A Fasciclin 2 morphogenetic switch organizes epithelial cell cluster polarity and motility

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Summary

Little is known about how intercellular communication is regulated in epithelial cell clusters to control delamination and migration. We investigate this problem using *Drosophila* border cells as a model. We find that just preceding cell cluster delamination, expression of transmembrane immunoglobulin superfamily member, Fasciclin 2, is lost in outer border cells, but not in inner polar cells of the cluster. Loss of Fasciclin 2 expression in outer border cells permits a switch in Fasciclin 2 polarity in the inner polar cells. This polarity switch, which is organized in collaboration with neoplastic tumor suppressors Discs large and Lethal-giant-larvae, directs cluster asymmetry essential for timing delamination from the epithelium. Fas2-mediated communication between polar and border cells maintains localization of Discs large and Lethal-giant-larvae in border cells to inhibit the rate of cluster migration. These findings are the first to show how a switch in cell adhesion molecule polarity regulates asymmetry and delamination of an epithelial cell cluster. The finding that Discs large and Lethal-giant-larvae inhibit the rate of normal cell cluster movement suggests that their loss in metastatic tumors may directly contribute to tumor motility. Furthermore, our results provide novel insight into the intimate link between epithelial polarity and acquisition of motile polarity that has important implications for development of invasive carcinomas.

Key words: Epithelial cell cluster, Cluster motility, Cluster polarity, Cell adhesion, Tumor suppressor, Fasciclin 2, *Drosophila*

Introduction

Individual cells rely on intrinsic polarity to migrate along shallow gradients of signals in vivo (Bretscher, 1996; Iijima et al., 2002; Lauffenburger and Horwitz, 1996; Mitchison and Cramer, 1996). Although considerable work has shown how individual cells establish intrinsic polarity (Locascio and Nieto, 2001), little is known about how polarity is established or used in migrating multicellular clusters during normal development. Specialized membrane domains connect cells in migrating clusters (Friedel et al., 1995; Kolega, 1981; Trinkaus, 1988), suggesting that intercellular communication is crucial for determining cluster organization, and thus may regulate cluster motility. However, the specific mechanisms by which individual cells organize into a cluster to regulate movement remain largely unknown. Migrating clusters are also a common vehicle for tumor cell movement (Friedel et al., 1995; Hegerfeldt et al., 2002; Kolega, 1981; Wang et al., 2000). Identifying the cell communication molecules that regulate cluster motility, and how their misregulation leads to aberrant movement, is thus likely to generate insights into the differentiation of invasive carcinomas.

Drosophila border cells (BCs) provide a simple in vivo model for deciphering the mechanisms of cell cluster movement (Montell, 2003). BCs are a cluster of six to eight somatic follicle cells that differentiate within the anterior follicular epithelium during mid oogenesis, maintaining some aspects of epithelial polarity (Niewiadomska et al., 1999), while losing others (this work). The BCs then delaminate from the follicular epithelium, a process that requires the polarized cluster to coordinately break contact from adjacent epithelial cells, while simultaneously directing invasion between the germ cells. Once the BCs exit the epithelium, they take ~6 hours to migrate roughly 150 μm between the nurse germ cells to the oocyte (Spradling, 1993).

The BC cluster includes two polar cells (PCs) that reside at the center of the cluster and that do not contact the migration substrate. Preceding BC differentiation, PCs secrete Unpaired, which determines how many adjacent epithelial cells will activate the Jak-Stat pathway, and thus become BCs (Bai et al., 2000; Beccari et al., 2002). Stat is sufficient for expression of the C/EBP transcription factor, Slow Border Cells (Slbo) (Beccari et al., 2002; Montell et al., 1992; Silver and Montell, 2001). Slbo directs BC differentiation and upregulation of DE-Cadherin, a cell-adhesion molecule essential for movement (Liu and Montell, 2001; Niewiadomska et al., 1999). Two tyrosine kinase receptors that appear to function redundantly, Pvr and Egfr, guide the BCs to the oocyte (Duchek and Rorth, 2001; Duchek et al., 2001). In contrast to these genes, Discs large (Dlg) (Woods and Bryant, 1991; Woods and Bryant, 1993) prevents BCs from reaching the oocyte prematurely (Goode and Perrimon, 1997).

Dlg is a member of a large family of conserved membrane-associated guanylate kinases (MAGuKs) that lack intrinsic kinase activity (Anderson, 1996). MAGuKs have multiple protein-binding domains, including three PDZ domains, an SH3 domain, and a GuK domain that act as scaffolding modules to assemble specific combinations of signaling, adhesion and cytoskeletal molecules at cellular junctions (Sheng, 1996). In *Drosophila*, Dlg scaffolding is crucial for suppressing local tumor invasion (Goode and Perrimon, 1997) and metastasis (Woodhouse et al., 1998). It is not known if these requirements reflect Dlg functions in controlling epithelial polarity, delamination, migration or any combination of these activities. The mechanisms by which Dlg inhibits migration and tumor invasion are likely to be conserved across species as two human MAGuKs, DLG and ZO1, are also implicated in oncogenesis. Human DLG binds to APC, the most commonly mutated gene in colorectal cancer (Matsumine et al., 1996), and is a target for E6 oncoprotein in human papillomavirus transformation (Gardiol et al., 2002). ZO1 is lost specifically at the transition from in situ to invasive breast cancer (Hoover et al., 1998).

How MAGuKs suppress tumorigenesis is not known. In humans, PSD-95 binds a RasGAP molecule (Kim et al., 1998), suggesting that MAGuKs may signal through small G proteins. In *Drosophila*, Dlg colocalizes and cooperates with two additional tumor suppressors at epithelial junctions, Lethal-giant-larvae (Lgl) and Scribbled (Scrib). Loss of either Lgl or Scrib causes a loss of epithelial polarity and over-proliferation that phenotypically resembles a loss of Dlg (Bilder et al., 2000). Lgl integrates membrane and cytoskeletal organization by binding and repressing Myosin 2 activity (Peng et al., 2000; Strand et al., 1994), and regulating vesicular trafficking (Lehman et al., 1999). Scrib is a scaffolding protein containing four PDZ domains and 16 leucin-rich repeats (Bilder and Perrimon, 2000). The putative Dlg-Lgl-Scrib complex is believed to mediate cellular interactions important for epithelial polarity, signaling and adhesion by clustering selected signaling and adhesion receptors with specific regulatory, cytoskeletal and trafficking molecules at cellular junctions.

To understand how Dlg scaffolding integrates multiple protein activities to regulate epithelial polarity and movement, we are analyzing proteins that bind to distinct Dlg domains. One such protein, Fasciclin 2 (Fas2), is a transmembrane cell-adhesion molecule (CAM) of the immunoglobulin superfamily. Fas2 binds Dlg PDZ1+2 domains, and is homologous to vertebrate neural cell-adhesion molecule (NCAM) (reviewed by Goodman et al., 1997). The Fas2 C terminus -SAV-COOH sequence selectively recruits Fas2 to neuromuscular junctions by binding Dlg PDZ1+2 (Thomas et al., 1997; Zito et al., 1997). In the absence of the Fas2 C terminus -SAV-COOH, or Dlg, Fas2 is diffusely localized, resulting in abnormal development of synapse structure (Thomas et al., 1997; Zito et al., 1997). The precise spatial and temporal pattern of Fas2 is crucial for targeted membrane growth, as demonstrated by axon guidance defects resulting from Fas2 loss or misexpression (Goodman et al., 1997).

