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Genetic Interaction Screens Identify a Role for Hedgehog Signaling in *Drosophila* Border Cell Migration

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Abstract

Background—Cell motility is essential for embryonic development and physiological processes such as the immune response, but also contributes to pathological conditions such as tumor progression and inflammation. However, our understanding of the mechanisms underlying migratory processes is incomplete. *Drosophila* border cells provide a powerful genetic model to identify the roles of genes that contribute to cell migration.

Results—Members of the Hedgehog signaling pathway were uncovered in two independent screens for interactions with the small GTPase Rac and the polarity protein Par-1 in border cell migration. Consistent with a role in migration, multiple Hh signaling components were enriched in the migratory border cells. Interference with Hh signaling by several different methods resulted in incomplete cell migration. Moreover, the polarized distribution of E-Cadherin and a marker of tyrosine kinase activity were altered when Hh signaling was disrupted. Conservation of Hh-Rac and Hh-Par-1 signaling was illustrated in the wing, in which Hh-dependent phenotypes were enhanced by loss of *Rac* or *par-1*.

Conclusions—We identified a pathway by which Hh signaling connects to Rac and Par-1 in cell migration. These results further highlight the importance of modifier screens in the identification of new genes that function in developmental pathways.

Keywords

cell migration; border cells; *Drosophila*; Hedgehog; Par-1; Rac

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Introduction

Cell migration is integral to normal processes such as the formation and remodeling of tissues during development. Because misregulated migration contributes to birth defects and cancer, gaining a better understanding of the mechanisms that control normal migration is vital. While more is known about single cell motility, cells frequently migrate in small to large collectives throughout development and in tumor metastasis (Friedl and Gilmour, 2009; Friedl et al., 2012). Our knowledge of how individual cells receive and integrate outside signals to coordinate group level migration within their native tissue environment is still incomplete. Studies using the *Drosophila* border cell model have provided recent insight into the cellular and molecular mechanisms governing *in vivo* collective cell migration (Yilmaz and Christofori, 2010; He et al., 2011). Border cells are a specialized group of cells that migrate during ovarian development (He et al., 2011; Montell et al., 2012). The *Drosophila* ovary is composed of strings of progressively developing egg chambers, each of which contains an oocyte and 15 supportive nurse cells surrounded by a monolayer follicle cell epithelium (Spradling, 1993; He et al., 2011). Border cells are specified in the epithelium at early stage 9 of oogenesis (Fig. 1A). A specialized pair of follicle cells at the anterior end of the egg chamber, the polar cells, recruit 4 to 8 additional cells surrounding the polar cells to become migratory border cells. Border cells detach from the epithelium as a cohesive cluster, migrate between the germline-derived nurse cells over a distance of ~150 μm and stop at the oocyte border by stage 10 (Fig. 1A).

The stereotypical migration of border cells is precisely regulated by the action of at least four known signaling pathways. Signaling through the JAK/STAT pathway specifies border cell identity and regulates border cell motility (Silver and Montell, 2001; Beccari et al., 2002; Ghiglione et al., 2002; Silver et al., 2005). In parallel, steroid hormone signaling through the Ecydsone receptor coordinates the timing of migration (Bai et al., 2000; Jang et al., 2009). JNK signaling promotes proper levels of cell adhesion between border cells to maintain the cluster during migration (Llense and Martin-Blanco, 2008; Melani et al., 2008). Finally, multiple growth factor ligands secreted by the oocyte activate two receptor tyrosine kinases (RTKs) expressed on border cells, Platelet Derived Growth Factor Receptor/ Vascular Endothelial Growth Factor Receptor-related (PVR) and Epidermal Growth Factor Receptor (EGFR), to direct border cells to the oocyte (Duchek et al., 2001; McDonald et al., 2003; McDonald et al., 2006). A current challenge is to understand how all of these signals are integrated at the spatial-temporal level to promote border cell detachment, to maintain cluster cohesiveness, and to establish directional guidance.

Like most migrating cells, border cells require the small GTPase Rac for movement. Rac regulates the actin cytoskeleton to promote cellular protrusions at the leading edge of single cells or sheets of cells. These protrusions allow cells to grip their migratory substrate and/or enable cells to sense directional signals in the environment for navigation within tissues. In border cells, RTK signaling promotes the formation of actin-rich cellular protrusions at the leading edge of the migrating cluster through a localized increase in the activation of Rac (Bianco et al., 2007; Prasad and Montell, 2007; Wang et al., 2010). Moreover, activation of Rac in one border cell is sufficient to polarize the cluster and direct their migration (Wang et al., 2010). However, the mechanism by which asymmetric Rac activation is interpreted between cells in the cluster to promote organized forward movement is still unclear. To help elucidate these and other mechanisms of border cell migration, we performed a screen to identify genes required for Rac-dependent cell motility. We report here that *hedgehog* (*hh*) is a strong suppressor of dominant-negative Rac-induced migration defects.

Hh was originally identified in a mutant screen designed to identify genes required for *Drosophila* embryonic patterning (Nusslein-Volhard and Wieschaus, 1980). Homologs are

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found in many species and play vital roles in a wide range of developmental processes, including cell fate determination, patterning, and cell proliferation (Ingham et al., 2011). Hh is a secreted morphogen that is capable of both short- and long-range signaling. Because Hh is a potent signaling molecule, its production, secretion, movement, and reception are highly regulated. The binding of Hh to its receptor, Patched (Ptc), alleviates Ptc-dependent repression of the protein Smoothened (Smo) to activate downstream signaling events. Thus, Hh and Ptc act antagonistically and *hh* loss-of-function phenotypes are similar to those observed when *ptc* is overexpressed (Ingham et al., 2011).

The known transcriptional effects of Hh signaling are mediated by the zinc-finger transcription factor Cubitus interruptus (Ci). This protein is a member of the vertebrate Gli family of transcription factors that can either activate or repress target gene transcription (Huangfu and Anderson, 2006). Upon Hh stimulation, the full-length activated form of Ci, known as Ci-155, translocates to the nucleus to activate transcription of downstream targets such as *ptc* and *decapentaplegic* (*dpp*). However, in the absence of Hh signaling, the C-terminal region of Ci undergoes a series of phosphorylation events that inhibits Ci activity (Aza-Blanc et al., 1997). After initial phosphorylation by cAMP-dependent protein kinase (PKA), both glycogen synthase kinase 3 (GSK-3) and casein kinase 1 (CK1) are capable of phosphorylating Ci (Aza-Blanc et al., 1997; Price and Kalderon, 1999; Price and Kalderon, 2002). These sequential modifications result in protein cleavage to generate a short form called Ci-75 which when ectopically overexpressed represses Hh activity and target genes (Aza-Blanc et al., 1997). Thus, modulation of Hh signaling at the level of signal pathway initiation or the control of post-translational modifications of downstream components is important to maintain proper gene expression in a wide variety of tissues.

While Hh functions as a chemoattractant of migrating cells and axons (Lin et al., 2012), the role (if any) of *hh* and its vertebrate homologs in cell motility apart from chemotactic responses is less understood. Moreover, the extent to which Hh signaling is generally required for cell migration during *Drosophila* and/or vertebrate development is unclear. Among its many biological functions, Hh promotes follicle cell differentiation during *Drosophila* oogenesis (Forbes et al., 1996a; Forbes et al., 1996b). Mutations in the negative regulators *ptc, cos-2* and the *Pka* catalytic subunit (*Pka-C1*) result in ectopic polar cells, which in some cases recruits cells to form additional border cell clusters (Liu and Montell, 1999; Zhang and Kalderon, 2000; Bai and Montell, 2002). Although a few of these extra border cell clusters complete their migration to the oocyte/nurse cell boundary, other clusters exhibit delayed migration. Whether this is due to a direct role for the Hh pathway in cell migration or is a consequence of altered cell fate remains to be determined. In this study, we present data that highlight a role for Hh signaling in border cell migration apart from border cell fate specification. Based upon the identification of Hh pathway components in two independent genetic screens, we propose that Hh signaling intersects with both Rac and the polarity protein Par-1 to mediate proper migration. Moreover, Hh may be required to maintain the subcellular localization of E-cadherin and polarized tyrosine phosphorylation, a marker of active tyrosine kinase signaling, to facilitate cell migration.

Results

Identification of *hh* in a Gain-of-Function Screen for Genes that Modify Rac-Dependent Border Cell Motility

Rac is a major regulator of border cell migration and promotes the actin-rich protrusions needed for their efficient collective motility (Murphy and Montell, 1996; Geisbrecht and Montell, 2004; Wang et al., 2010). However, relatively little is known about the genetic and molecular pathways that interact with Rac in this highly regulated process. We previously described a screen to identify genes that when overexpressed, modify the Rac-dependent cell

migration defect (Geisbrecht and Montell, 2004). We report here the complete results of the screen and describe one candidate, *hh*, in more detail (Table 1; Fig. 1B-G). An inactive form of Rac (*RacN17*) was expressed in border cells using *slow border cells*- (*slbo*-) GAL4, which drives expression in all border cells of the cluster, but not the central polar cells, prior to and throughout their migration (Fig. 1A, 1E). Expression of UAS-*RacN17* driven by *slbo*-GAL4 resulted in a complete block in border cell migration and significantly reduced female sterility (Fig. 1B, C) (Murphy and Montell, 1996; Geisbrecht and Montell, 2004). Therefore, we performed a screen to identify genes that when overexpressed have the ability to overcome this collective cell migration defect.

We crossed flies of the genotype *slbo*-GAL4; UAS-*RacN17* to an available collection of 2273 randomly inserted EP (enhancer/promoter) “target” P-element lines designed to drive overexpression of genes located near the insertion site (Rorth et al., 1998; Geisbrecht and Montell, 2004). Ovaries from the progeny of misexpressed EP lines that significantly rescued fertility were dissected and immunostained to directly examine the extent of border cell migration in stage 10 egg chambers. We then determined the migration index (MI), which is calculated from the weighted average migration distance of each border cell cluster from the anterior end of the egg chamber to the nurse cell/oocyte border (Fig. 1A; 0 = no migration; 100 = complete migration). The MI allowed us to determine which EP lines significantly rescued the *RacN17*-mediated border cell migration defects (Fig. 1B; Table 1). We verified that expression of additional *UAS* constructs did not non-specifically titrate out the amount of GAL4 protein required to maintain expression of inactive Rac. Expression of UAS-*mCD8:GFP* or UAS-*slbo* resulted in a MI of 4 and 7, respectively, which was not significantly different from *slbo*-GAL4, UAS-*RacN17* alone with a MI of 4 (Fig. 1B) (Geisbrecht and Montell, 2004).

