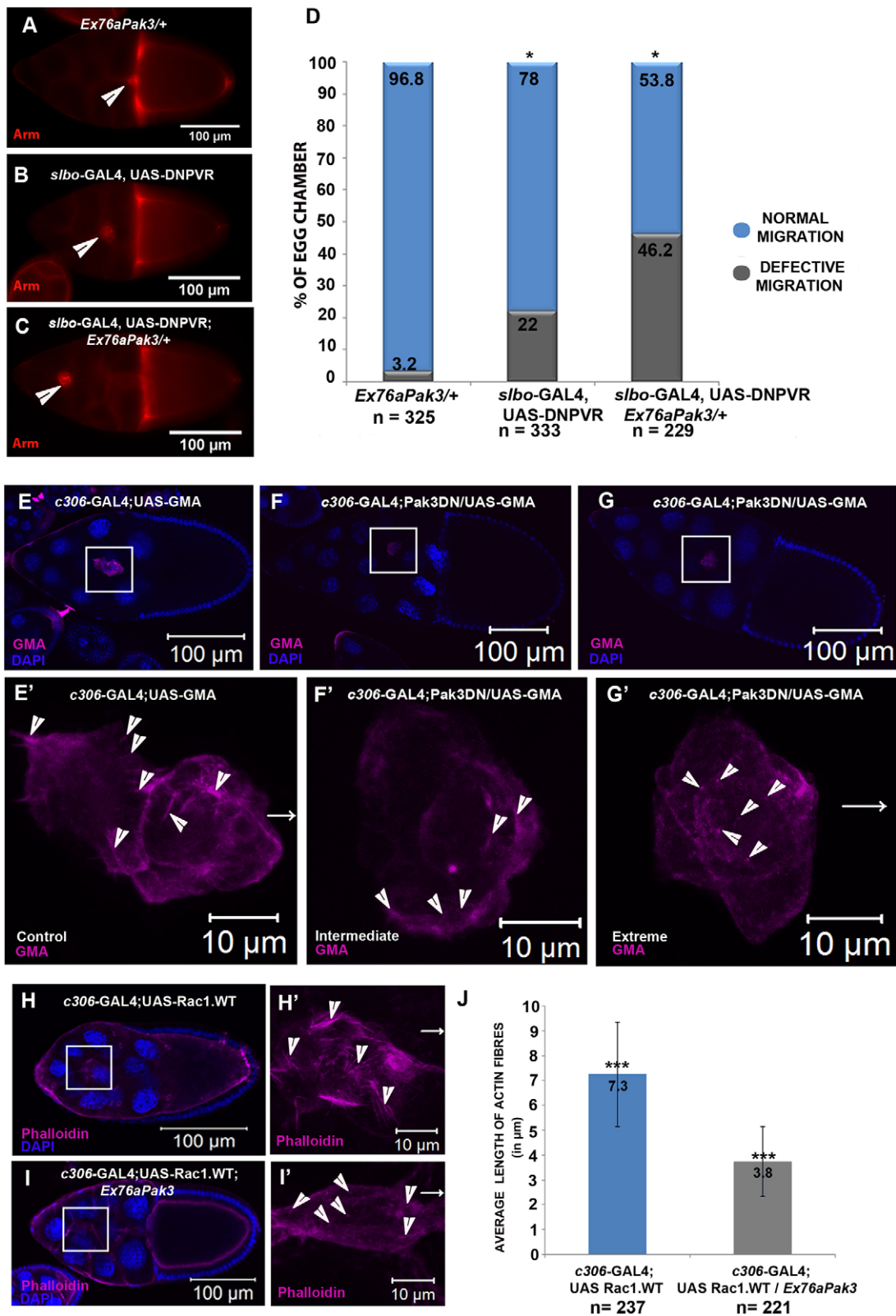
Fig. 3. Pak3 interacts with Pvr and affects F-actin. (A-C) Stage 10 egg chambers of the indicated genotypes stained for Arm (red). Arrowhead marks border cells. (D) Quantification of the migration defect for the indicated genotypes. *n*, number of egg chambers analyzed. *$P<0.05$. (E-G′) Merged confocal *z*-sections of egg chambers of the indicated genotypes expressing moesin-GFP (magenta). (E′,F′,G′) Magnified images of border cells (square) in E,F,G. (E′) Control border cells with long F-actin fibers (GMA). (F′,G′) Pak3DN clusters showing examples with (F′) an intermediate phenotype in which clusters exhibit few fibers and (G′) an extreme phenotype with distinct actin puncta. Arrowheads mark actin fibers or puncta. Arrows indicate direction of migration. (H-I′) Egg chambers of the indicated genotype stained with phalloidin. Border cells (square) are magnified in H′,I′. Arrowheads mark actin filaments. (J) The average length of actin fibers in border cells of the indicated genotypes. *n*, number of fibers quantified. Note the significant decrease in actin fiber length upon Rac1 overexpression in the *Ex76aPak3/+* versus wild-type background. ***$P<0.001$; error bars are s.d.
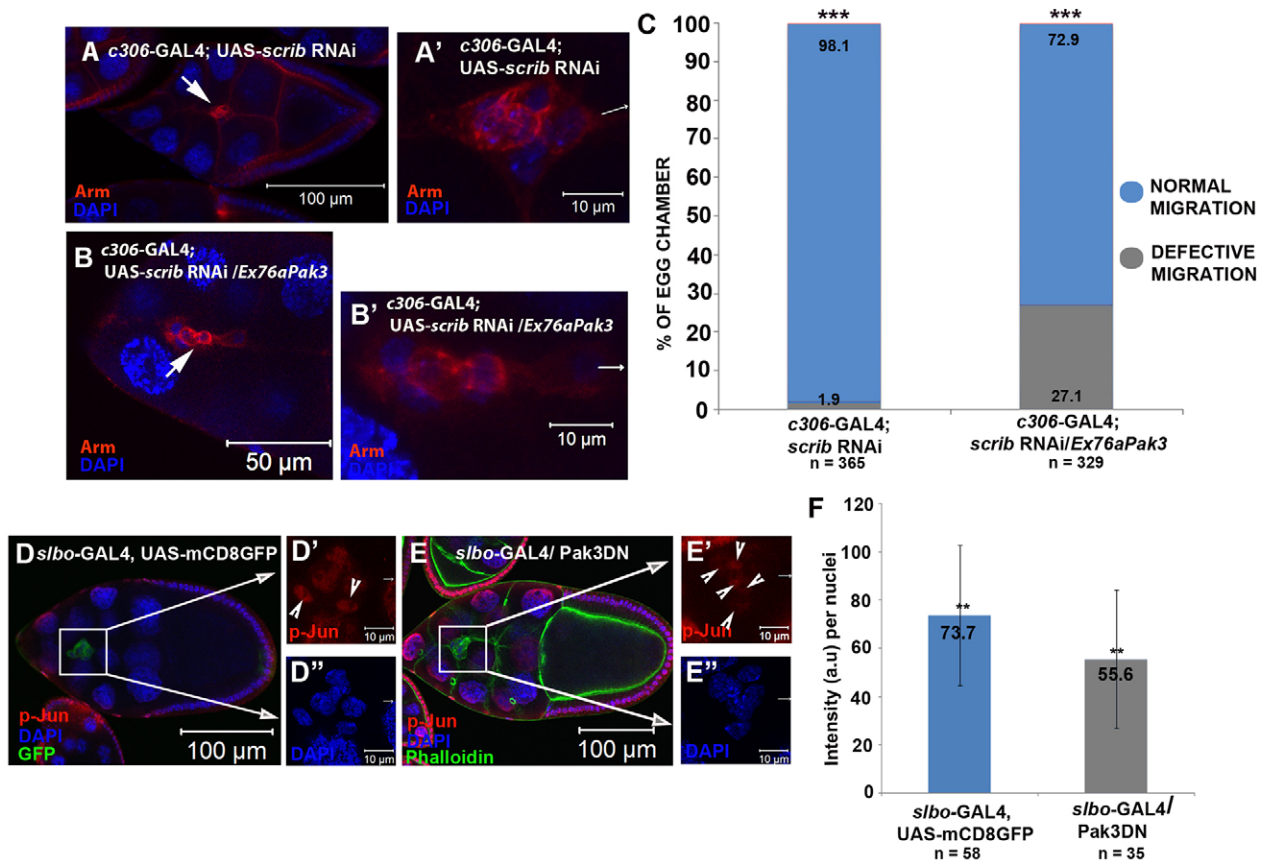
Fig. 4. Pak3 interacts with Scrib and regulates JNK signaling. (A-B′) Merged *z*-sections of egg chambers of the indicated genotypes stained for Arm (red) and with DAPI (blue). (A′,B′) Higher magnification of the border cells shown in A and B, respectively. Thin arrows indicate the direction of migration. Bold arrows mark border cells. (C) Inhibition of border cell movement by *scrib* RNAi is enhanced in the *Ex76aPak3*/+ background (***P<0.001). *n*, number of stage 10 egg chambers. (D-E″) Merged *z*-sections of (D-D″) *slbo*-GAL4; UAS-mCD8GFP and (E-E″) *slbo*-GAL4; Pak3DN/+ egg chambers stained with p-Jun antibody (red). (D′,D″ and E′,E″) Higher magnification of border cell cluster indicated in D and E. Arrowheads indicate the levels of p-Jun in the border cells. Note the reduction in the level of p-Jun between D′ and E′. (F) Quantification of p-Jun intensity for control and Pak3DN nuclei. **P<0.01. Error bars represent s.d.