In the present study, we have examined the role of Fas2, Dlg and Lgl in regulating the motility of an organized cell cluster. We employ a novel method of measuring BC motility that enables us to distinguish the function of Fas2, Dlg and Lgl in regulating the delamination of BCs out of the follicular epithelium from their roles in regulating BC migration. Furthermore, we introduce the use of reproducibly oriented clusters, which enables us to assess the importance of protein localization during delamination and migration. Combined with genetic mosaic analysis, and targeted rescue experiments, these data provide the first model describing the role of neoplastic tumor suppressors in BC movement.

We find that while Dlg and Lgl are constitutively expressed in all follicle cells, Fas2 expression is selectively lost, precisely at the time of BC differentiation, from the anterior follicle epithelium, including the BCs. Fas2 expression is maintained in PCs at the center of the BC cluster. Loss of Fas2 expression in BCs permits a reorganization of Fas2, Dlg and Lgl epithelial polarity, to a motile polarity in PCs, which is crucial for efficient delamination. At the same time, PC Fas2 signals Dlg and Lgl maintenance in BCs, which inhibits the rate of migration. Our data thus demonstrate how dynamic Fas2 expression and polarity regulate epithelial junctions to control cluster motility with temporal precision. Furthermore, our observation that Dlg and Lgl inhibit the rate of movement of a developmentally regulated cell cluster suggests that their loss in metastatic tumors not only facilitates the transition from epithelial to motile polarity, but also directly contributes to tumor motility. The reorganization of molecules important for epithelial polarity to achieve motile cluster polarity has important implications for the coordinate misregulation of epithelial polarity and motility during carcinoma invasion.

Materials and methods

Genetics

The following alleles were used: $Fas2^{rd1}$, $Fas2^{MB2225}$ (enhancer-trap line with P{lArB} inserted into the first exon of *Fas2*) (Cheng et al., 2001), $Fas2^{EB112}$ (null) (Grenningloh et al., 1991), dlg^{hf321} (temperature-sensitive), dlg^{lv55}, dlg^{m52} (null) (Woods and Bryant, 1991), lgl^{ts3} (temperature-sensitive), lgl^{4} (null) (Manfruelli et al., 1996) and $slbo^{1310}$ (Montell et al., 1992). UAS lines were Fas2 (Schuster et al., 1996), $Fas2^{29C}$ (Fas2Δ3) (K. Zito and C. S. Goodman, unpublished), CD8-Fas2 (Zito et al., 1997), Dlg and Dlg^{C17}(Dlg PDZ1-3) (Hough et al., 1997). Gal4 lines were BA3 (gift of Trudi Schüpbach) and Slbo (Rørth et al., 1998). The FRT^{101} $hsFLP^{22}$ *tub-lacZ* chromosome was generated by meiotic recombination and verified by PCR and β-gal staining. *Fas2* mosaics were induced in $Fas2^{EB112}$ FRT^{101}/FRT^{101} $hsFLP^{22}$ *tub-lacZ* third instar larvae by heat shock at 37°C for 2 hours on two consecutive days. Strong loss of function *dlg* and *lgl* phenotypes were examined by rearing dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} flies at the permissive temperature (18°C), then shifting them to restrictive temperature (25°C) for 8 hours.

Histochemistry and imaging

Flies were reared on fresh yeast at 25°C. Antibody, phalloidin and β-gal activity staining were as previously described (Goode and Perrimon, 1997). The following primary antibodies were used: rabbit anti-Amphiphysin (number 9906, 1:500) (Zelhof et al., 2001), rat anti-Crb (1:1000; U. Tepass), rabbit anti-Dlg (1:500; K.-O. Cho), mouse anti-Fas2 (1D4, ppIg 1:1000; DSHB), mouse anti-Fas3 (7G10, ppIg 1:1000; DSHB), rabbit anti-β-gal (1:2000; Cappel), mouse anti-α-Tubulin (1:500; Sigma). Polyclonal anti-Slbo (1:2000) was produced at Bethyl Laboratories in rabbits with HPLC-purified C-terminal Slbo peptide 429-VSRVCRSFLNTNEHSL-444, followed by affinity purification. Anti-Lgl (1:4000) was raised in a sheep against C-terminal Lgl peptide 1146-DNKIGTPKTAPEESQF-1161. Specificity of antibodies was confirmed by ELISA, western blotting of ovarian

proteins and staining wild-type and mutant egg chambers with immune and preimmune sera. Cy5-, FITC- or rhodamine red-X-conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories) were used (1:2000). Alexa^{488}- or Alexa^{568}-phalloidin (1:10; Molecular Probes) were used to visualize Actin. Images were acquired with a Zeiss LSM 510 confocal microscope or with a conventional epifluorescence Zeiss Axioplan 2 microscope equipped with a Hamamatsu ORCA digital camera. Confocal images were processed using Photoshop software (Adobe).

Cell migration profiles

The traditional method for determining if a mutation perturbs BC migration is to compare mutant BC migration with migration of the surrounding follicle epithelium (Lee at al., 1996). Our purpose for choosing an alternative methodology, by using the oocyte as a clock to measure the rate of BC migration, is that the genes we examined are expressed in both BCs and the follicular epithelium, so we could not be assured the epithelium migrated like wild type. Furthermore, using oocyte growth as a clock allows us to clearly distinguish the effects of the mutation on delamination versus migration, and thus to determine the primary cause of the motility defects. Images of stage 9 phalloidin-stained egg chambers were captured with a Zeiss Axioplan-2 microscope equipped with a Hamamatsu ORCA digital camera. Images were measured as described in Fig. 3B using AxioVision 3.1 software (Carl Zeiss Vision). Morphometric analysis was similar to that described (Zarnescu and Thomas, 1999). However, instead of plotting percent BC migration completed as a function of percent oocyte length, we plotted percent BC migration completed as a function of percent oocyte area. This gives more reproducible profiles because oocyte length, but not oocyte area, is expected to oscillate, owing to expansion and contraction of the surrounding muscle layer. None of the mutations we analyzed affected linear progression of oocyte growth. Growth of the oocyte was converted to time by taking wild-type migration as a standard 6 hours (Spradling, 1993). To ensure that we measured an unbiased sample, we were careful to include every s9 egg chamber, even those in which the BCs had just started to penetrate between the nurse cells, so that all of our analyses are based on a uniformly distributed set of border cell migration distances (see Fig. 3E). Tumor development in *dlg* and *lgl* temperature-sensitive allele egg chambers was not apparent because the flies were shifted to the restrictive temperature (25°C) for only 8 hours preceding BC analysis (furthermore, the temperature shift applied to wild-type flies did not affect oocyte growth or BC migration). SYSTAT 10 software (SSI) was used to complete General Linear Model analysis of data points from on average 200 egg chambers per genotype. Statistical significance was taken at a value of $P<0.05$.