Twenty-nine EP lines that exhibited a MI   20 were kept for further study. Of these, nine genes were confirmed to be overexpressed by the upstream EP-line in a GAL4-dependent manner (Table 1). All of these lines were located either upstream or immediately downstream of the predicted ATG in the correct orientation. The roles of two genes identified in this screen, *Drosophila inihibitor of apoptosis 1* (*diap1*) and *actin 5C*, were previously described (Geisbrecht and Montell, 2004). To address specificity of the EP lines for rescue of *RacN17* migration defects, we further tested their ability to rescue the border cell migration defects of other known mutants (Table 1). The overall levels and spatial activity of Rac are important for border cell migration; uniform expression of constitutively active Rac (*RacV12*) in all border cells completely inhibits their migration (Murphy and Montell, 1996; Duchek et al., 2001; Geisbrecht and Montell, 2004). Notably, however, none of the EP lines significantly suppressed *RacV12*. Constitutively active Ras (*RasV12*), a different GTPase independently involved in border cell migration, also strongly disrupts border cell migration (Lee et al., 1996). However, only one EP line (CG3394) rescued *RasV12*. Slbo is a C/EBP transcription factor that regulates the expression of genes required for border cell motility (Montell et al., 1992; Borghese et al., 2006; Wang et al., 2006). Similar to *RacN17, slbo* mutant border cells fail to migrate and remain at the anterior tip of the egg chamber (Montell et al., 1992). Only overexpression of the EP lines inserted into the *actin5C* and *spt4* genes suppressed the *slbo* migration defects. Thus, while a few EP lines were able to rescue other border cell mutants, the majority of the EP lines identified in this screen specifically suppressed the *RacN17* phenotype.

The strongest suppressor identified in the screen was EP(3)3521 (Fig. 1B), which rescued the *RacN17* migration defects almost as effectively as the positive control, *UAS-RacWT* (Fig. 1B). This EP line is inserted in the 5’UTR of the *hh* gene (Fig. 1D). We performed *in situ* hybridizations to confirm that EP(3)3521 specifically overexpressed *hh* transcript in a *slbo*-GAL4 specific pattern (Fig. 1E-G). Moreover, expression of Hh using three different,

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independent UAS-*hh* transgenes rescued the *RacN17* migration defects, although to varying degrees. The least level of suppression occurred with UAS-*hh* (N), a truncated active form of Hh (Porter et al., 1996) possibly because too much Hh signal was induced (see below). This Hh-mediated rescue of border cell migration was specific for *RacN17*, because overexpression of EP(3)3521/Hh did not suppress the *RacV12, RasV12*, or *slbo* mutant migration defects (Table 1).

Identification of *ptc* in a Screen for Genes that Interact with *par-1* in border cell migration

Dominant genetic interaction screens often reveal components of genetic pathways or cellular processes that may be missed in other types of screens (St Johnston, 2002). We recently identified a critical role for the serine-threonine kinase and cell polarity protein Par-1 in border cell detachment and motility (McDonald et al., 2008). Heterozygotes of a strong loss-of-function allele of *par-1, par-1^{27C1}*, exhibit normal border cell migration (Table 2). However, *par-1^{27C1}* exhibits strong border cell migration defects in transheterozygous combination with mutant alleles of several genes required for border cell migration (Liu and Montell, 1999; McDonald et al., 2003). We reasoned that other genes required for border cell migration, and potentially those that function with *par-1*, might dominantly interact with *par-1* in a similar manner. Therefore, we performed an EMS mutagenesis screen of the second chromosome for alleles that caused border cell migration defects in heterozygous combination with *par-1^{27C1}*.

Out of 2,198 chromosomes screened, six complementation groups (seven mutant alleles) disrupted border cell migration when crossed to *par-1^{27C1}* (Table 2). These genes were termed *pdig*, for *par-1* dominant interacting genes. Two *pdig* complementation groups were allelic to known border cell migration genes: the *pdig1* allele disrupts *taiman* (*tai*) and the *pdig6* group is allelic to the *Drosophila* E-cadherin gene *shotgun* (*shg*). Since *tai* and *shg* are both essential for border cell migration (Niewiadomska et al., 1999; Bai et al., 2000), the isolation of mutations in these two genes validated the screen approach. The four other *pdig* genes (*pdig2, pdig3, pdig4* and *pdig5*) were subsequently mapped to specific chromosomal regions using the cytologically defined Bloomington deficiency kit (Table 2). A mutation in *pdig4* maps to *sec5*, which encodes a member of the exocyst complex and has recently been implicated in border cell migration (Assaker et al., 2010). The other three *pdig* alleles map to regions that do not contain genes known to regulate border cells, suggesting we have identified mutations in new genes involved in border cell migration.

To identify the gene mutated by *pdig5^{31A7}*, the allele was mapped to the cytological region 44D5-E3 by complementation with overlapping deficiencies (Table 2). Available lethal mutant alleles for this region were tested for complementation with *pdig5^{31A7}*. Two strong loss of function alleles of the Hh receptor Ptc, *ptc^7* and *ptc^9*, were lethal in combination with *pdig5*, suggesting that they are allelic. Moreover, *par-1^{27C1}* exhibited comparable border cell migration defects in heterozygous combination with *ptc^9* (12% of egg chambers; n = 396). Therefore, *pdig5^{31A7}* is an allele of *ptc* and we identified a genetic interaction between *par-1* and *ptc*. Surprisingly, we also observed a genetic interaction between *par-1* and a positive regulator of the Hh pathway, *smo*. In egg chambers heterozygous for both *par-1^{27C1}* and *smo^3*, 11% of border cells did not complete their migration (n = 254 egg chambers). We observed a weaker interaction with *hh*; 7% of egg chambers heterozygous for *par-1^{27C1}* and *hh^{AC}* had a border cell migration defect (n = 338 egg chambers). These results suggest that reduction of *par-1* sensitizes border cells to changes in the overall levels of Hh signaling. Since Hh was identified as a suppressor of dominant-negative Rac, we also tested the ability of Par-1 to modify *RacN17* phenotypes in this assay (Fig. 1B). Overexpression of *par-1* suppressed Rac-mediated border cell migration defects, although to a lesser extent than Hh.

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Members of the Hh Pathway are Expressed in Border Cells During Late Oogenesis

The identification of members of the Hh pathway in two independent genetic screens strongly suggested a role for Hh signaling in border cells. While the expression and function of the Hh pathway has been examined at earlier stages of oogenesis (Forbes et al., 1996a; Forbes et al., 1996b; Zhang and Kalderon, 2000; Zhang and Kalderon, 2001; Hartman et al., 2010), it has not been described during later oogenesis. Thus, we determined the expression pattern of key members of the Hh pathway during stages 8 to 10 of oogenesis when border cells are recruited and undergo migration. We first investigated the expression of a nuclear-*lacZ* enhancer trap in the *hh* gene locus, which has been shown to report the endogenous localization of *hh* in various tissues including the early ovary (Forbes et al., 1996a). *hh-lacZ* is expressed in the germarium (Forbes et al., 1996a) and then turns off until later oogenesis, when it specifically labels border cells but not central polar cells (Fig. 2A). We confirmed the expression of *hh* in border cells via *in situ* hybridization using an anti-sense probe (Fig. 1G).

We next determined the expression of the Hh receptor Ptc, which was assayed first with a nuclear-*lacZ* enhancer trap in the *ptc* gene. ptc^{H84}-*lacZ* reflects *ptc* endogenous expression and reports Hh activity (Forbes et al., 1996b). ptc^{H84}-*lacZ* was expressed at very low levels at stage 7 in all cells but was expressed at high levels in border cells starting when they form at late stage 8 (Fig. 2B). At stages 9 and 10, border cells had detectable ptc^{H84}-*lacZ* with low expression in other follicle cells including the polar cells (Fig. 2B, 2C). These data were confirmed with two *lacZ* reporter constructs encompassing 10.8 kb (Fig. 2D) and 5.5 kb (Fig. 2E), respectively, of the *ptc* 5' upstream regulatory region. These constructs are expressed in the normal *ptc* RNA and protein patterns in the wing disc (data not shown) (Chen and Struhl, 1996). *ptc-lacZ* 10.8 was highly expressed in polar cells and at low levels in border cells and follicle cells (Fig. 2D). *ptc-lacZ* 5.5 was expressed at similarly high levels in the polar cells but at higher levels in border cells and follicle cells beginning at late stage 8 and continuing through stage 10 (Fig. 2E). We observed slight differences in the expression levels of the *ptc* reporters in border cells, which could be due to insertion site variability and/or difference in the constructs themselves. Nonetheless, the observed patterns of *ptc-lacZ* are consistent with *ptc* expression turning on within border cells prior to and during their migration. We confirmed the *ptc* expression pattern using *in situ* hybridization; signal was observed early in the germarium, as previously reported (Forbes et al., 1996b), as well as in border cells (Fig. 2F). Ptc protein is normally found in intracellular vesicles and undergoes active trafficking within the cell (Zhu et al., 2003; Torroja et al., 2004). Using an antibody to Ptc, we detected uniform but low Ptc protein in cytoplasmic punctae in all follicle cells including border cells (Fig. 2G). Ptc protein was found in a broader pattern than that of *ptc-lacZ*, but agrees with previously published results and the RNA expression pattern (Fig. 2F) (Forbes et al., 1996b).

Next, we examined the localization of Smo, the obligate transducer of Hh signal (Fig. 2H, 2I). Smo protein was broadly expressed in egg chambers, with high levels in the germline and lower levels in follicle cells. When we examined the localization of Smo in border cells in further detail, we observed Smo in cytoplasmic-associated punctae within border cells (Fig 2I). This subcellular localization of Smo is consistent with its known association with intracellular vesicles in Hh signaling-responsive cells (Zhu et al., 2003; Torroja et al., 2004).

Finally, we analyzed the expression of the transcription factor Ci in later-staged egg chambers. A GFP protein trap in the first intron of the *ci* gene (Quinones-Coello et al., 2007) showed high expression in follicle cells and border cells (Fig. 2J). To confirm this expression, we used an antibody to detect full-length Ci (Ci-FL), which is the form of Ci that translocates to the nucleus and activates the Hh pathway (Aza-Blanc et al., 1997). Ci-FL undergoes shuttling between the cytoplasm and the nucleus and under normal antibody

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staining conditions appears cytoplasmic (Aza-Blanc et al., 1997; Hooper, 2003). Notably, high levels of Ci-FL were detected in the cytoplasm of follicle cells with lower levels in border cells (Fig. 2K), in agreement with a report by Sun and Deng (Sun and Deng, 2007).

STAT Regulates *hh* and *ptc* Expression in Border Cells

The transcription factor Stat92E regulates expression of multiple downstream targets specifically required for border cell migration, including *slbo* (Silver and Montell, 2001; Beccari et al., 2002). Since *hh* and *ptc* are both expressed at relatively high levels in border cells, we wanted to know whether JAK/STAT signaling was important for their expression. In egg chambers homozygous for a hypomorphic allele of *unpaired (upd)*, the activating ligand for the JAK/STAT pathway, *hh-lacZ* expression was decreased in border cells compared to wild-type (Fig. 3A-B'). The expression of ptc^{H84}*-lacZ* was also decreased in egg chambers mutant for a viable combination of *stat* alleles, $stat^{397}/stat^{3391}$ (Fig. 3C-D'). These data suggest that STAT signaling is necessary for *hh* and *ptc* expression in migratory border cells. In agreement with this idea, we observed genetic interactions between *stat* and members of the *hh* pathway (Fig. 3E). Consistent with previous results, loss of one copy of *stat* revealed mild border cell migration defects (Silver and Montell, 2001). Viable females that were trans-heterozygous for $stat^{397}$ and a known interactor, $slbo^{1310}$, showed border cell migration defects in approximately 30% of egg chambers (Fig. 3E) (Silver and Montell, 2001). Similarly, we observed genetic interactions between *stat* and mutations in several genes in the *hh* pathway, including *Pka-C1, cos-2* and *hh* itself; the border cell migration defects ranged from 25-60% when these mutant alleles were trans-heterozygous with $stat^{397}$. We did not observe a genetic interaction between *smo* and *stat*, which may indicate that this genetic combination is not dosage sensitive or the alleles chosen for this analysis did not reveal an interaction. No border cell migration defects were observed in egg chambers trans-heterozygous for hh^{AC} and $slbo^{1310}$. In addition, overexpression of *hh* was unable to suppress the *slbo* mutant phenotype (Table 1). Therefore, *hh* may be a *slbo*-independent downstream target of STAT.