with a weak rosette pattern observed in less than 20% of the clusters. Unlike in the wild type, the distribution of Sdt and Patj proteins at the bc-bc interface is weak or partial and observed in less than 85% of the Pak3-depleted clusters (*n*≥15). Remarkably, we observe distinct enrichment of Sdt and Patj proteins at the bc-nc interface in 85% and 73% of the clusters, respectively (Fig. 5B,B′,D,D′, Movie 3A,B). Similar enrichment at the bc-nc interface was observed for Crb protein in all the Pak3-depleted clusters analyzed (*n*=10; Fig. S4B,B′). Overall, these results suggest that the localization and distribution of apical proteins is regulated by Pak3 in the moving border cells.

We next examined the lateral markers Cora and Dlg1 in 3D reconstructions of wild-type clusters (*n*=23 and *n*=15 reconstructions, respectively). Cora and Dlg1 were asymmetrically localized and covered more than two-thirds of the circumference of the pc-bc interface in 90% of the clusters analyzed (Fig. 5E,E′, Fig. S4C,C′). In Pak3-depleted clusters, the distribution of Cora (*n*=24) and Dlg1 (*n*=16) proteins was restricted to the pc-bc interface but, unlike in the wild type, their expression domain was drastically reduced in 90% and 63% of the clusters, respectively (Fig. 5F,F′, Fig. S4D,D′). In addition, we observed mislocalization of Cora protein to the bc-bc and bc-nc interface in Pak3-depleted clusters (Movie 4A,B). We also analyzed the distribution of the basal protein βps-integrin in the migrating cluster. In wild-type clusters (*n*=15), βps-integrin is expressed at high levels at the bc-bc interface (Fig. 5G,G′) and less than 20% of the clusters exhibit some mislocalization to the bc-nc

interface. By contrast, 72% of the Pak3-depleted border cell clusters (*n*=18) exhibited mislocalization of βps-integrin to the bc-nc junction (Fig. 5H,H′). Since depletion of Pak3 affects the localization of apical, lateral and basal markers, this suggests that Pak3 modulates the apical-basal polarity of the migrating cluster.

Next we evaluated the distribution of the transmembrane protein E-cadherin in the migrating cluster. It has been shown that E-cadherin is mislocalized in Par-6-deficient border cells (Pinheiro and Montell, 2004). We reasoned that if Pak3 indeed affects the polarity of the migrating cluster, its downregulation should also affect the distribution of E-cadherin, as observed for Par-6-deficient clusters. In wild-type clusters, E-cadherin is enriched at bc-pc and bc-bc interfaces (Niewiadomska et al., 1999) and is maintained at a low level at the bc-nc interface (Bai et al., 2000; Niewiadomska et al., 1999). Consistent with our expectation, and unlike in the wild type, the E-cadherin distribution was enriched at the bc-nc interface in 70% of Pak3-depleted clusters (*n*=16; Fig. 5I-J′, Movie 5). This phenotype was recapitulated in MARCM experiments using the *Ex76aPak3* allele (Fig. S4E,E′). From the above results, it is clear that Pak3 modulates apical-basal polarity in the migrating border cells and that perturbing its function impedes the efficient movement of the cluster.