Protein levels and Fas2 polarity measurements

Relative Fas2 levels in *Fas2*^{null}/+ and *Fas2*^{rd1} ovaries were estimated by western blot analysis of ovarian protein using a chemiluminescence-based ECL system (Amersham). Fas2 levels in *Fas2*^{null}/+, *Fas2*^{rd1} and *Fas2*^{null}/*Fas2*^{rd1} ovaries were also estimated from Axioplan images of anterior polar cells in late s8 egg chambers. All images were taken at the same settings for both the microscope and camera, within the linear range of the photo detector. NIH Image software was used to quantitate the fluorescent signals. The quantitation agreed with the western data. Fas2 estimates were additionally confirmed by quantitative RT-PCR on total RNA prepared from whole ovaries, which agreed with relative Fas2 levels obtained by both western and microscopic image analyses. Relative Dlg and Lgl levels in BCs adjacent to Fas2^{+} and *Fas2*^{null} PCs were estimated from confocal images of mosaic clusters.

Fas2 polarity was measured on PC images by determining the pixel densities of Fas2 staining in the leading and trailing halves of PCs using NIH Image software. Polarity is presented as a ratio of leading Fas2 to trailing Fas2.

Results

Expression and localization of Fas2 and Dlg during BC differentiation

To determine if Fas2 plays a role in Dlg-mediated control of BC cluster motility, we compared patterns of Fas2 and Dlg expression during oogenesis. We found that Dlg is constitutively expressed in follicle cells during oogenesis (Fig. 1B). By contrast, Fas2 is expressed in all follicle cells during the early growth stages (stages 1-6), but expression decreases following cessation of follicle cell proliferation (from stage 7 onwards, Fig. 1C). Most strikingly, Fas2 is completely lost from anterior epithelial cells, except PCs, specifically at the time of BC differentiation (Fig. 1C,E′-E‴). A *Fas2* enhancer-detector is expressed in the same pattern as Fas2 protein (Fig. 1H,I), indicating that Fas2 expression is regulated at a transcriptional level. These data suggest that Dlg function might be modulated by differential Fas2 expression specifically at the time of BC movement.

To obtain evidence for Fas2-Dlg cooperativity in cluster movement, we characterized the correlation between subcellular localization of Fas2 and Dlg and BC differentiation. Prior to BC differentiation, Fas2 and Dlg colocalize on the lateral surface of the presumptive BC epithelial cells, and around the circumference of the PCs (Fig. 1D-D″). As the BCs differentiate, Fas2 expression is lost from BCs (Fig. 1E′), and Dlg becomes localized around the circumference of the BCs (Fig. 1E,E″,E‴). Simultaneously, Fas2 and Dlg switch from a circumferential localization around the PCs (Fig. 1D-D″), to a sharply polarized pattern at the front of the PCs, facing the leading edge of the BC cluster (Fig. 1E-E‴). As the BCs delaminate from the epithelium, one BC extension encapsulating the leading edge of the PCs pioneers invasion (Fig. 1F,F′). Fas2-Dlg polarization is maintained in PCs as the BCs move to the oocyte (Fig. 1G). These data suggest that dynamic changes in Fas2 expression and localization may modulate Dlg to control BC movement.

Loss of Fas2 expression in BCs permits a shift in Fas2 polarity in PCs

Polarization of Fas2 to the leading half of the PCs, precisely at the time when Fas2 expression is lost in BCs, suggests that Fas2 loss in BCs might be crucial for establishing its polarity. To test this hypothesis, we expressed Fas2 in BCs using the UAS-Gal4 system (Brand and Perrimon, 1993). Targeting Fas2 to BCs using Slbo-Gal4 (expressed in BCs but not PCs, Fig. 2D) causes Fas2 to accumulate around the circumference of PCs (Fig. 2B), in a pattern resembling that found in wild-type clusters preceding BC differentiation. This experiment demonstrates that loss of Fas2 expression in BCs is crucial for Fas2 polarization. As a control, we targeted chimeric CD8-Fas2 to BCs. CD8-Fas2 has the extracellular Ig domain of Fas2 replaced with the extracellular Ig domain of human CD8. CD8-Fas2 does not significantly affect Fas2 polarity in PCs (Fig. 2C). Thus, loss of Fas2 homophilic interactions between BCs and PCs appears to be crucial for establishing Fas2 polarity.

In addition to shifting Fas2 polarity, targeting Fas2 to BCs dramatically reduces Fas2 at the contact site between PCs (compare Fig. 2A with 2B, arrow). A similar redistribution is not observed when CD8-Fas2 is expressed in BCs (Fig. 2C), indicating that reduction of Fas2 between PCs is driven by