Hh Signaling is Required for Efficient Border Cell Migration

The expression of Hh pathway members in border cells along with the observed genetic interactions with known border cell migration genes suggested that Hh signaling may regulate border cell migration. To test this directly, we employed multiple genetic approaches to knock down the function of multiple members of the Hh pathway in border cells. To test if the loss of Hh signaling proteins results in defective border cell migration, we obtained transgenic RNAi lines for *smo, ci* and *hh* that have been used previously to knock down the respective genes (Hartman et al., 2010; Su et al., 2011). Specific knockdown of these genes in all border cells using *slbo*-GAL4 caused migration defects in 10-22% of egg chambers (Fig. 4A-C). This is similar to the results achieved by knockdown of *par-1* using RNAi with the same GAL4 driver (Fig. 4A) (McDonald et al., 2008). RNAi approaches can lead to incompletely penetrant phenotypes due to partial knockdown. Therefore, to confirm these results we took several complementary approaches to disrupt Hh activity. Expression of a *smo* mutant in which all PKA serine phosphorylation sites are mutated to alanines ($smo^{PKA\text{-}SA}$) competes with endogenous Smo and thus reduces Hh signaling *in vivo* (Apionishev et al., 2005; Su et al., 2011). Expression of this phosphorylation-defective UAS-$smo^{PKA\text{-}SA}$ in border cells driven by *slbo*-GAL4 inhibited their migration to a similar extent as *smo* RNAi (Fig. 4A). Overexpression of wild-type *ptc* greatly reduces Hh signaling (Johnson et al., 1995). We found that overexpression of *ptc* B1 (wild-type *ptc*) in border cells disrupted migration in 32% of egg chambers (Fig. 4A, 4D). The stronger phenotypes caused by *ptc* overexpression compared to RNAi knockdown of *ci, smo* and *hh* likely reflects the uniformly high inhibition of Hh activity by *ptc*; RNAi, though useful, commonly results in variable efficiency of gene knockdown (Perrimon et al., 2010).

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Together, these results indicate that knockdown of the Hh pathway disrupts border cell migration.

We next utilized available mutant alleles to confirm the role of Hh signaling components in border cell migration. We first tested the function of this pathway in border cells by mosaic clonal analysis using alleles of *smo*. We only obtained small clones with a strong loss-of-function allele, *smo*^{2}. Therefore, we induced clones of the cold-sensitive *smo* allele, *smo*^{3}. We incubated the flies at 25°C, which has been used previously to recover *smo* clones in the imaginal wing disc (Rodriguez and Basler, 1997). Border cells homozygous mutant for *smo*^{3} exhibited defective migration, although the phenotype was incompletely penetrant (Fig. 4E). Clusters with one to four *smo*^{3} mutant border cells exhibited migration defects in 28% of egg chambers (n = 43); border cells that did not reach the oocyte stalled between 50-75% of the normal migration distance (Fig. 4E). We did not observe completely mutant *smo* border cell clusters, possibly due to reduced proliferation of mutant cells in the clones (Duman-Scheel et al., 2002). Similar results were obtained when we analyzed additional Hh pathway members using mosaic clone analysis. Clusters that contained at least one border cell mutant for *hh* (*hh*^{AC} allele), *dispatched* (*disp*; *disp*^{S037707} allele), which is required for release of Hh from signaling cells (Burke et al., 1999), or the transcription factor *ci* (*ci*^{94} allele) also exhibited migration defects (n 8 migration-defective egg chambers for each genotype). We note that the incompletely penetrant phenotypes suggest that Hh may function in parallel to one or more other pathways that regulate border cell migration.

We next examined the effects of increasing the levels of Ci, the downstream transcription factor for Hh signaling, in border cells. Overexpression of either wild-type *ci*, or an activated version (*ci* m1-4), severely disrupted the ability of border cells to complete their migration (Fig. 5). Most border cells expressing high levels of wild-type *ci* either did not migrate or only migrated a short distance away from the anterior tip of the egg chamber (Fig. 5A, 5C), in agreement with Sun and Deng (Sun and Deng, 2007). However, border cells overexpressing activated *ci* were more severely affected (Fig. 5B, 5C). These results suggest that having the correct spatial-temporal organization and/or level of Hh signaling is important for migratory border cells, similar to Rac, Slbo, Upd and other genes required for border cell migration (Rorth et al., 2000; Duchek et al., 2001; Silver and Montell, 2001). Alternatively, overexpression of Ci could disrupt the normal Hh signaling circuit in border cells or indirectly affect other signaling pathways, thus accounting for the observed migration defects.

Loss of Hh Signaling Disrupts Distribution of E-cadherin and Phosphorylated Tyrosine

To determine which aspect of border cell migration was disrupted in the absence of Hh signaling, we analyzed several key markers of the cytoskeleton, border cell identity and membrane organization. We overexpressed *ptc* to consistently and maximally decrease the levels of Hh signaling in all border cells. Two markers of the cytoskeleton, α-tubulin to detect microtubules and phalloidin to detect F-actin, were each normal in border cells lacking Hh activation (Fig. 6A-D). We next analyzed the distribution of STAT in border cells to determine whether decreased Hh signaling affects STAT localization or protein levels. STAT localizes to the nucleus when STAT is active (Meyer and Vinkemeier, 2004) and is a marker of differentiated border cells (Silver et al., 2005). Both control and UAS-*ptc* border cells had similar levels of nuclear-localized STAT (Fig. 6E, 6F), consistent with Hh activity being downstream of STAT signaling (Fig. 3).

In contrast, the distribution/levels of several membrane-associated markers were altered when *hh* signaling was disrupted. Wild-type border cells have enriched levels of the cell adhesion protein E-cadherin (E-cad) at the membrane interfaces between border cells and at polar cell membranes (Fig. 6G) (Niewiadomska et al., 1999; Bai et al., 2000).

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Overexpression of *ptc* disrupted the normally organized membrane E-cad localization; 64% of clusters had increased localization around the polar cells and uneven localization between border cells (Fig. 6H; n = 11). Finally, we examined the localization of phosphorylated tyrosine (p-Tyr), which has been used to analyze levels of tyrosine kinase activity, including the RTKs PVR and EGFR, in border cells (Jekely et al., 2005; Assaker et al., 2010). Wild-type border cells normally have membrane-enriched p-Tyr staining at the front of the cluster at stage 9 prior to their detachment from the follicular epithelium and subsequent migration (Fig. 6I, 6I'; 91%, n = 11 egg chambers). However, when *ptc* was overexpressed we observed aberrant p-Tyr staining localization (Fig. 6J, 6J'). Border cells overexpressing *ptc* had either membrane-enriched p-Tyr at both the front and back of the cluster (Fig. 6J, J'; 32%, n = 19 egg chambers) or p-Tyr was no longer enriched at either the front or back (16%). Thus, a marker of overall border cell identity and the organization of the cytoskeleton appear to be normal when Hh signaling is disrupted, but the localization of specific membrane-enriched proteins are altered.

Rac and *par-1* Interact with *hh* in the Wing

To determine the extent to which the *hh, Rac* and *par-1* pathways interact in other tissues, we chose to focus on the patterning of the adult *Drosophila* wing blade. The role of Hh signaling in this tissue is well-defined and genetic interactions are commonly used to ascertain whether mutants enhance or suppress pathway-specific wing phenotypes. Compared to wild-type wing blades (Fig. 7A), expression of *ptc* under control of the developing wing *71B*-Gal4 driver at 25°C resulted in a reduction in wing blade size, a slight narrowing between the L3-L4 longitudinal veins and a fusion of the L3-L4 veins proximal to the anterior crossvein (ACV) (Fig. 7B). This is a mild *ptc* overexpression phenotype (Johnson et al., 1995). At higher temperatures (29°C), *71B*-GAL4 confers a higher level of *ptc* overexpression (and disruption of Hh signal), resulting in a more severe phenotype (Fig. 7C) (Johnson et al., 1995). Removal of one copy of *par-1* at 25°C enhances the mild veination phenotype, further reducing the space proximal to the ACV (Fig. 7D; compare to Fig. 7B). Furthermore, loss of one copy each of the three *Drosophila Rac* genes (*Rac1, Rac2* and *Mtl*) upon *ptc* overexpression at 25°C abolished the ACV and partially deleted L3 (Fig. 7E, 7F), which closely resembles the 29°C *ptc* overexpression phenotype (Fig. 7C). These data demonstrate that both *par-1* and *Rac* interact genetically with the Hh signaling pathway in the developing wing. This suggests that there is a conserved relationship amongst these pathways in the wing and ovary, although the precise biochemical functions may differ in the two tissues. Moreover, the observation that *Rac* mutations severely enhance the wing vein phenotypes associated with decreased Hh signaling is consistent with overexpression of Hh suppressing the *RacN17* phenotypes in border cells.

Discussion

In this work, a role for the Hh signaling pathway in collective migration of the border cells was uncovered in two independent genetic screens. Previous genetic mosaic screens in border cells identified a role for *cos2* in polar cell differentiation (Liu and Montell, 1999), but had yet to reveal a role for Hh signaling components in border cell migration. It has long been recognized that alternative screening methods are advantageous in uncovering genes that may be required earlier in development and/or for those genes with redundant functions. Both of these explanations are supported by published literature and the data presented here. First, ectopic Hh signaling, either by overexpression of *hh* itself or loss of the downstream components *ptc, cos2*, or *Pka-C1*, produces early ovarian phenotypes that include oocyte mis-positioning and excess polar cells (Forbes et al., 1996a; Liu and Montell, 1999; Zhang and Kalderon, 2000; Bai and Montell, 2002). These events occur prior to border cell recruitment and migration and thus may complicate analyses of Hh signaling in subsequent

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oogenic processes. We bypassed this potential issue by inducing downregulation of the Hh pathway specifically in border cells just prior to their migration. Second, the migration defects due to loss of Hh pathway components appear to be incompletely penetrant. Despite considerable reduction of Hh signaling due to overexpression of *ptc*, most border cells were able to complete their migration. However, the significant suppression of *RacN17* motility defects by overexpression of Hh particularly indicates an important functional role for this pathway in border cells. Our data are thus consistent with other, at present unknown, signaling pathways functioning in concert with Hh for proper cell migration.