This observation is especially interesting considering that apical polarity proteins are known to cooperate with Rho GTPases to spatially regulate cellular protrusion in epithelial cells (Georgiou and Baum, 2010). Coupling the above results with our live imaging
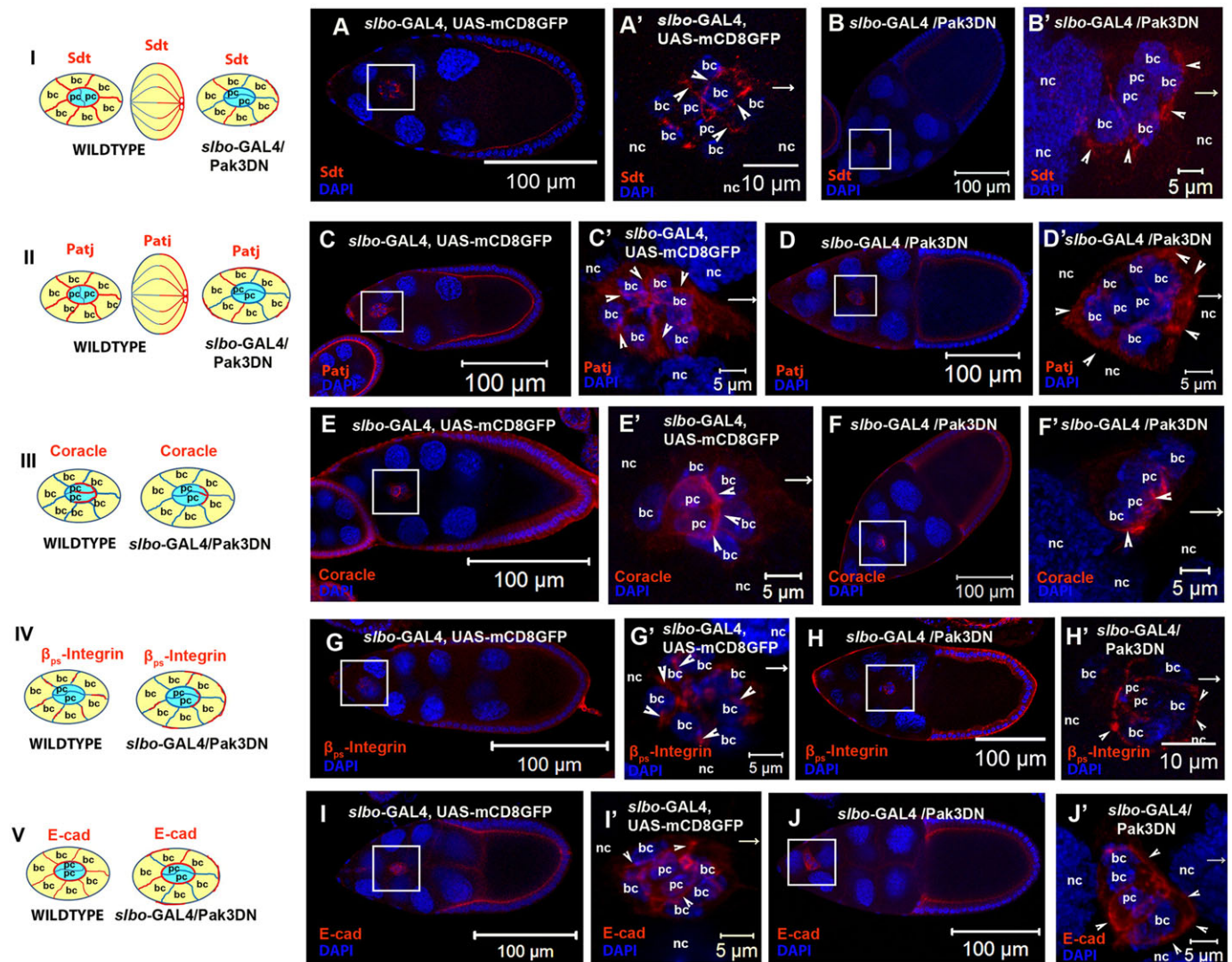
Fig. 5. Pak3 affects the apical-basal polarity of border cell clusters. (I-V) Schematic representations of protein distribution in control (*slbo*-GAL4, UAS-mCD8GFP) and Pak3-depleted (*slbo*-GAL4/Pak3DN) clusters. Bold red lines depict protein localization. pc, polar cell; bc, border cell; nc, nurse cell. (A-J′) Merged z-sections of egg chambers stained for Sdt, Patj, Cora, βps-integrin and E-cadherin (red) and with DAPI (blue). (A,A′,C,C′,E,E′,G,G′,I,I′) Control; (B,B′,D,D′,F,F′, H,H′,J,J′) Pak3 depleted. (A′-J′) Magnified border cell cluster shown (square) in A-J. In the control, Sdt and Patj mark the bc-bc and bc-pc interface (arrowheads in A′,C′). In Pak3-depleted clusters, Sdt and Patj are mislocalized to bc-nc interface (arrowheads in B′,D′). In the control, Cora is localized between the bc-pc and pc-pc interfaces (arrowheads in E′). Pak3 depletion reduces the domain of Cora expression (arrowheads in F′). In the control, βps-integrin expression marks the bc-bc interface (arrowheads in G′). In Pak3-depleted clusters, βps-integrin is mislocalized to bc-nc interface (arrowheads in H′). E-cadherin is localized to bc-bc and pc-bc cell interfaces in the control (I′). In Pak3-depleted clusters, E-cadherin is mislocalized to the bc-nc interface (arrowheads in J′).

data, it appears that loss of apical-basal polarity in Pak3-depleted clusters might result in the improper activation of GTPases, thus causing misdirected protrusion. In addition, we observed mislocalization of E-cadherin to the bc-nc interface in Pak3-depleted clusters. We know that the adhesive contact at the bc-nc interface is dynamic and needs to be turned over frequently for efficient forward movement. Having a higher level of E-cadherin at the bc-nc boundary should therefore result in stronger adhesions, thus impeding the efficiency and speed of forward movement.

In the context of all the results above, it appears that Pak3 functions downstream of guidance receptor signaling to regulate the apical-basal polarity of the migrating border cells. We were intrigued by the fact that our data suggest that guidance receptor signaling might also regulate apical-basal polarity in collectively moving border cells. To confirm this, we examined the polarity of border cells deficient in guidance receptor signaling, comparing

the distribution of the apical polarity marker Patj in control and guidance receptor-depleted border cells. Consistent with our expectation, and unlike in the control, downregulation of guidance receptor function resulted in mislocalization of Patj to the bc-nc interface (Fig. S6). This phenotype is similar to that observed for Pak3-depleted border cells, supporting our hypothesis that guidance receptor signaling modulates the distribution of the apical polarity marker in the moving border cells.

Next, we examined the role of Rac GTPase in apical-basal polarity, as it is known to be an important mediator of guidance receptor signaling and to mediate front-rear polarity in migrating border cells.

Rac1 mediates apical-basal polarity in moving border cells
Our result and previous reports suggest that Pak3 functions downstream of Rac GTPase in various developmental contexts in

flies (Duan et al., 2012; Baek et al., 2012). Since depletion of Pak3 or guidance receptor function affects the apical-basal polarity of migrating border cells, we proposed that if Rac GTPase functions in the same biochemical pathway as Pak3 then it should also affect the apical-basal polarity of the migrating cluster.