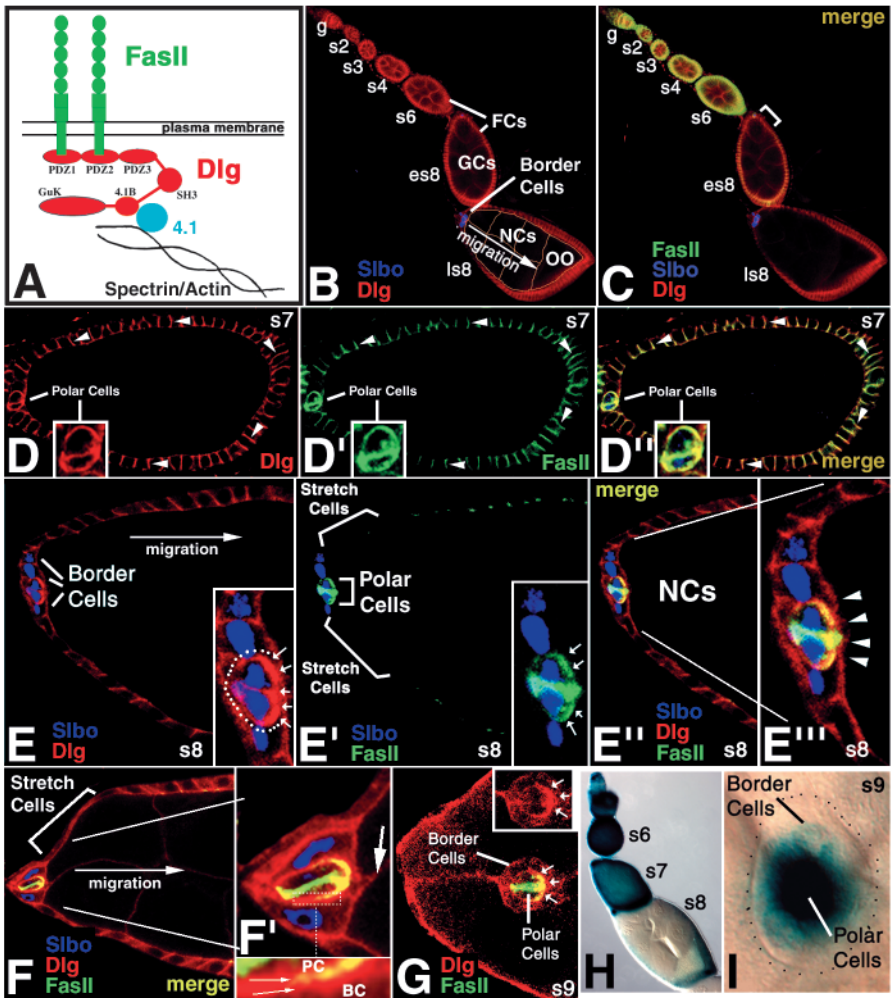

Fig. 1. Fas2, Dlg and Slbo expression during oogenesis. (A) Transmembrane Fas2 binds to PDZ1 and PDZ2 domains of cortical Dlg. (B) Dlg is expressed in egg chamber germ cells (GCs) and follicle cells throughout oogenesis (g, germarium; s, stage of oogenesis; e, early; l, late). Border cell (BC) differentiation at stage 8 is marked by Slbo expression. At stage 9, the BCs migrate between the nurse cells (NCs) to the front edge of the oocyte (OO) (arrow). (C) Same egg chambers as (B). Fas2 is expressed in all follicle cells through stage 7 (see also D′). At stage 8, Fas2 expression is lost in anterior follicle cells that include the BCs (bracket), precisely at the time when the BCs differentiate. (D-D″) At stage 7, just preceding BC differentiation, Fas2 and Dlg are uniformly distributed around the circumference of the polar cells (PCs, inset). Fas2 and Dlg predominantly colocalize in all follicle cells (arrowheads), but at late stage 7, Fas2 starts becoming localized more apically (completes apical localization at stage 8, see E′), while Dlg remains uniformly distributed on the lateral membrane. (E-E‴) Anterior of a stage 8 egg chamber. (E) When the BCs differentiate (Slbo expression), Dlg redistributes in the PCs toward the leading edge of the cluster (inset, arrows). (E′) Fas2 expression is lost in stretch cells and BCs, but continues in PCs at the center of the cluster, where it becomes precisely colocalized with Dlg in a graded leading-to-trailing pattern (inset, arrows). BC extensions that express Dlg on their surface encapsulate the PCs at the leading edge of the cluster (E‴, arrowheads). These extensions pioneer BCs invasion at stage 9 (F,F′, arrow). Fas2 is expressed in PC, but not BC membranes (F′, inset, arrows). (G) Fas2 and Dlg (inset) continue to be polarized in PCs toward the leading edge of the BCs as the cluster migrates (arrows). (H,I) *Fas2* enhancer-trap expression during oogenesis. The *Fas2* transcription pattern (H) resembles Fas2 protein pattern (C). At stage 9, Fas2 is expressed in PCs but not BCs (I).

homophilic interactions between endogenous PC Fas2 and ectopic BC Fas2. We reasoned that because Fas2 is a homophilic cell-adhesion molecule (Goodman et al., 1997), when Fas2 is misexpressed in BCs, binding between PC and BC Fas2 competes for PC-PC Fas2 binding, thus decreasing level of Fas2 in the interface between PCs (Fig. 2A). If so, then loss of Fas2 from BCs provides a mechanism to sequester most Fas2 between PCs, thus facilitating clustering of Fas2 along the leading half of the PCs. This model predicts that excess Fas2 would prevent polarization. To test this prediction, we targeted excess Fas2 to PCs using BA3-Gal4 (for BA3-Gal4 expression, see Fig. 3D). We found that Fas2 accumulates around the entire circumference of the PCs, rather than selectively at the leading half of the PCs (Fig. 6H). We believe that this observation further indicates that sequestration of Fas2 through homophilic binding between PCs facilitates its polarization.

To determine whether Fas2 sequestration between PCs occurs via homophilic binding, we analyzed the consequence of disrupting Fas2 interactions by removing Fas2 from one of the PCs. To remove Fas2 from one of the PCs, we generated *Fas2*^{null} clones. When one PC is Fas2^{+} and the other is *Fas2*^{null}, Fas2 no longer accumulates at the interface between the PCs (Fig. 2E, arrow; compare with Fig. 2A). This result indicates that Fas2 homophilic binding occurs between wild-type PCs. Furthermore, we observe that Fas2 redistributes to the site of contact with BCs, in the direction where BCs lead invasion (Fig. 2E,E′). We therefore reasoned that Fas2 might be binding a BC receptor. We never observe a complete loss of Fas2 from the Fas2^{+} PC, as would be expected if Fas2 did not interact with a BC receptor (Hortsch et al., 1998). The putative BC receptor is clearly not residual Fas2, as Fas2 is present in PC membranes even when they contact *Fas2*^{null} BCs (Fig. 2H).

We asked if the putative BC receptor is expressed preceding BC differentiation. We reasoned that if the putative receptor is expressed before BC differentiation, then Fas2 should be present in PC membranes even when they contact *Fas2*^{null} undifferentiated BCs. We generated *Fas2*^{null} clones and found that loss of Fas2 from presumptive BCs at stage 7 causes loss of Fas2 from the adjacent membrane of the Fas2^{+} PCs (Fig. 2G), indicating that putative receptor is not expressed before BC differentiation. We also analyzed whether the putative BC