Function of Hh Signaling in Border Cell Migration

The Hh pathway is capable of regulating a wide variety of cellular responses through transcriptional regulation of downstream target genes (Ogden et al., 2004). In most tissues, Hh is secreted from a local source, but the downstream effects occur only in *ptc*-receiving cells that may reside up to ten cell diameters away (Ingham and McMahon, 2001). In migrating border cells, the results presented here suggest an autocrine mechanism where Hh is both produced and received by the same cells. Both the *hh-lacZ* and multiple *ptc-lacZ* enhancer traps/reporters reveal transcriptional activity in the outer, migratory cells of the cluster. Furthermore, we show that *hh* expression is regulated by JAK/STAT signaling and is independent of Slbo regulation (Fig. 8). It remains a distinct possibility that the Hh signal is relayed between border cells within the cluster. Nonetheless, our data favor a role for Hh specifically in border cells rather than receiving Hh signal from other cells in the ovary. This idea is supported by our findings that migration was impaired when Hh and proteins required for its signal reception and transduction were knocked down by RNAi selectively in the border cells using *slbo*-GAL4. Furthermore, border cells mutant for *disp* did not complete their migration. As Disp is required in Hh-secreting cells for release of lipid-modified active Hh (Burke et al., 1999) this further indicates that border cells produce Hh signal. It is still unclear whether paracrine versus autocrine Hh signaling is biologically important. However, a number of studies have reported roles for autocrine Hh activity in the *Drosophila* wing disc and optic primordium in the embryo and the salivary gland in the larva, as well as autocrine Sonic hedgehog (Shh), a vertebrate Hh homolog, in neural stem cells, B-cell lymphoma, and interferon-stimulated cerebellar dysplasia during brain development (Zhu et al., 2003; Wang et al., 2004; Cai et al., 2008; Biehs et al., 2010; Singh et al., 2010). This type of Hh signaling mechanism also occurs in a variety of human tumors, where abnormal Hh pathway activation in an autocrine fashion increases cell proliferation and invasion (Katoh and Katoh, 2005; Datta and Datta, 2006).

A question raised by our study is what role the Hh signaling pathway plays in border cell migration. As depicted in our proposed model (Fig. 8), border cells with reduced Hh activation exhibited altered localization of E-cad and depolarized p-Tyr, either of which could affect border cell motility. E-cad is required for border cell migration by promoting proper adhesion with the nurse cell substrate. Importantly, disruption of E-cad localization contributes to migration defects caused by loss of steroid hormone signaling and cell polarity genes (Niewiadomska et al., 1999; Bai et al., 2000; Pinheiro and Montell, 2004). Loss of one of the guidance ligands, *pvf1*, disrupts E-cad localization in border cells similar to what we observed when Hh activity was impaired (McDonald et al., 2003). Given that PVF1 signals to the receptor PVR on border cells, this suggests a connection between Hh and RTK signaling. Indeed, wild-type border cells exhibit polarized activation of the RTKs PVR and EGFR prior to migration as assayed by global tyrosine phosphorylation and specific phosphorylation of PVR (Tyr-1428) (Jekely et al., 2005; Janssens et al., 2010). Disruption of this polarized RTK by several mechanisms, as shown by reduced or mislocalized p-Tyr, impairs border cell migration (Jekely et al., 2005; Assaker et al., 2010; Quinones et al., 2010). Our data suggest that Hh signaling restricts polarized p-Tyr to the

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front of the border cell cluster. Interestingly, overexpression of vertebrate Shh in keratinocytes increased activation of EGFR and invasion through matrix (Bigelow et al., 2005). Moreover, there is evidence for synergism between Hh and EGFR signaling to activate Gli (Ci homolog) transcription targets in human cells (Kasper et al., 2006). Nonetheless, proteins other than RTKs (and/or their targets) can be phosphorylated on tyrosines and thus recognized by the p-Tyr antibody; thus, the role for Hh signaling in border cells may be independent of the RTK pathways. Regulators of endocytosis are also required for localized, high levels of p-Tyr in border cells (Jekely et al., 2005; Assaker et al., 2010). However, in contrast to loss of *hh*, loss of endocytic pathway members do not significantly impair E-cad levels or localization (Assaker et al., 2010). Thus, Hh likely regulates border cell migration by a distinct mechanism. Further experiments will be needed to determine if Hh signaling is required for the proper levels or distribution of an unknown protein(s) that affects tyrosine phosphorylation and E-cad localization during border cell migration.

Connection(s) between Rac, Par-1, and Hh Pathways

The data presented here suggest a link between the Rac, Hh, and Par-1 signaling pathways. However, our understanding of how these proteins function together to modulate migration remain a mystery. The results from our suppression screen indicate that overexpression of Hh can overcome Rac-dependent migration defects. In fact, Hh was the strongest suppressor obtained from our screen. A simple explanation for this observation is that Ci induces transcription of one or more as yet unknown downstream target genes required in the migratory process (Fig. 8). However, the entire repertoire of Ci targets has yet to be elucidated and specific targets (apart from *ptc* itself) in border cells are unknown. Interestingly, Rac2 was recently uncovered as a potential target of the Hh signaling pathway in the *Drosophila* embryo (Biehs et al., 2010). Both Rac1 and Rac2 are essential for border cell migration (Geisbrecht and Montell, 2004). Thus, it is intriguing to speculate that upregulation of Rac2 by Hh is a possible mechanism to overcome the *RacN17* migration phenotype. Alternatively, the Hh pathway may directly or indirectly affect regulation of Rac protein activity either by increasing the amount of active Rac-GTP or by regulating the subcellular localization of activated Rac within the border cell cluster (Fig. 8). Hek 293 cells exposed to Shh had increased levels of the active form of the small GTPase RhoA (Kasai et al., 2004). Similarly, Shh stimulated RhoA and Rac via phosphoinositide 3-kinase (PI3K) signaling during chemotaxis of fibroblasts (Polizio et al., 2011). Because border cells do not rely on PI3K activity (Fulga and Rorth, 2002), another mechanism is likely involved.

What is the connection between Par-1 and the Hh signaling pathway? One possibility is the emerging requirement for microtubules in Hh signaling. Disruption of microtubules with the drug nocodazole prevents downstream transcriptional responses, possibly due to nuclear translocation of Gli proteins (Tukachinsky et al., 2010). Furthermore, Cos2-mediated subcellular motility and translocation of its cargo Ci requires microtubules in *Drosophila* (Farzan et al., 2008). Par-1 is a central player in mediating microtubule polymerization and dynamics (Hayashi et al., 2011). More specifically, phosphorylation of microtubule-associated proteins (MAPs) by the Par-1 kinase induces detachment of MAPs from microtubules. It is interesting to speculate that the kinase activity of Par-1 is essential in the Hh pathway to regulate Cos2 or MAP proteins for Ci mobility. Another possibility is that Par-1 functions through Rac. Overexpression of Par-1 partially suppressed the *RacN17* migration defect, similar to overexpression of Hh. Although it is unclear why Par-1 rescued the Rac phenotype, it is possible that Par-1 acts in parallel to Hh signaling to promote Rac-mediated border cell motility. Notably, mammalian MARK2 (Par-1 homolog) promotes microtubule growth downstream of Rac1 at the leading edge of migrating cells (Nishimura

et al., 2012). Further studies, however, are needed to determine the precise molecular relationships amongst the Rac, Hh and Par-1 pathways in collectively migrating border cells.

A Conserved Role for Hh in Other Morphogenic Processes

Cell shape changes are important for most aspects of morphogenic processes, including cell contractility and cell migration. Hh signaling induces cell shape changes in the developing *Drosophila* eye via regulation of non-muscle myosin II (Corrigall et al., 2007; Escudero et al., 2007). Thus, the role of Hh in border cells may be to regulate cell shape during migration. Accumulating evidence points to a non-canonical role for Hh in mediating mammalian cell migration. Shh can function as a chemoattractant in migrating cells and guidance of axons independent of Gli-induced gene transcription (Jenkins, 2009; Lin et al., 2012). In axon guidance, Shh stimulates phosphorylation and activation of Src kinase and thereby facilitates axon turning through regulation of the actin cytoskeleton (Yam et al., 2009). Specifically, Shh induced phosphorylation of Src at Tyr-418, an activating site, and polarization of Src family kinases within the axon. This appears to be consistent with our finding that loss of Hh activity depolarized global p-Tyr distribution. In other migratory cell types, Shh also acts as a chemoattractant that induces cytoskeletal rearrangements and migration independent of the canonical transcriptional response (Hochman et al., 2006; Bijlsma et al., 2007; Bijlsma et al., 2012). However, the mechanism of Hh function in border cell migration is likely to be different for several reasons. First, Hh is unlikely to function as a long-range chemoattractant, because border cells are the likely source of Hh signal (as discussed above). Second, a role for Src in border cell migration is unknown at present, so Hh may mediate tyrosine phosphorylation of other substrates in border cells. Third, we have evidence that Ci is involved in border cell migration and therefore canonical Hh-induced transcription is predicted to be important. Nonetheless, our results are consistent with a conserved role for the Hh pathway in regulating cytoskeletal-mediated events in migrating cells, which in border cells likely functions through the Rac GTPase.

Experimental Procedures

Drosophila Genetics and Mosaic Analysis

Fly culture and crosses were performed according to standard procedure at 25° C, except where indicated. The following mutant and transgenic fly stocks were used for the initial analysis of RacN17 overexpression and specificity controls: *slbo*-Gal4; UAS-*RacN17* (Geisbrecht and Montell, 2004); UAS-*mCD8-GFP* (Bloomington Stock Center); UAS-*slbo* (Geisbrecht and Montell, 2004); UAS-*RacWT* (Bloomington Stock Center); UAS-*RacV12* (Bloomington Stock Center); UAS-*RasV12* (Lee et al., 1996). Additional lines utilized: *slbo*^{1310}/*CyO* (*slbo-lacZ*; (Montell et al., 1992)); *hh-lacZ*/TM6B (Forbes et al., 1996a); *ptc-lacZ* reporter lines encompassing the upstream regulatory region of *ptc: ptc-lacZ* 5.5 and *ptc-lacZ* 10.8 (gifts of Joan Hooper); *ptc*^{H84lacZ}/Cyo (*P(A92)ptc*^{H84} enhancer trap; (Ingham et al., 1991)); Ci-GFP^{YD0681} (Quinones-Coello et al., 2007); UAS-*hh* N (N-terminus active form of *hh*; (Porter et al., 1996)); UAS-*hh* A (full-length *hh*; (Porter et al., 1996)); UAS-*hh* I (full-length *hh*; (Ingham and Fietz, 1995)); *upd*^{sisC5} (Sefton et al., 2000); *stat*^{3391} (*Stat92E*^{EP3391}; (Silver and Montell, 2001)); UAS-*ptc B1* (wild-type Ptc; (Johnson et al., 1995)); UAS-*smo*$^{PKA\text{-}SA}$ (Apionishev et al., 2005); UAS-*ci WT* (HA-tagged; (Chen et al., 1999)); UAS-*ci (m1-4)* (HA-tagged; (Chen et al., 1999)); *par-1*^{27C1} (McDonald et al., 2008); *par-1*$^{\Delta 16}$ (Cox et al., 2001); *71B*-GAL4 (wing GAL4 driver; (Brand et al., 1994)); *Rac*^{J10}*, Rac2*$^{\Delta}$*, Mtl*$^{\Delta}$ and *Rac*^{J11}*, Rac2*$^{\Delta}$*, Mtl*$^{\Delta}$ (Bloomington Stock Center; (Hakeda-Suzuki et al., 2002)). Other alleles used for genetic interactions: *stat*^{397} (Silver and Montell, 2001); *smo*^{3} (from D. Kalderon; (Quirk et al., 1997)); *Pka-C1*^{H2} (Zhang and Kalderon, 2000); *cos-2*^{52B7} (Liu and Montell, 1999); *hh*^{AC} (null allele from P. Beachy; (Lee et al., 1992)); *ptc*^{7} (*ptc*^{9B};

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(Phillips et al., 1990)); *ptc*^{9} (*ptc*^{IN108}; (Schuske et al., 1994)); *tai*^{61G1} (Bai et al., 2000); *shg*^{R69} (Godt and Tepass, 1998). *w*^{1118} was used as the wild-type strain.