Rac1 regulates border cell movement, and downregulation of Rac1 function impedes migration in 98.6% of the clusters (Geisbrecht and Montell, 2004). We examined the distribution of apical-basal polarity markers in Rac1-deficient border cells. In wild-type clusters, the apical markers Sdt and Patj are enriched at the bc-bc and bc-pc interfaces (Fig. 6A,A′,C,C′), whereas in Rac1-compromised border cell clusters Sdt and Patj (n=10 clusters each) were mislocalized and enriched at the bc-nc interface in ≥90% of the clusters (Fig. 6B,B′,D,D′). This pattern was very similar to that observed for Pak3-depleted border cell clusters. In addition, the distribution of the lateral markers Cora (n=10) and Dlg1 (n=11) was altered. In wild-type clusters, these lateral markers were asymmetrically localized at the bc-pc interface and spanned two-thirds of the circumference of the pc-bc interface in 90% of the clusters (Fig. 6E,E′, Fig. S5A,A′). By contrast, the lateral domain in the Rac1-compromised cluster was severely reduced as we observed a reduction in the domain of expression of Cora and Dlg1 in 90% and 63% of the clusters, respectively (Fig. 6F,F′, Fig. S5B,B′). Similar to the Pak3-deficient clusters, we observed mislocalization of Cora protein to bc-bc and bc-nc interfaces in 80% of the clusters

analyzed. In addition, the border cells overexpressing the RacN17 construct exhibited mislocalization of E-cadherin to the bc-nc interface in 80% of the clusters (n=10; Fig. 6G-H′). These results suggest that impairment of Rac1 function results in loss of apical-basal polarity in migrating border cells.

Consistent with our expectation, downregulation of Rac1 function affects apical-basal polarity in the moving border cells. Although Rac GEF (guanine exchange factor) and polarity proteins such as Baz are known to function together to spatially regulate cellular protrusion in epithelial cells, this is probably the first evidence to suggest that Rac GTPase can affect apical-basal polarity in collectively moving border cells (Georgiou and Baum, 2010). This observation is fascinating, as it appears that guidance receptor signaling functioning through Rac GTPase and Pak3 controls both front-rear and apical-basal polarity in migrating border cells.