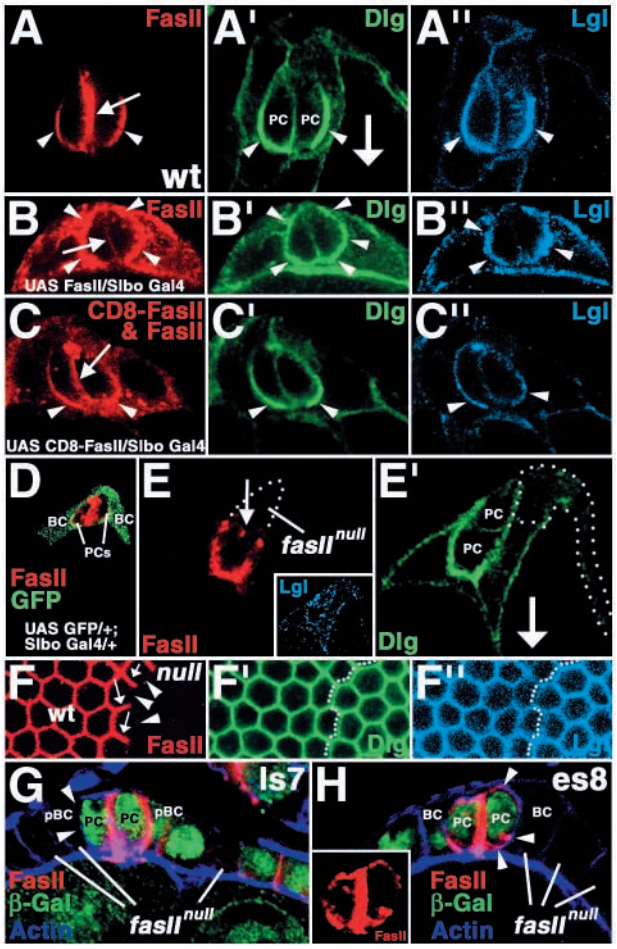

Fig. 2. Fas2 polarization and signaling. (A-A″) Wild-type BC cluster. Fas2, Dlg and Lgl are polarized to the leading half of the PCs (arrowheads; leading-to-trailing Fas2 polarity ratio: 8.6±1.6; see also Fig. 6A-A″). Fas2 is localized at the highest level in the interface between the PCs (A, arrow). (B-B″) Targeting Fas2 to BCs using Slbo-Gal4 (Slbo-Gal4 is not expressed in PCs; D) causes Fas2 to become localized around the circumference of the PCs (leading-to-trailing Fas2 polarity ratio: 1.1±0.2), and dramatically reduced at the interface between the PCs (arrow, compare with A). Dlg and Lgl colocalize in a similar pattern. (C) Targeting chimeric CD8-Fas2 to BCs does not change Fas2 polarity (leading-to-trailing polarity ratio: 6.1±2.0; anti-Fas2 antibody partially recognizes also CD8-Fas2). (E-E′) *Fas2* mosaic, in which one PC has no Fas2. The PCs and cluster are turned perpendicular to the normal orientation for initiating movement, with the $Fas2^{+}$ PC oriented in the direction of migration (arrow in E′, compare with A′). High Fas2 level between PCs is lost (E, arrow, compare with A). Leading-to-trailing Fas2 polarity ratio is 6.8. (E′) Dlg has cortical localization in BCs that contact the $Fas2^{+}$ PC, but is mislocalized in the BC in contact with the $Fas2^{null}$ PC (outline). Lgl shows the same distribution (inset, E), indicating that Fas2 signals maintenance and localization of Dlg and Lgl in BCs. (F-F″) *Fas2* mosaic epithelium (at the time of BC delamination). Dlg and Lgl levels, and localization are similar in both $Fas2^{+}$ and $Fas2^{null}$ follicle cells. In $Fas2^{+}$ follicle cells, Fas2 is lost from the membranes that contact $Fas2^{null}$ cells (arrowheads), but remains at the site where they contact $Fas2^{+}$ cells (arrows). (G) A late stage 7 *Fas2* mosaic, in which loss of Fas2 from the presumptive BC epithelial cell (pBCs) causes loss of Fas2 from the membrane of the adjacent $Fas2^{+}$ PC (arrowheads). (H) In an early stage 8 *Fas2* mosaic, Fas2 is present in the PC membrane (arrowheads) adjacent to a $Fas2^{null}$ BC. This suggests the presence of a Fas2 receptor in stage 8 BCs (see text).

receptor is present in other stage 8-9 follicle cells. In mosaics of $Fas2^{null}$ and $Fas2^{+}$ follicular epithelia, Fas2 is localized only at cell membranes where a $Fas2^{+}$ cell contacts another $Fas2^{+}$ cell, and not where $Fas2^{+}$ cells contact $Fas2^{null}$ cells (Fig. 2F). Thus, there does not appear to be an alternate Fas2 ligand (BC receptor) in other follicle cells.

The above experiments indicate that the putative BC receptor is required to maintain Fas2 in PC membranes. These experiments do not address if the putative BC receptor directs Fas2 polarization to the leading half of the PCs. To test the importance of the putative BC receptor for Fas2 polarization, we targeted CD8-Fas2 to wild-type and $Fas2^{rd1}$ PCs. CD8-Fas2 is polarized in PCs (Fig. 6J; and data not shown). This suggests that the putative BC receptor does not polarize Fas2, but rather polarization depends on the Fas2 cytoplasmic domain, and presumably interaction with Dlg PDZ domains. In support of this model, dramatic decrease of Dlg or Lgl in dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} PCs causes loss of Fas2 polarity (see Fig. 6E,G). Thus, Dlg and Lgl, which are polarized to the leading half of PCs (Fig. 2A′,A″, Fig. 6A′,A″), appear to be crucial for establishing Fas2 polarity.

The simplest synthesis of these results is that developmentally programmed loss of Fas2 expression couples three events crucial for establishing BC polarity: (1) Fas2 homophilic interactions are lost between PCs and BCs, thus permitting formation of Fas2 heterophilic interactions with a putative BC receptor (essential for maintaining Fas2 in PC membranes); (2) Fas2 polarity is directed by Dlg and Lgl polarity in PCs; and (3) accumulation of Fas2 between PCs through homophilic interactions ensures that Fas2 level is kept sufficiently low at the site of contact with BCs to permit its polarization. This novel, precisely timed morphogenetic switch for Fas2 polarization suggests that Fas2 plays a crucial role in regulating cell cluster movement.

Fas2 regulates efficient BC delamination, but inhibits the rate of BC migration

Previous studies suggested that *dlg* mutant BCs reach the oocyte prematurely (Goode and Perrimon, 1997). The method used in that study did not allow us to distinguish if the defect resulted from premature BC delamination or faster BC migration. To address these alternatives, we used oocyte growth as a clock to measure the rates of BC delamination and migration (Fig. 3; Materials and methods).

We characterized a *Fas2* allelic series by measuring Fas2 levels in the following egg chambers: $Fas2^{null}/+$ (46±11% Fas2), $Fas2^{rd1}$ (6±3% Fas2), $Fas2^{rd1}/Fas2^{null}$ (3±2% Fas2) and $Fas2^{null}$ (0% Fas2) (Materials and methods). In 46% Fas2 clusters, BCs delaminate ~15% faster (~1 hour) (P=0.066, compared with wild type), but migration time is essentially wild type (Fig. 4). Six percent Fas2 BCs delaminate faster (not statistically significant) and migrate 35% faster (P=0.008, compared with wild type) (Fig. 4). The faster rate of migration of these clusters is thus quantitatively similar to 50% faster migration seen when negative regulator of migration Ena/Vasp is removed from the leading edge of mammalian fibroblasts

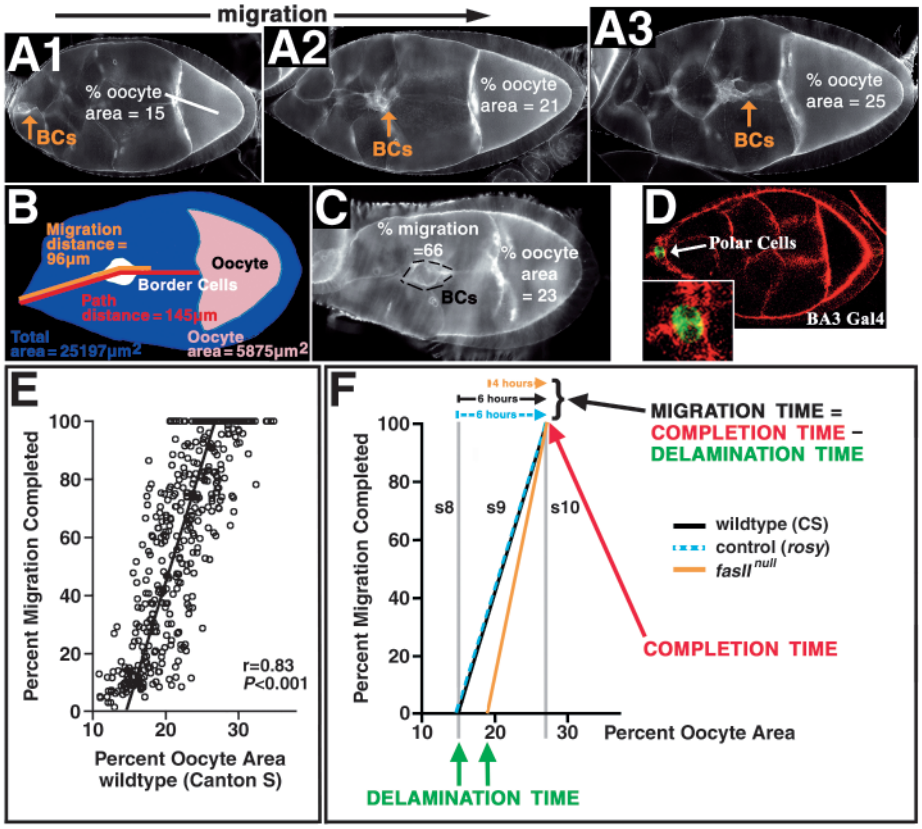

Fig. 3. BC motility assay. (A1-A3) Wild-type stage 9 egg chambers of increasing maturity. As the border cells (BCs, arrow) move to the oocyte, oocyte area increases relative to the rest of the egg chamber (B,C,E). This oocyte growth can thus be used as a clock to measure the rate of BC migration. The % oocyte area (=oocyte area divided by the total area of the egg chamber; 23%) was plotted against the % migration (migration distance divided by the path distance; i.e. 66%). The results of from ~400 wild-type egg chambers are shown in E. For all genotypes: $0.75 \leq r \leq 0.88$, $P<0.001$. (F) Graphs for wild-type, rosy and *Fas2*^{null} BC clusters are shown. Delamination time is the point at which the BCs completely leave the epithelium (0% migration; green arrows). Migration time is the difference between the completion time (100% migration; red arrow) and the delamination time (green arrows). (D) BA3-Gal4 expression pattern. Slbo-Gal4 expression pattern is shown in Fig. 2D.