UAS-RNAi lines were obtained from the VDRC and Bloomington Stock Center (TRiP Harvard collection): UAS-dsRNA to GFP (GFP RNAi); UAS-*par-1* RNAi (McDonald et al., 2008); UAS-*smo* RNAi (VDRC line v9542); UAS-*smo* RNAi (TRiP line JF02363); UAS-*ci* RNAi (TRiP line JF01715); UAS-*hh* RNAi (TRiP line JF01804). GAL4-UAS overexpression and transgenic RNAi was performed after overnight incubation at 29°C. *slbo*-Gal4 (recombined with UAS-*mCD8:GFP*) was used throughout these studies (Rorth et al., 1998). *slbo*-GAL4, UAS-*mCD8:GFP* was outcrossed to *w*^{1118} and used as a control. The more widely-expressed *c306*-GAL4 (Murphy and Montell, 1996) produced similar phenotypes, but was not used further since its expression early in oogenesis could interfere with early Hh functions.

Mutant clones were generated by mitotic recombination using the FLP/FRT system using an X-chromosome *hs-flp* (Xu and Rubin, 1993). Adult flies of the correct genotype were heat shocked for 1 hour, twice a day at 37°C and incubated at 25°C to enable recovery of clones. Females were fattened and dissected at 25°C 4-7 days after the last heat shock. Clones were marked by loss of the *ubi-nlsGFP* marker. The following alleles were used: *FRT82B, hh*^{AC}; *smo*^{3}, *FRT40A;* and *FRT82B, disp*^{S037707}. *ci* mutant clones (marked by loss of GFP) were generated by analyzing females of the genotype *hs-flp; FRT42D P[ci*$^{+}$*] hsp70-GFP/ FRT42D; ci*^{94}*/ci*^{94} (Methot and Basler, 1999).

For the wing genetic interaction studies, *71B*-GAL4 was recombined with UAS-*ptc* B1 and crossed to the relevant mutant alleles at 25°C. Female progeny were collected and analyzed as described (Johnson et al., 1995; Zhu et al., 2003). Wing phenotypes were 100% penetrant (n > 30 for each genotype).

Rac Modifier Screen

The *slbo*-GAL4; UAS-*RacN17* stock was crossed to 2273 EP lines (Bloomington Stock Center) (Rorth et al., 1998). The progeny from these crosses were initially screened for rescue of female fertility and secondarily screened for the extent of border cell migration. Lines that exhibited a MI 20 were kept for further analysis and specificity tests as described in the Results section. Nine lines were confirmed to be in the correct orientation for gene induction using sequence annotation information available through FlyBase. *In situ* hybridization was performed for a subset of the candidates to confirm that the respective EP line driven by *slbo*-GAL4 overexpressed the downstream gene.

par-1 Dominant Genetic Interaction Screen

Mutant alleles that exhibited dominant genetic interaction in viable heterozygous combination with *par-1*^{27C1} were identified as part of a previous mutagenesis screen for new *par-1* alleles (McDonald et al., 2008). Briefly, isogenized *dp, cn, bw* males were fed 22 mM EMS (ethyl methanesulfonate; Sigma) in 1% sucrose for 24 hours (Bokel, 2008). Mutagenized males were allowed to recover for 24 hours and then crossed to virgin *w*; Sco/CyO females for 3-4 days. 2198 mutagenized mutant male *dp, cn, bw, */Sco* progeny (where the asterisk indicates the mutagenized chromosome) were crossed to virgin *PZ6356, par-1*^{27C1}*/CyO* females to test for genetic interaction. Female *dp, cn, bw, */PZ6356, par-1*^{27C1} progeny were fattened overnight. Ovaries were dissected, fixed in 96-well plates and stained for β-galactosidase activity to detect *PZ6356*, an enhancer trap that labels border cells and the oocyte nucleus (Liu and Montell, 1999). Lines with a putative phenotype were identified first using a stereo microscope and then confirmed at higher magnification. F2 generation *dp, cn, bw, */Sco* males were backcrossed to virgin *w*; *Sco/CyO* females to

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establish a balanced stock. *Inter se* crosses were used to establish complementation groups. Once lines were rescreened and the phenotype confirmed, they were crossed to the cytologically defined Bloomington chromosome 2 deficiency kit to map the location of homozygous lethal mutations. Those deficiencies that were lethal in combination with the mutant allele were then crossed to *par-1*^{27C1} to determine whether the deficiency had a comparable dominant interaction with *par-1*.

In Situ Hybridization, Immunofluorescence and Microscopy Analysis

Ovary dissections and *in situ* hybridizations were carried out as described in Wang, et al. (Wang et al., 2006) and S. Zimmerman, N. Peters, A. Altaras, and C. Berg (in preparation). Probes for *in situ* hybridization were made from *hh* and *ptc* cDNA clones (Porter et al., 1996; Su et al., 2011) by *in vitro* transcription; the *ptc* anti-sense probe was previously used to analyze *ptc* in the wing disc (Su et al., 2011).

Antibody staining was performed as described (McDonald and Montell, 2005). Fixation was generally performed in 4% formaldehyde/0.1 M potassium phosphate buffer, pH 7.4 for 10 min at room temperature; except fixation for anti-Ci and anti-Smo antibody staining was performed for 15 min on ice followed by 15 min at room temperature. Antibodies used were: rabbit anti-β-galactosidase at 1:2000 (Cappel); mouse anti-Fas III at 1:10 (7G10, Developmental Studies Hybridoma Bank, DSHB); concentrated rat anti-Ci at 1:150 (2A1; DSHB) (Motzny and Holmgren, 1995); mouse anti-Smo at 1:10 (20C6; DSHB) (Lum et al., 2003); concentrated mouse concentrated anti-Ptc at 1:600 (Apa1; DSHB) (Torroja et al., 2004); mouse anti-α-Tubulin at 1:800 (DM1A, Sigma); rat anti-E-Cadherin at 1:25 (DCAD2; DSHB) (Uemura et al., 1996); rabbit anti-STAT at 1:1000 (a gift from S. Hou); and mouse anti-phosphotyrosine at 1:1000 (p-Tyr-100; Cell Signaling Technology). Secondary antibodies or phalloidin (to label F-actin) conjugated to Alexa Fluor 488 or Alexa Fluor 568 (1:400; Invitrogen) were used. DAPI (0.05 μg/mL; Sigma) was used to stain nuclei. Samples were mounted on slides in Vectashield (Vector Labs) or Aqua-Poly/Mount (Polysciences, Inc.). Imaging was performed on a Zeiss AxioImager Z1 motorized epifluorescent microscope using the ApoTome System and a MRm CCD camera. Either a Plan-Apochromat 20x 0.75 numerical aperture (NA) objective or a Plan-Neofluar 40x 1.3 NA oil objective was used. Image adjustments (e.g. brightness, contrast and/or color) were performed using Photoshop CS4 or ImageJ. Figures were assembled in Adobe Photoshop CS4 or Illustrator CS4. GraphPad Prism 4 was used to compile graphs and calculate SEM.

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Bullet points

- Two independent genetic screens uncover a role for Hh signaling in cell migration.
- Overexpression of Hh rescues Rac-dependent cell migration defects.
- Disruption of Hh signaling affects E-cadherin and phosphorylated-tyrosine localization in the migrating border cells.
- The Rac, Par-1 and Hh signaling pathways intersect in wing vein development.

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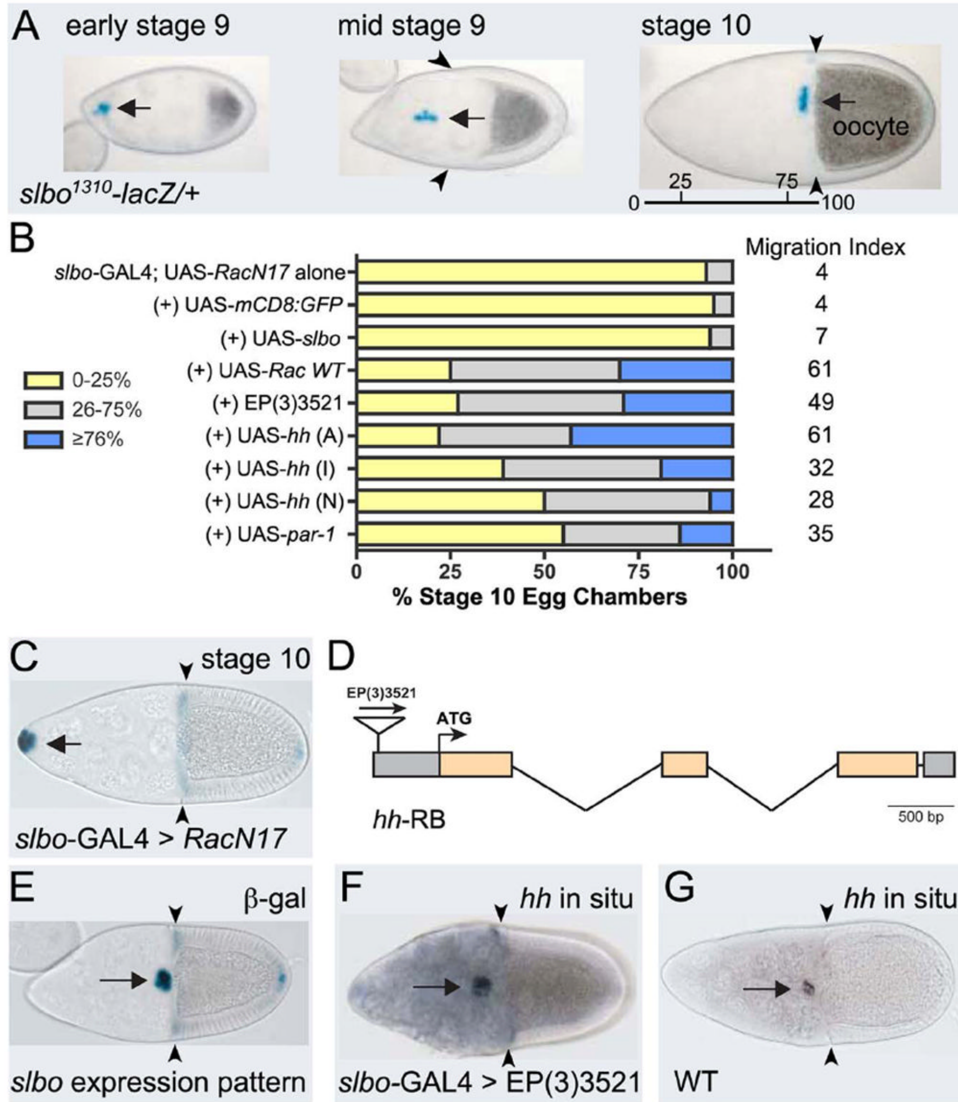

Fig. 1. Overexpression of *hh* rescues *RacN17* border cell migration defects

(A) *slbo*^{1310}-*lacZ/+* egg chambers at indicated stages stained for β-galactosidase activity (blue) to show border cells at beginning (left), during (middle) and end of migration when they reach the oocyte (right); migration distance indicated (bar). (B) Quantification of border cell migration at stage 10, with relative MI, following overexpression of indicated genes in a *slbo*-Gal4; UAS-*RacN17* background (controls from (Geisbrecht and Montell, 2004)). Migration is shown as the percentage of border cells that migrated 0-25% (yellow), 26-75% (gray), or 76% (blue) of the distance to the oocyte. N > 100 egg chambers for each genotype. (C) Stage 10 *slbo*-Gal4; UAS-*RacN17* egg chamber stained for *slbo*^{1310}-*lacZ*; border cells did not migrate. (D) Diagram of the *hh* gene with coding exons (orange). EP(3)3521 is inserted 424 bp upstream of the ATG. (E) Stage 10 *slbo*^{1310}*/+* egg chamber stained to show *slbo* expression pattern (blue). (F, G) RNA *in situ* hybridization to detect *hh* expression at stage 10. (F) *slbo*-GAL4 driving EP(3)3521 shows increased *hh* expression in the *slbo* pattern. (G) Wild-type egg chamber. Arrows indicate border cells; arrowheads show extent of follicle cell rearrangement. Anterior to the left in this and subsequent figures.