Increasing JNK signaling rescues the polarity defect associated with Pak3DN

Having established that Pak3 functions downstream of guidance receptor signaling, we investigated how Pak3 affects apical-basal polarity in migrating border cells. As JNK signaling has been shown to affect localization of the polarity marker Baz in the migrating cluster, we examined whether Pak3 functions through the JNK pathway to modulate apical-basal polarity in the moving border cells (Llense and Martin-Blanco, 2008). We compared the
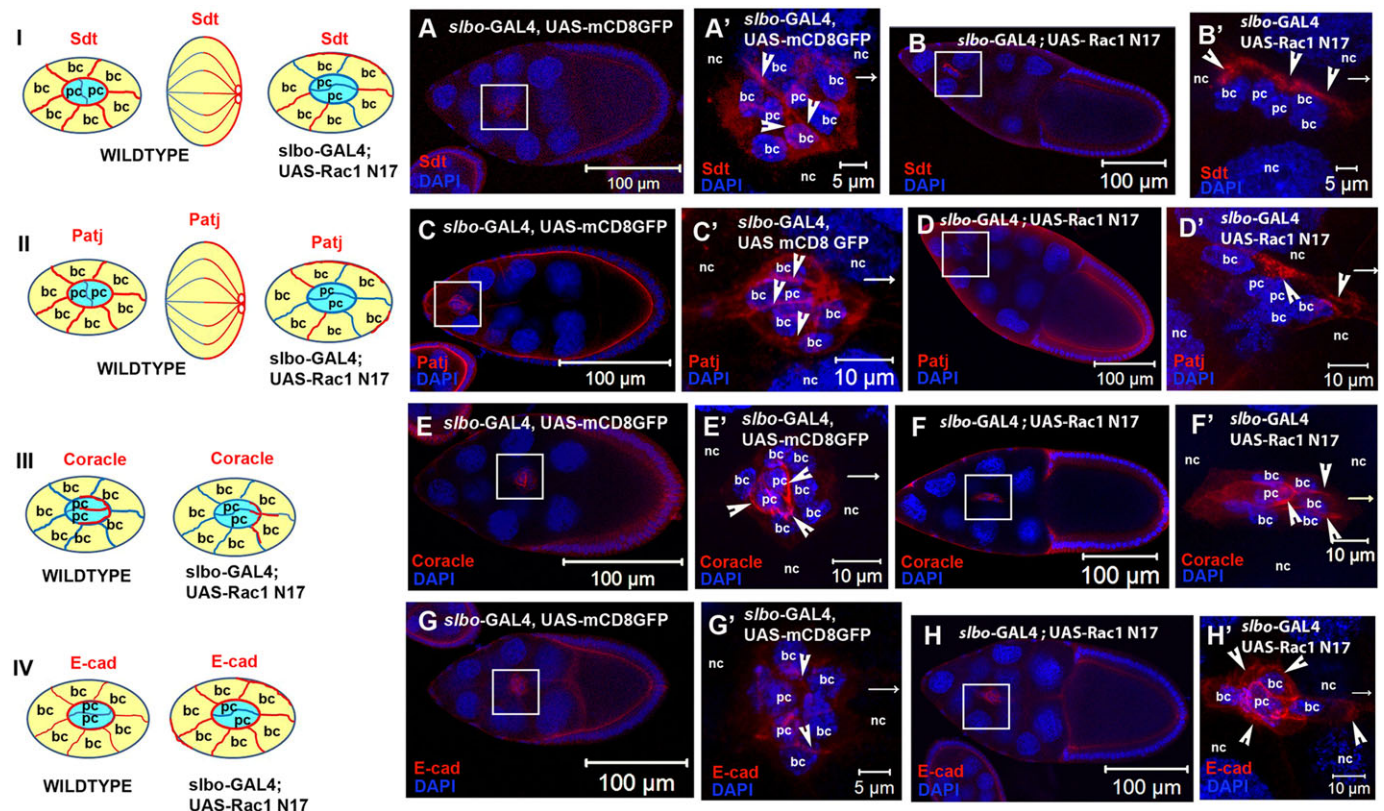
Fig. 6. Rac1 affects apical-basal polarity in migrating border cells. (I-IV) Schematic representations of protein distribution in control (*slbo*-GAL4, UAS-mCD8GFP) and Rac1-depleted (*slbo*-GAL4; UAS-RacN17) clusters (see Fig. 5 legend for key). (A-H′) Merged *z*-sections of clusters stained for the polarity markers Sdt, Patj, Cora and E-cadherin (red) and with DAPI (blue). (A,A′,C,C′,E,E′,G,G′) Control; (B,B′,D,D′,F,F′,H,H′) Rac1 depleted. (A′-H′) Magnified border cell cluster shown (square) in A-H. Sdt and Patj are localized between the bc-bc and bc-pc interfaces in control clusters (arrowheads in A′,C′), whereas in Rac1-depleted clusters Sdt and Patj are mislocalized to the bc-nc interface (arrowheads in B′,D′). In the control, Cora is localized between bc-pc and pc-pc interfaces (arrowheads in E′). In Rac1-depleted