(Bear et al., 2000). Three percent and 0% Fas2 BCs also have faster migration similar to 6% Fas2 BCs (P=0.006 and 0.031, respectively, compared with wild type), but delamination is progressively delayed by 13% (P<0.0005, compared with wild type) and 35% (P<0.0005, compared with wild type), respectively (Fig. 4).

These findings indicate that Fas2 has two functions, one that inhibits rate of BC migration and another that promotes delamination. Although this coupling of inhibitory and promoting functions is unusual, a precedent for this genetic behavior exists from previous studies of Fas2 function. Goodman et al. (Goodman et al., 1997) have shown that in 50% Fas2 synaptic buttons, there is a twofold increase in synaptic growth, whereas further reduction to 10% Fas2, or less, causes a twofold decrease in synaptic growth, similar to the pattern we see for delamination. By contrast, fasciculation and synapse formation are normal at 50% Fas2, but are lost at 10% or lower Fas2, similar to the quantitative pattern that we observe for increased migration rate.

Fas2 migration defects may result from Fas2 loss in PCs, or from premature loss of Fas2 in early follicle cells (Fig. 1C). To determine if Fas2 is required in PCs, we targeted it to PCs in *Fas2*^{rd1} and *Fas2*^{null} egg chambers using the BA3-Gal4 driver. We observe complete rescue of *Fas2* migration defects when Fas2 is targeted to PCs using BA3-Gal4, indicating that PC Fas2 regulates migration of the cluster (rescue of *Fas2*^{rd1}: P=0.001, compared with *Fas2*^{rd1}; rescue of *Fas2*^{null}: P=0.037, compared with *Fas2*^{null}) (Fig. 4). Moreover, we did not observe rescue of *Fas2* migration when BCs were targeted with Fas2 using Slbo-Gal4 (Fig. 4), further demonstrating that Fas2 acts in PCs not BCs to control migration.

To determine if the PDZ-binding domain or extracellular domain of Fas2 is involved in regulating cluster migration, we targeted Fas2Δ3 or chimeric CD8-Fas2 to *Fas2* PCs. The Fas2Δ3 molecule is missing the last three amino acids that bind PDZ1 and PDZ2 domains of Dlg (Thomas et al., 1997; Zito et al., 1997). None of these two Fas2 derivatives was able to rescue *Fas2*^{rd1} or *Fas2*^{null} migration defects (Fig. 4). This indicates that both the extracellular domain of Fas2 that interacts with a putative BC receptor and the intracellular domain that binds Dlg are essential for Fas2 function. This assertion is further supported by targeting dominant-negative Fas2Δ3 to wild-type PCs, which increases the rate of BC migration by about 10% (P=0.006, compared with UAS-Fas2Δ3/+). Likewise, CD8-Fas2 appears to act in a dominant-negative manner, increasing migration by 10% (UAS-CD8-Fas2/+; BA3-Gal4/+: P=0.025, compared with UAS-CD8-Fas2/+) (see below and Fig. 4). As PC Fas2 does not contact the migration substrate, we conclude that Fas2 inhibits rate of migration by signaling to BCs.

Fas2 signals Dlg and Lgl localization in BCs

Fas2 is required for Dlg localization in synapses (Thomas et al., 1997), and Dlg is required for Lgl localization in epithelial cells (Bilder et al., 2000). We therefore asked if *Fas2* motility defects might result from Dlg and Lgl mislocalization. In wild-type clusters, polarized Fas2 colocalizes with Dlg and Lgl in PCs, while Dlg and Lgl colocalize in the cortex of BCs (Fig. 6A-A″). In both 6% and 0% Fas2 clusters, Dlg and Lgl levels are lower in the cortex of the BCs, while cytoplasmic levels of Dlg and Lgl increase (Fig. 2E′, Fig. 6B′,B″,C′,C″). This suggests that the level of Dlg and Lgl did not change, but that