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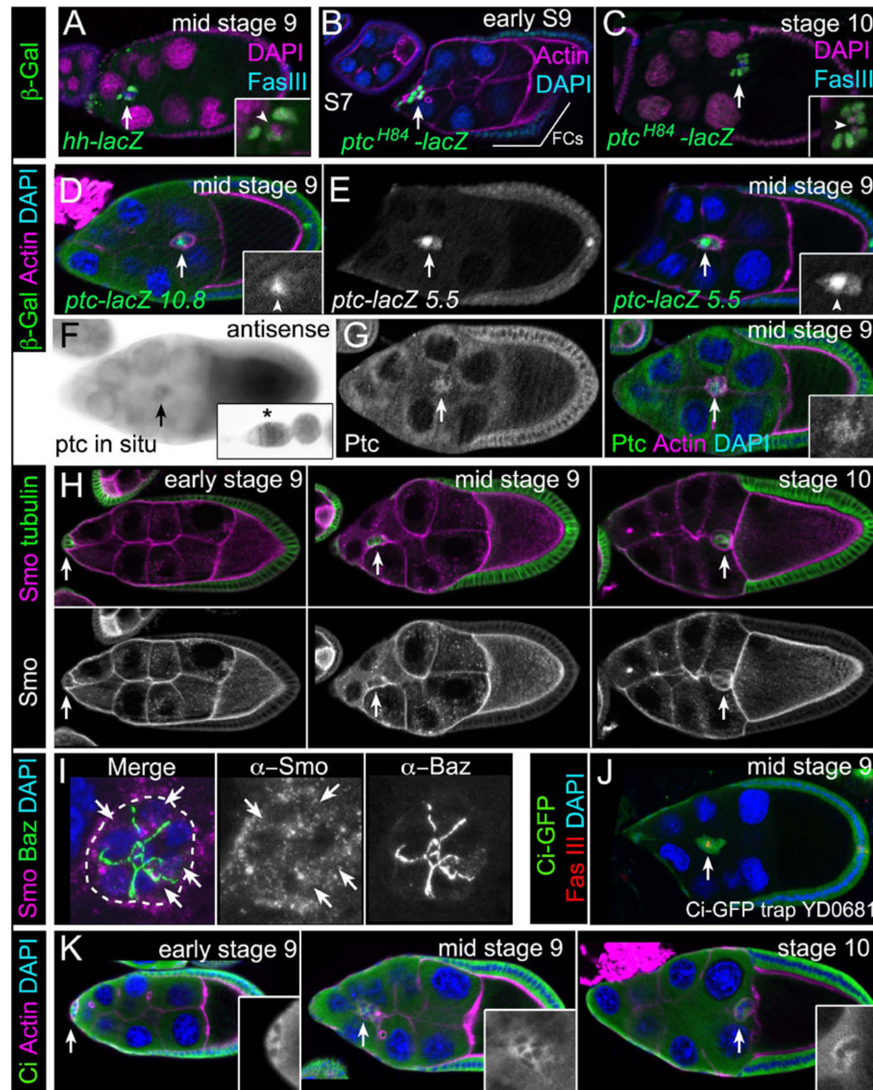

Fig. 2. Expression of Hh pathway components in wild-type border cells

Representative images of egg chambers. Arrows indicate border cells. (A-E) Immunostaining for β-galactosidase (green) to detect *hh-lacZ* (A) or *ptc-lacZ* (B-E) enhancer trap (A-C) and promoter (D, E) reporters. Co-stains are Fas III (polar cells; blue in [A, C]), DAPI (nuclei; magenta in [A, C], blue in [B, D, E]) and F-actin (magenta [B, D, E]). Insets, magnified views of border cells (*lacZ* and DAPI in [A, C]); arrowheads, polar cells; FCs, follicle cells. (F) *ptc* mRNA expression. Inset, *ptc* expression in germarium. (G) Egg chamber immunostained for Ptc (green) and co-stained for F-actin (magenta). Inset, magnified view of border cells (Ptc). (H, I) Immunostaining for Smo (magenta). (H) Smo protein localization before, during, and after migration. α-Tubulin (green) labels all follicle cells. (I) Merged *z*-stack of border cells stained for Smo (magenta) and Bazooka (green). Smo localizes to cytoplasmic punctae (arrows); cluster outer border is outlined. (J, K) Ci protein (green) is present in border cells as detected by a GFP protein trap in *ci* (J) and using an antibody to Ci-FL protein (K; insets). Egg chambers were co-stained for Fas III (red [J]), F-actin (magenta [K]) and DAPI (blue).

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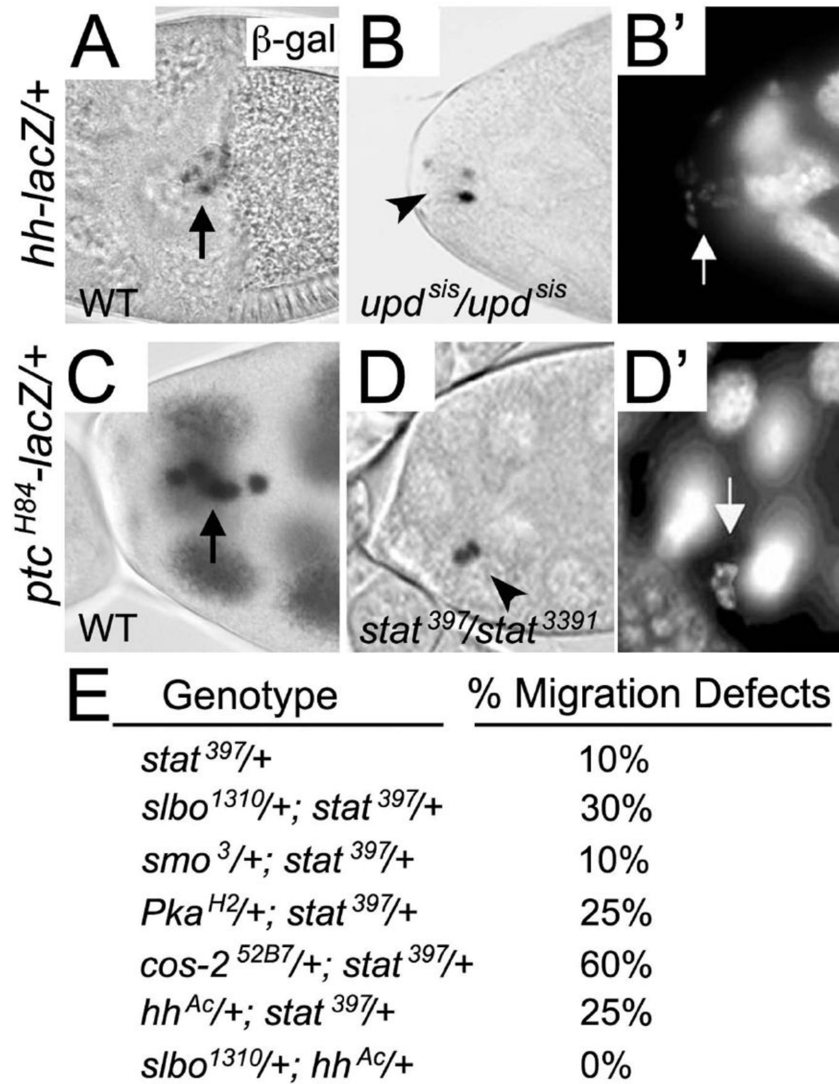

Genotype	% Migration Defects
stat^{397}/+	10%
slbo^{1310}/+; *stat*^{397}/+	30%
smo^{3}/+; *stat*^{397}/+	10%
Pka^{H2}/+; *stat*^{397}/+	25%
cos-2^{52B7}/+; *stat*^{397}/+	60%
hh^{Ac}/+; *stat*^{397}/+	25%
slbo^{1310}/+; *hh*^{Ac}/+	0%

Fig. 3. JAK/STAT signaling regulates expression of *hh* and *ptc* in border cells

(A-D') Stage 10 egg chambers stained for nuclear lacZ activity in the border cells (arrows) and counterstained with DAPI (B', D') to mark nuclei. (A) LacZ reporter activity is present in all border cells in *hh-lacZ* egg chambers. (B, B') Homozygous *upd*^{sis} mutant egg chamber in which the border cell cluster remained at the anterior tip of the egg chamber. Few border cell nuclei show *hh-lacZ* expression (arrowhead in B) even though border cells are present (arrow in B'). (C) LacZ reporter activity is present in all border cells in *ptc-lacZ* egg chambers. This egg chamber was slightly overexposed and thus staining in the nurse cells is more apparent. (D, D') *stat*^{397}/*stat*^{3391} mutant egg chamber in which border cells failed to migrate (arrowhead in D). *ptc-lacZ* expression is only present in two border cells, but all cells are visible by nuclear DAPI staining (arrow in D'). (E) Table showing results of dominant *stat* genetic interaction tests, expressed as the percentage of border cell migration defects found in trans-heterozygous flies of the indicated genotypes; 42 n 190 egg chambers for each genotype.