clusters the domain of Cora expression is reduced and also mislocalized to the bc-bc interface (arrowheads in F′). E-cadherin is localized to bc-bc and pc-bc interfaces in the control (arrowheads in G′). Similar to the Pak3-deficient cluster, the downregulation of Rac1 function mislocalizes E-cadherin to the nc-bc interface (arrowheads in H′).
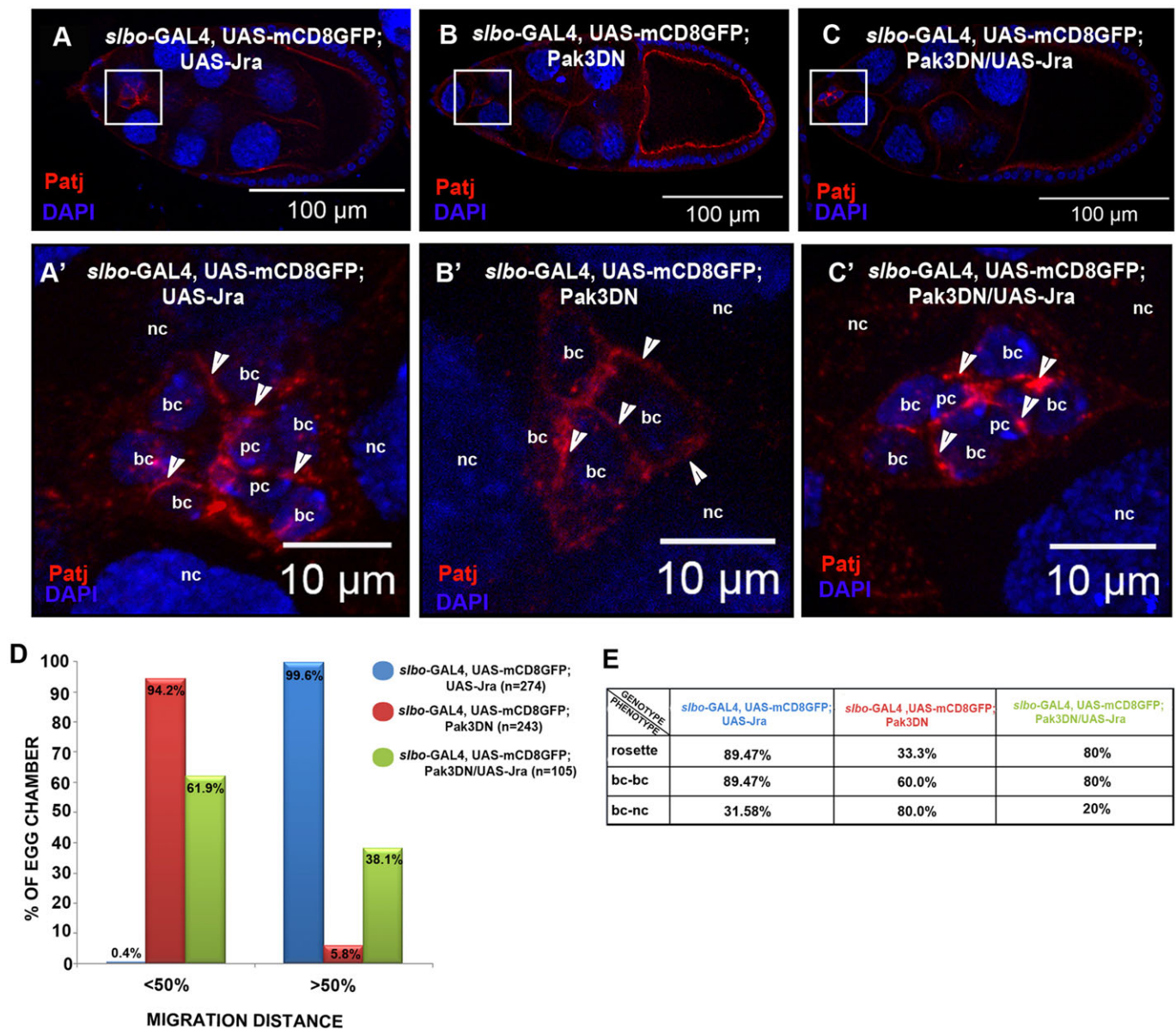
Fig. 7. Increasing JNK signaling rescues the distribution of the apical marker Patj in Pak3DN clusters. (A-C′) Merged z-sections of egg chambers of the indicated genotype stained for Patj (red) and with DAPI (blue). (A′-C′) Magnified images of the border cell cluster shown (square) in A-C. Arrowheads indicate the expression pattern of Patj. Clusters overexpressing Jra mimic the Patj distribution in controls, i.e. rosette distribution with the bc-bc interface highlighted (A′). Pak3DN clusters exhibit loss of the rosette pattern and enrichment at the bc-nc interface (B′). (C,C′) Clusters co-expressing Pak3DN and Jra. Note the rescue in the rosette distribution of Patj compared with Pak3DN. (D) Migration efficiency of the indicated genotypes. n, number of clusters analyzed. (E) Quantification of the distribution of Patj in clusters of the indicated genotype. Note the rescue (compare green with red) in the migration efficiency (D) and distribution of Patj (E) in Pak3DN upon co-expression of Jra.