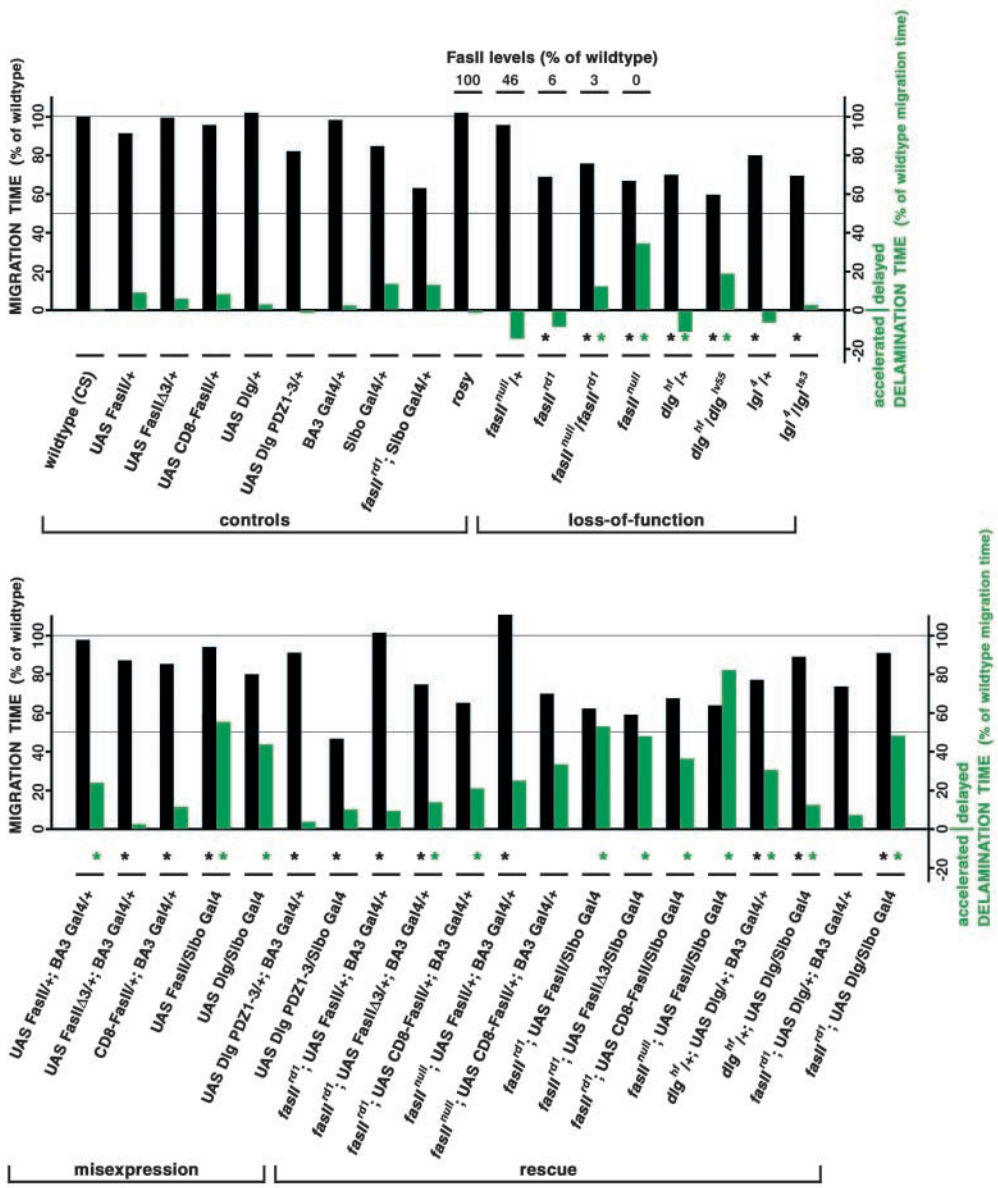

Fig. 4. Quantitative analysis of the role of Fas2, Dlg and Lgl in BC motility. Bar graphs show time of delamination (green) and migration (black) of BCs. Migration is faster when the level of Fas2, Dlg or Lgl is lower. These defects are rescued when Fas2 is targeted to PCs in *Fas2* clusters using BA3-Gal4, or when Dlg is targeted to BCs in *Fas2* or *dlg* clusters using Slbo-Gal4. Slower delamination of BCs correlates with the decrease of Fas2 polarity (see Fig. 6). Asterisks indicate statistically significant differences (General Linear Model analysis, $P<0.05$) from control (loss-of-function and misexpression experiments), or from *Fas2* or *dlg* mutants (rescue experiments).

the proteins became redistributed. We confirmed this by quantifying Dlg and Lgl levels in Fas2$^+$ and *Fas2^{null}* BCs (Materials and methods). We find that the levels of Dlg and Lgl do not decrease more than 20%. Further, these defects are specific, as Crumbs (Crb), a transmembrane protein that organizes apical polarity, and α-Spectrin (α-Spec), a cortical molecule that interacts with the cytoskeleton, show no changes in localization in *Fas2^{null}* BCs (P.S. and S.G., unpublished).

Mislocalization of Dlg and Lgl in BCs in *Fas2* clones might result from loss of Fas2 in PCs, or from premature loss of Fas2 in early follicle cells. To distinguish these alternatives, we analyzed *Fas2^{null}* mosaic clusters in which only one PC expresses Fas2. Significantly, Dlg and Lgl localize normally in BCs that contact Fas2$^+$ PCs, but mislocalize in BCs that contact 0% Fas2 PCs (Fig. 2E,E′). This suggests that PC Fas2 maintains Dlg and Lgl localization in BCs. However, premature loss of Fas2 in presumptive BCs might also contribute to mislocalization of Dlg and Lgl in BCs, if *Fas2^{null}* PC clones also include *Fas2^{null}* BCs. To address this possibility, we analyzed Dlg and Lgl localization in other Fas2$^+$ and *Fas2^{null}* follicle cells at the time of BC delamination. Dlg and Lgl localization and levels are normal in *Fas2^{null}* follicle cells (Fig. 2F′,F″). PC Fas2 thus ensures Dlg and Lgl localization in BCs, but not in other follicle cells. This interpretation is consistent with the previous observation that the putative BC receptor is expressed in BCs, but not other follicle cells (Fig. 2F,H).

Dlg and Lgl collaborate with Fas2 to regulate delamination and inhibit migration

To determine if *Fas2* clusters migrate faster due to mislocalization of Dlg and Lgl in BCs, we examined migration rates of *dlg* and *lgl* mutant BCs. In *dlg^{hf}*/+ or *lgl^4*/+ clusters, the BCs delaminate prematurely (*dlg^{hf}*/+: $P=0.001$, compared with wild type; *lgl^4*/+: not statistically significant) and migrate faster (*dlg^{hf}*/+: $P<0.0005$ compared with wild type; *lgl^4*/+:

$P<0.0005$ compared with wild type) (Fig. 4). This pattern is similar to motility of 6% Fas2 BCs (Fig. 4), suggesting that mislocalization of Dlg and Lgl in *Fas2* BC clusters causes faster migration.

If decrease in Dlg function in BCs causes faster migration, then targeted expression of Dlg to dlg^{hf}/+ BCs should rescue faster migration. Targeting Dlg to dlg^{hf}/+ BCs completely rescues faster migration ($P<0.0005$, compared with dlg^{hf}/+) (Fig. 4). Targeting Dlg to PCs rescues migration only weakly (Fig. 4). Dlg thus acts in BCs to inhibit migration. Moreover, targeting wild-type BCs with a truncated Dlg molecule containing only three PDZ domains, Dlg PDZ1-3, expected to compete with endogenous Dlg for interaction with PDZ-binding proteins, causes significant increase in migration rate (UAS-Dlg PDZ1-3/Slbo-Gal4: $P=0.031$, compared with Slbo-Gal4/+), and is quantitatively comparable with 6% Fas2 or dlg^{hf}/+ clusters (Fig. 4). Targeting expression of Dlg PDZ1-3 to PCs does not significantly affect cluster motility, perhaps because of the higher level of Dlg in PCs compared with BCs (Fig. 4). These data support the hypothesis that Dlg acts in BCs to inhibit movement. To obtain further evidence that mislocalization of BC Dlg causes faster migration of *Fas2* clusters, we asked if targeting Dlg to BCs could rescue *Fas2* clusters. Targeting Dlg to $Fas2^{rd1}$ BCs completely rescues faster migration of $Fas2^{rd1}$ clusters ($P<0.0005$, compared with $Fas2^{rd1}$; Slbo-Gal4/+), but no rescue occurs by targeting Dlg to PCs ($P=0.910$, compared with $Fas2^{rd1}$) (Fig. 4). Moreover, targeting *Fas2* BCs with Fas2, Fas2Δ3 or CD8-Fas2 does not rescue faster migration of BC clusters (Fig. 4). These experiments indicate that faster migration of *Fas2* clusters results from lower levels of Dlg in the BC cortex.

Dlg recruits Lgl to the membrane in epithelial cells and neuroblasts (Bilder et al., 2000; Peng et al., 2000). To determine if Dlg and Lgl collaborate in BCs, we analyzed Dlg and Lgl localization in dlg^{hf}/+ and lgl^{4}/+ clusters. Whereas levels of cortical Dlg and Lgl are lower in dlg^{hf}/+ clusters (Fig. 6D′,D″), only Lgl is lower in lgl^{4}/+ clusters (Fig. 6F′,F″). Dlg thus recruits Lgl to BC plasma membranes (see also Fig. 6C′,C″), but Lgl does not recruit Dlg.