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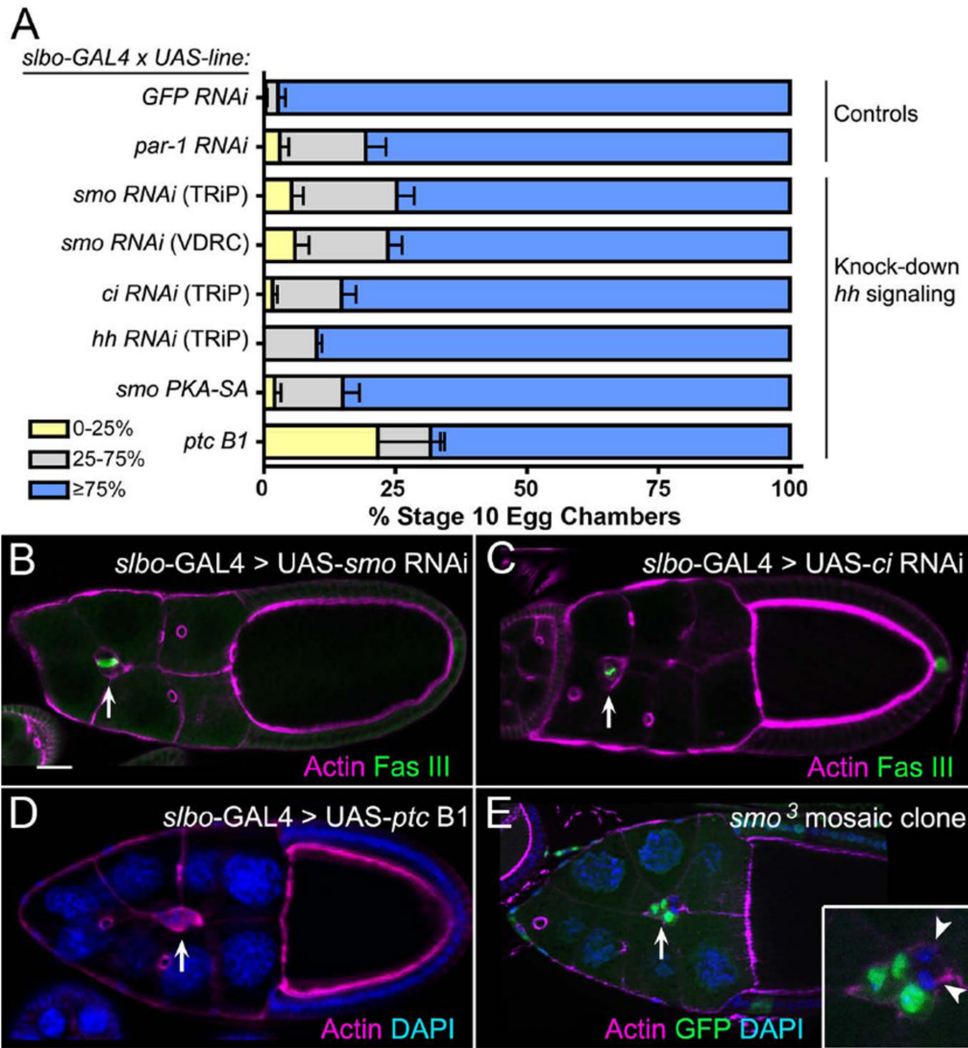

Fig. 4. Migration defects upon loss of Hh pathway members in border cells

(A) Quantification of border cell migration at stage 10 upon knockdown of Hh pathway components using RNAi or overexpression approaches. Migration is shown as the percentage of border cells that migrated 0-25% (yellow), 26-75% (gray), or 76% (blue) of the distance to the oocyte. N 193 egg chambers for each genotype; at least 3 experiments were performed. Error bars represent standard error of the mean (SEM). (B-E) Fixed stage 10 egg chambers stained for phalloidin to mark F-actin (magenta) at cell membranes and DAPI (blue) to mark nuclei. GFP (green) marks wild-type cells in (E) and Fas III (green) marks polar cell membranes in (B, C). Arrows point to border cells. (B-D) Examples of egg chambers in which RNAi knockdown of *smo* (B) or c*i* (C) or in which *ptc* overexpression (D) is driven by *slbo*-GAL4; all show incomplete border cell migration. (E) Loss of *smo* by mosaic clonal analysis reveals a migration defect in a stage 10 egg chamber. Inset shows that two cells in the border cell cluster are mutant (GFP-negative; arrowheads). Scale bar in (B) represents 20 μm.

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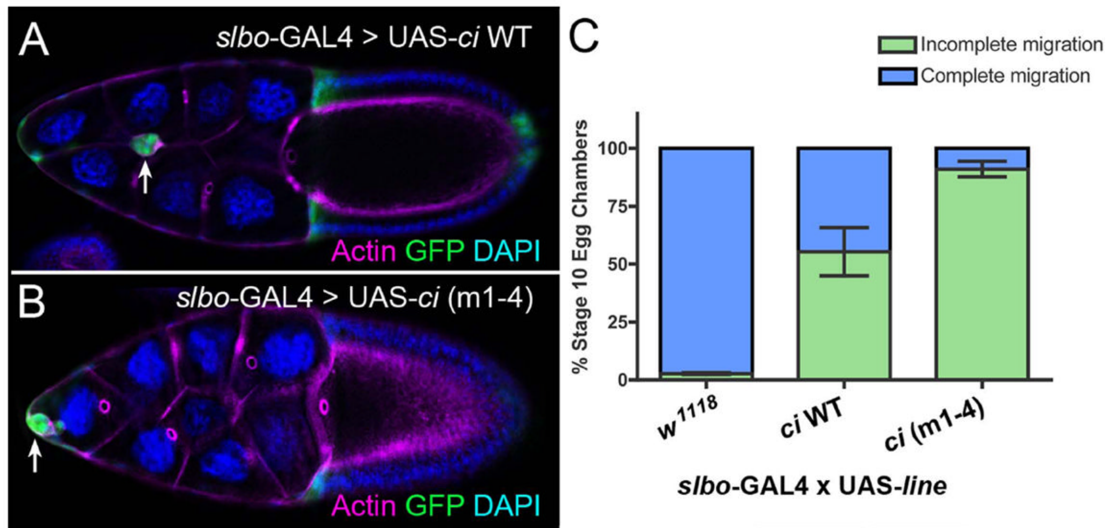

Fig. 5. Overexpression of *ci* disrupts border cell migration

(A, B) Stage 10 egg chambers stained for phalloidin to mark F-actin (magenta) at cell membranes. GFP (green) marks the GAL4 expression pattern and DAPI (blue) marks the nuclei. Overexpression of WT *ci* (A) or an activated version of *ci* (B), *ci* (m1-4), driven by *slbo*-GAL4 results in a strong migration defect. (C) Quantification of stage 10 border cells that did not reach the oocyte (incomplete; green) versus those that reached the oocyte (complete; blue) upon overexpression of *ci* in the border cells. N 200 egg chambers for each genotype; at least 3 experiments were performed. Error bars represent SEM.

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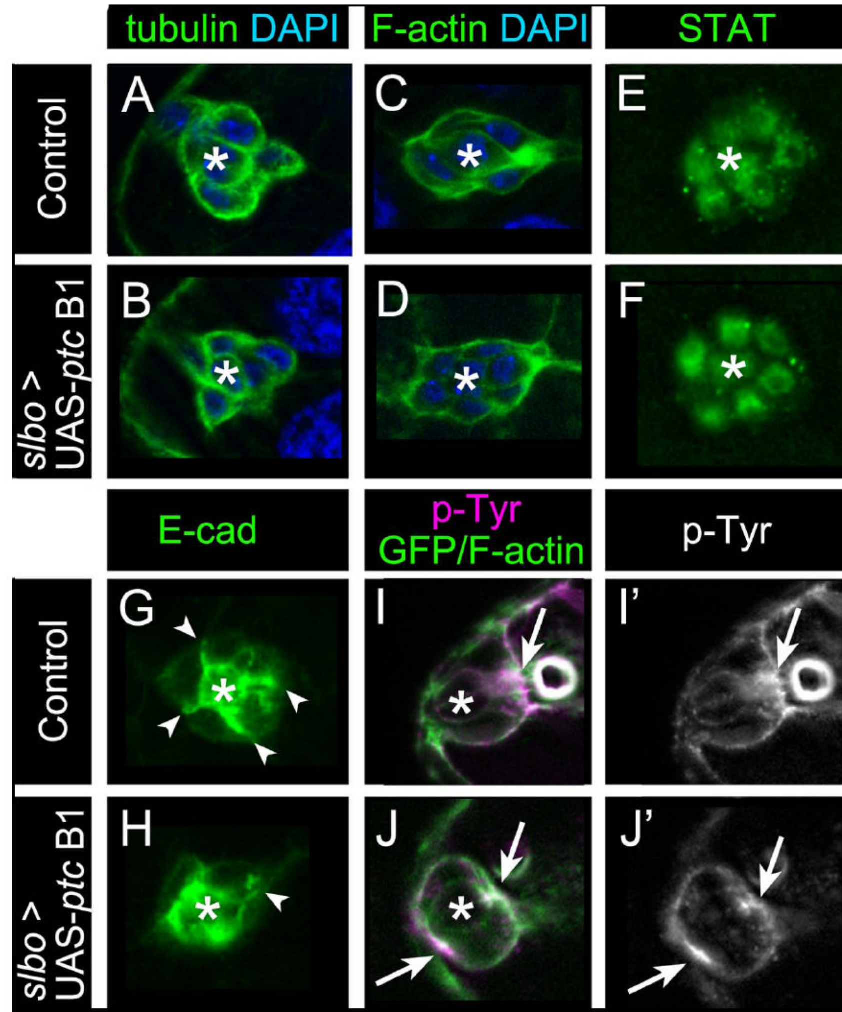

Fig. 6. Loss of Hh signaling affects Ecad and p-Tyr distribution within border cells

Representative images of stage 9 control *slbo*-GAL4/+ (A, C, E, G, I) or *slbo*-GAL4/UAS-*ptc* B1 (B, D, F, H, J) egg chambers stained for border cell markers; asterisks mark polar cells. (A, B) Border cells labeled for α-tubulin (green) to mark microtubules and DAPI (blue) to label nuclei. Microtubule organization is normal. (C, D) Border cells stained for phalloidin to mark F-actin (green) and DAPI (blue). F-actin localization is normal. (E, F) Border cell nuclei stained for STAT (green). Nuclear STAT is normal. (G, H) Border cells stained for E-cad (green). (G) In control, E-cad is enriched at membranes between border cells (arrowheads) and polar cells (asterisk). (H) *ptc* B1 cluster in which E-cad distribution is disrupted at border cell membranes; one cell has normal E-cad (arrowhead). (I-J') Border cells prior to migration stained for p-Tyr (magenta [I, J]; white [I', J']) and F-actin/GFP (green in I, J). (I, I') p-Tyr is enriched at the front of control border cell clusters (arrow). (J, J') *ptc* B1 border cell cluster in which p-Tyr is enriched at the front and rear (arrows). N 10 egg chambers for each genotype.