distribution of the apical marker Patj in Pak3-depleted clusters overexpressing either Jra or the mCD8GFP reporter. Patj expression in the border cell cluster overexpressing Jra is similar to that of the wild type. We observed a distinct rosette pattern for Patj protein, with the bc-bc interface conspicuously highlighted in 80% of the clusters (n=15). In less than 20% of the clusters Patj was mislocalized at the bc-nc interface (Fig. 7A,A′). None of the Pak3-depleted clusters exhibited a conspicuous rosette pattern, although we did observe some weak or partial rosette distribution in a few clusters. However, all Pak3-depleted clusters exhibited distinct enrichment of Patj at the bc-nc interface (n=15 clusters; Fig. 7B,B′). Interestingly, we observed a distinct rosette pattern for Patj protein in more than 80% of the clusters co-expressing Jra and

Pak3DN (n=15 clusters; Fig. 7C,C′). In addition, we observed some degree of rescue in the migration efficiency of the clusters, as 38% of border cell cohorts co-expressing Jra and Pak3DN covered more than 50% of the migration distance, as compared with 5.8% for Pak3-depleted clusters (Fig. 7D). This suggests that overexpression of Jra rescues the Patj distribution and the migration efficiency of Pak3-depleted clusters, supporting our hypothesis that Pak3 modulates apical-basal polarity through JNK signaling in moving border cells.

The result above is consistent with a previous report that JNK signaling modulates apical-basal polarity in migrating border cells to regulate cluster integrity and efficient forward movement (Llense and Martin-Blanco, 2008). Curiously, our rescue at the level of distribution

of the apical polarity protein Patj was much more penetrant than the restoration of migration efficiency of the cluster, suggesting that Pak3 might affect multiple processes in moving border cells, one of which is the modulation of apical-basal polarity in the migrating cluster.

DISCUSSION

Collective cell migration plays an important role in several processes, including tissue morphogenesis, wound healing and tumor cell dissemination. Hallmarks of collective cell migration are the dynamic maintenance of cell-cell junctions and the partial retention of apical-basal polarity in the moving cells. Although these characteristics are very important for efficient group cell movement, the underlying molecular mechanism controlling these traits and how they effectively orchestrate migration are not well understood. We have employed the model of border cell migration in *Drosophila* oogenesis to examine the molecular machinery that regulates the asymmetric distribution of polarity proteins in the migrating cluster and how this is likely to influence their migratory behavior.