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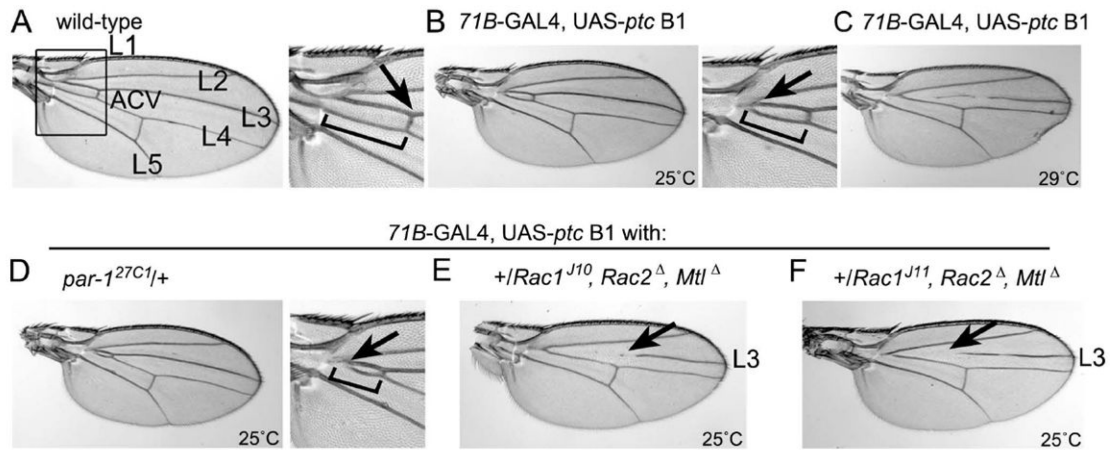

Fig. 7. Loss of one copy of *par-1* or the *Rac* genes enhances adult wing defects caused by reduced Hh signaling

(A) Wild-type wing in which the longitudinal veins L1-L5 and anterior crossvein (ACV) are indicated (left panel). The boxed region is expanded (right panel) to show the normal length of veins L3-L4 (bracket) on the proximal side of the ACV (arrow) located between L3 and L4. (B) Reduced Hh signaling via overexpression of *ptc* at 25°C results in mild wing phenotypes (Johnson et al., 1995); reduced length of the L3-L4 longitudinal veins proximal to the ACV (bracket) and fusion of the proximal end (arrow) are indicated. (C) Severe wing vein phenotype caused by overexpression of *ptc* at 29°C. (D) Enhancement of the mild *ptc* overexpression vein fusion phenotype (arrow, bracket) upon loss of one copy of *par-1* at 25°C. (E, F) Removal of one copy of all three *Rac* genes, using two different *Rac1* alleles, at 25°C strongly enhances the mild *ptc* overexpression phenotype. The ACV and portions of the L3 longitudinal vein (arrows) are eliminated, which phenocopies severe *ptc* overexpression at 29°C (compare to [C]). Note that overexpression of *ptc* reduces overall wing size (Johnson et al., 1995).

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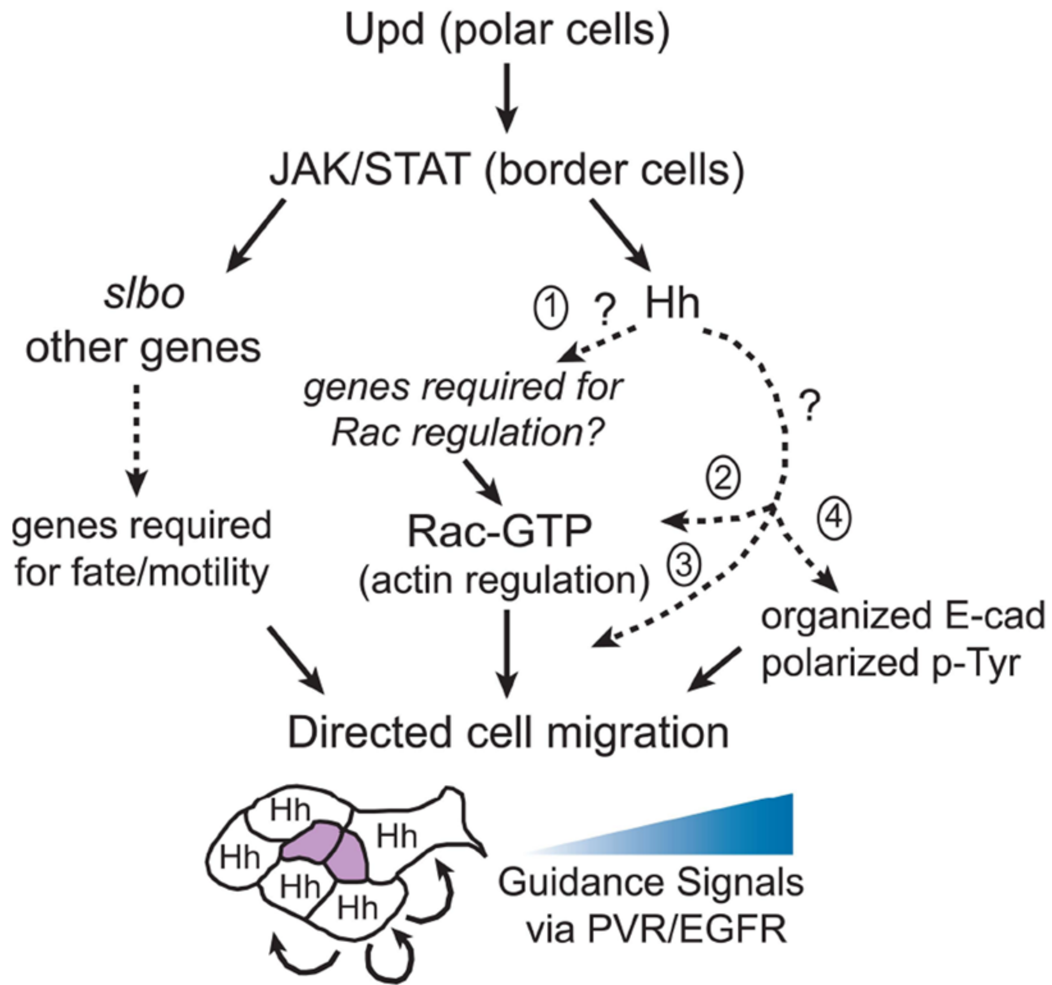

Fig. 8. Model for Hh signaling in border cells

Upd signaling from the polar cells activates JAK/STAT in surrounding border cells (reviewed in Montell et al., 2012). JAK/STAT triggers expression of *slbo* and other genes required for border cell fate and motility. Hh is a downstream target of JAK/STAT but is independent of Slbo. Hh may be upstream (1) of active Rac (Rac-GTP), for example if it regulates the expression of genes required for Rac regulation such as a guanine-nucleotide exchange factor (GEF). Alternatively, the genetic data are consistent with Hh functioning with (2, 3), or in parallel to (4) Rac-GTP, possibly through Par-1 (for simplicity not shown). Hh could upregulate Rac levels in border cells (2), act downstream of Rac on presently unknown targets (3) and/or regulate E-cad/p-Tyr localization in parallel to Rac (4). Together, the border cell signaling pathways converge to direct collective border cell migration in response to a gradient of guidance signals secreted from the oocyte via the RTKs, PVR/EGFR. Hh is active in each border cell and likely received by the same cell, although autocrine signaling between cells in the cluster could also occur (arrows, border cell schematic). See text for additional details.

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Table 1

EP lines that suppress *RacN17* border cell migration defects

EP line	Map position	Rescue strength	Gene over-expressed	EST/cDNA	Orientation[a]/molecular information[b]	Rescue *RacV12*[c]	Rescue *RasV12*[c]	Rescue *slbo*[1310 c]
EP(X)1451	11A1	I/S	CG1806 (novel)	LP03706	(+/+) -4300 bp of ATG	–	–	–
EP(X)1569	5C7	S	*actin5C*[d, e]		(+/+) -828 bp of ATG	–	–	+
(X)1604	5C7	S	*actin5C*[d, e]		(+/+) -833 bp of ATG	–	–	+
EP(2)2172	23B7	S	CG3059 (NTPase)	LD11641	(+/+) -6508 bp of ATG	–	–	–
EP(2)2604	49B10	S	*spt4*	LD44495	(+/+) +84 bp of ATG	–	–	+
EP(2)2633	60C5-6	S	CG3394	GH22220	(+/+) -163 bp of ATG	–	+	ND
EP(3)3203	98A14	W	*RpS10a*	LD32148	(+/+) -508 bp of ATG	+/-	–	–
(3)3597	98A14	S	*RpS10a*	LD32148	(+/+) -497 bp of ATG	–	ND	–
EP(3)3279	72D1	S	*diap1*[d, e]	GH15335	(+/+) -8737 bp of ATG	–	–	–
(3)3308	72D1	I/S	*diap1*[d, e]	GH15335	(+/+) -8154 bp of ATG	–	–	–
EP(3)3521	94E2	S	*hedgehog*[d]	hh FL cDNA[f]	(+/+) -424 bp of ATG	–	–	–
EP(3)3556	66F1	I	*smaug*	LD07551	(+/+) -4237 bp of ATG	–	–	–

[a]Orientation of EP line with respect to coding strand (+/+ or +/-).

[b]Distances calculated from genomic sequence.

[c]EP lines were crossed to *slbo*-GAL4;UAS-*RacV12*, *slbo*-GAL4;UAS-*RasV12* or $slbo^{1310}/slbo^{1310}$ to determine specificity of *RacN17* suppression. The ability to suppress (+) or to not suppress (–) the respective border cell migration defects was determined.

[d]Enhancer traps show expression in border cells: *G(1)0177 (actin5C)*; th^{j5C8} *(diap1)*; hh^{90E} *(hh)*.

[e]Data from Geisbrecht and Montell (2004).

[f]*hh* full length cDNA obtained from P. Beachy.

Abbreviations: S, strong; I, intermediate; W, weak; ND, not determined.

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Table 2

Complementation groups isolated in *par-1* dominant interaction screen

Gene	Allele(s)	*par-1*^{27C1} interaction (% migration defects)[a]	Chromosome location[b]	*par-1* interaction with known allele	Synonyms/homologs	Role in border cell migration
par-1^{27C1}/+ (control)	–	3	–	–	–	–
taiman (*tai*; *pdig1*)	*4A2*	27	30A2-30A6	Yes (*tai*^{61G1})	DAIB1/AIB1	Bai et al., 2001
pdig2	*11H1*	11	35E1-35F7	–	Unknown	–
pdig3	*25G8*	10	59B1-59B3[c]	–	Unknown	–
pdig4 (*sec5*)[d]	*30E5*	19	23F2-3[e]	ND[f]	Sec5	Assaker et al., 2010
patched (*ptc*; *pdig5*)	*31A7*	13	44D5-44E1	Yes (*ptc*^{9})	Ptc1	*This study*
shotgun (*shg*; *pdig6*)	*31D8*	24	57B15-57B16	Yes (*shg*^{R69})	DE-cadherin/E-cadherin	Niewiadomska et al., 1999
	46G1	15				

[a]Dominant interaction with *par-1* (*pdig, +/+, par-1*^{27C1}) scored as percentage of egg chambers in which border cells did not complete their migration to the oocyte by stage 10; n 100 egg chambers for each genotype. Alleles exhibited similar but milder interaction with *par-1*$^{\Delta 16}$.

[b]Chromosomal location based on lethal complementation with mutant alleles and/or overlapping deficiencies from the Bloomington Stock Center. In most cases the non-complementing deficiency exhibited a comparable interaction with *par-1*.

[c]Second lethal mutation mapped to 40A5-40D3 (Df(2L)Exel6049), but *par-1* did not interact with this deficiency.

[d]Missense point mutation mapped to the *sec5* open reading frame: Q393K (nucleotide 1178 C to A).

[e]Based on lethality; *par-1* had a milder interaction with Df(2L)BSC31, which removes this region (23E5;23F4-5).

[f]ND, not determined.