Our study demonstrates that the group I p21-activated kinase Pak3 is a crucial modulator of apical-basal polarity in migrating border cells. Based on our analysis combined with previous findings (Baek et al., 2012; Duan et al., 2012), we propose that Pak3 functions downstream of Rac GTPase in guidance receptor signaling to modulate efficient border cell movement. In Pak3-compromised border cells, the distribution of polarity proteins is significantly altered. In control clusters, apical markers such as Sdt, Patj and Crb are asymmetrically localized and decorate the bc-bc and bc-pc interfaces. However, in Pak3-depleted clusters the apical markers are mislocalized to the bc-nc interface. Moreover, the distribution of lateral domain markers such as Cora and Dlg1 was also affected. In wild type, the lateral markers asymmetrically span more than half the surface of the pc-bc interface. In Pak3-compromised border cells this domain is severely reduced, supporting the fact that apical-basal polarity is disrupted. Expression of βps-integrin, which marks the basal domain, was also altered and the protein mislocalized to the bc-nc interface instead of the bc-bc junction. These results suggest that Pak3 modulates the apical-basal polarity of the migrating border cells and, when its function is compromised, the migration efficiency of the cluster is hampered. Our data suggest that Pak3 functions via JNK signaling to regulate the asymmetric distribution of polarity markers in migrating border cells.

Since disruption of polarity determinants, such as Baz and Par-6, impedes border cell movement (Pinheiro and Montell, 2004), it is intriguing as to how the retention of apical-basal polarity is also essential for the proper execution of collective cell movement. One hypothesis suggests that the partial retention of apical-basal polarity aids in cluster movement by the asymmetric restriction of proteins such as E-cadherin and actin. This polarized distribution probably aids in direction sensing and the formation of appropriately directed forward protrusions (Pinheiro and Montell, 2004). Our results support this hypothesis in the following ways. (1) In live cell imaging experiments, we observe large numbers of misdirected, short-lived protrusions from Pak3-depleted clusters. (2) Genetic interaction of Pak3 with the guidance receptor Pvr suggests that retention of apical-basal polarity helps in direction sensing and the generation of polarized cellular protrusions. (3) In Pak3-deficient border cell clusters, we observed mislocalization of E-cadherin to the nc-bc interface. In moving border cells, we know that E-Cadherin is maintained at low levels at the bc-nc interface. Thus, alteration in E-cadherin levels at the bc-nc interface might result in stronger adhesion between border cells and nurse cells, thus impeding protrusive behavior and the forward movement of the cluster.

Given that Rac GTPase regulates front-rear polarity during cell movement, our results suggest that it also has a similar role in the maintenance of apical-basal polarity in collectively migrating cells, although we do not understand very clearly whether Rac regulates both polarities independently or if setting up of one kind of polarity is prerequisite for the establishment of the other. It will be interesting to examine this further as it will provide important insight into how different polarities are generated and maintained in collective cell movement. In addition, since several tumor cells disseminate as small cohorts or clusters, it would be interesting to test whether they use the same mode as that described here for border cells, for efficient dispersal.

MATERIALS AND METHODS

Drosophila stocks
c306-GAL4 and *slbo*-GAL4 were employed for overexpression (Brand and Perrimon, 1993; Rørth et al., 1998). hsflp;P[Actin-<17b>GAL4], UAS-GMA (Prasad and Montell, 2007) was used for overexpression clonal analysis (see the supplementary Materials and Methods). The following fly lines were used: *dPak3^{zyg}* (Duan et al., 2012), *Ex76aPak3* (Bahri et al., 2010), UAS-Pak3V5(K322A) (Duan et al., 2012), UAS-GMA (Dutta et al., 2002), UAS-DNPVR, UAS-DNPVR; UAS-DNDER (Duchek et al., 2001), UAS Rac1N17 (Murphy and Montell, 1996), UAS-*scribble*RNAi (Bloomington Stock Center number 29552) and UAS-Pak3RNAi (v44607,VDRC). For MARCM, *c306*-GAL4; FRT82B *Ex76aPak3/Tb* was crossed to Hsflp;UAS GFP, FRT 82BeTubGal80 (BDSC).

Immunohistochemistry
2- to 3-day-old female flies were fattened at 29°C for all experiments with Pak3DN and *scrib* RNAi strains for 20-22 h. The remainder were fattened at 25°C for 14-16 h. Ovary dissection, fixation and staining were performed using standard protocols (Montell, 1999). For further details, see the supplementary Materials and Methods.

Live imaging and analysis
Flies used for live cell imaging of border cell migration were of genotype *c306*-GAL4; UAS-GMA/UAS-Pak3DN, with *c306*-GAL4; UAS-GMA/ UAS-GMA as control. Time-lapse microscopy was performed as described earlier (Prasad and Montell, 2007). Frames were taken at 2-min intervals. Culture conditions were the same for both control and experimental samples. On average, each movie lasted for ~3-4 h. Details concerning the calculation of protrusion parameters, actin fiber length and quantification of p-Jun are provided in the supplementary Materials and Methods.

Statistics
Statistical significance was analyzed by a two-tailed *t*-test of unequal variance.

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Supplementary information
Supplementary information available online at
http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.125682/-/DC1

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