Similar to partial loss of Fas2, partial loss of Dlg and Lgl both increase migration rate, and accelerate delamination (Fig. 4). These data, combined with the rescue and localization experiments, indicate that Fas2, Dlg and Lgl function in a common pathway to control BC movement. If Dlg and Lgl are key collaborators with Fas2 in cluster movement, then further reduction of Dlg and Lgl might cause delayed delamination, similar to that observed in 0% Fas2 clusters. Consistent with this hypothesis, clusters expressing temperature sensitive allele combinations dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} migrate slightly faster than dlg^{hf}/+ and lgl^{4}/+ clusters (dlg^{hf}/dlg^{lv55}: $P=0.012$, compared with wild type; lgl^{4}/lgl^{ts3}: $P=0.001$, compared with wild type) (Fig. 4). More strikingly, both dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} clusters have slower delamination (Fig. 4), although only dlg^{hf}/dlg^{lv55} cluster delaminate significantly slower ($P<0.0005$, compared with wild type). There are several possible interpretations for why the delamination of 0% Fas2 clusters is slower than for dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} clusters (Fig. 4), but the observation that they show the same trend towards slower delamination indicates that Dlg and Lgl collaborate with Fas2 in both delamination and migration. Further evidence for this conclusion comes from the high resolution cellular analysis described below.

Slower delamination of *Fas2*, *dlg*, and *lgl* clusters correlates with defective PC polarity

To determine if there is a common defect in clusters that delaminate slower, we compared Fas2, Dlg and Lgl localization in clusters with faster versus slower delamination. 6% Fas2, dlg^{hf}/+ and lgl^{4}/+ BCs delaminate faster, and Fas2, Dlg and Lgl remain polarized in PCs (Fig. 6B-B″,D-D″,F-F″). 0% Fas2, dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} BCs delaminate slower, and Fas2, Dlg and Lgl have an apolar pattern in PCs (Fig. 6C-C″,E-E″,G-G″; polarity is not restored as BC clusters migrate). Furthermore, loss of Fas2, Dlg and Lgl polarity, owing to misexpression of Fas2 in BCs in UAS-Fas2/Slbo-Gal4 clusters (see Fig. 2B for the loss of Fas2 polarity), causes dramatic delay of delamination ($P<0.0005$, compared with Slbo-Gal4/+), without affecting migration rate (Fig. 4). We conclude that when Fas2, Dlg and Lgl PC polarity is normal, BCs delaminate on time or prematurely, but when PC polarity is lost, BC delamination is slower. These data strongly suggest that PC polarity is crucial for organizing the cluster for efficient delamination.

Ectopic PC Fas2 specifically delays delamination

To further characterize the importance of Fas2 polarity in delamination, we examined the consequence of disrupting Fas2 polarity by misexpressing Fas2 in PCs. These clusters have Fas2 around the circumference of the PCs (Fig. 6H), as in dlg^{hf}/dlg^{lv55} and lgl^{4}/lgl^{ts3} clusters (Fig. 6E,G). As expected for a Fas2-binding protein, Dlg becomes localized in the same pattern as Fas2 (Fig. 6H′), together with Lgl (Fig. 6H″), providing further evidence that Fas2 can direct Dlg and Lgl localization (see also Fig. 6C′,C″). The BCs consistently migrate behind the epithelium (Fig. 5A). We found that, although migration rate is almost normal, delamination is dramatically delayed ($P<0.0005$, compared with UAS-Fas2/+) (Fig. 4). Consistent with these data, both the wild-type polarized pattern of Fas2, and timely delamination, are restored by targeting Fas2 to $Fas2^{rd1}$ PCs, which contain polarized Dlg and Lgl (Fig. 6K-K″). However, Fas2 targeted to $Fas2^{null}$ PCs, which have unpolarized Dlg and Lgl (Fig. 6C′,C″), fails to rescue polarity (leading-to-trailing Fas2 polarity ratio is 2.1±1.3), and these clusters have slower delamination (Fig. 4). To determine if the delamination defects resulting from Fas2 misexpression in PCs result from higher Fas2 levels, rather than disruption of Fas2 polarity, we targeted wild-type PCs with Fas2Δ3. Polarity of Fas2 is relatively normal in UAS-Fas2Δ3/+; BA3-Gal4/+ PCs (Fig. 6I). BCs migrate ahead of the epithelium when Fas2Δ3 is targeted to PCs (Fig. 5B), and this defect results from faster BC migration ($P=0.006$, compared with UAS-Fas2Δ3/+), not premature delamination (Fig. 4). Targeting CD8-Fas2 to PCs also does not disrupt Fas2 polarity (Fig. 6J), but causes faster BC migration ($P=0.025$, compared with UAS-CD8-Fas2/+), with no effect on delamination ($P=0.071$, compared with UAS-CD8-Fas2/+) (Fig. 4). These experiments reveal once again that when Fas2 polarity is maintained, delamination is not perturbed. Furthermore, as Fas2Δ3 is not expected to bind to Dlg PDZ domains, and CD8-Fas2 is expected to interfere with Fas2 interaction with the putative BC receptor, these data further

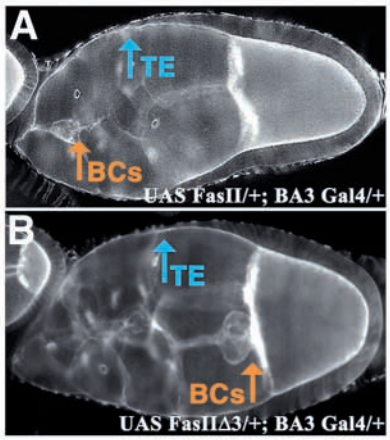

Fig. 5. Misexpression of Fas2 and Fas2Δ3 in PCs. (A) Misexpression of Fas2 in PCs causes BCs to initiate migration behind the trailing edge of the epithelium (TE) (see Fig. 3A1-3 for wild-type migration). Delamination is significantly delayed, but the rate of migration is normal (Fig. 4). (B) Misexpression of Fas2Δ3, which is unable to interact with Dlg, does not affect time of delamination, though it accelerates migration by 10% (Fig. 4).

suggest that Fas2 interactions with PC Dlg and the putative BC receptor are crucial for inhibiting BC migration. In conclusion, these results indicate that Fas2 is crucial for timely delamination through its function in establishing PC polarity, while it inhibits rate of BC migration through a distinct process that does not depend on PC polarity.

Fas2 polarity regulates cluster asymmetry during delamination

If PC polarity plays a crucial role during delamination, then that polarity must be translated into asymmetry within the BC cluster, as it is the BCs that mediate the coordinate delamination activities of breaking of contact with adjacent epithelial cells while simultaneously invading between adjacent germ cells. To explore the relationship between BC asymmetry and Fas2 polarity, we dramatically altered Fas2 polarity by removing Fas2 from one of the two PCs. In these *Fas2* mosaic clusters, the Fas2^{+} PC orients the cluster at the time of delamination, such that it rotates perpendicularly compared with wild type (Fig. 2E,E′). The entire cluster reorganizes such that both BCs contacting the Fas2^{+} PC lead delamination (Fig. 2E,E'). Fas2-Dlg PC polarity is thus a key organizer of the BC cluster asymmetry.

Additional evidence for BC cluster asymmetry comes from localization of Amphiphysin (Amph), a vesicle trafficking protein important for Dlg and Lgl localization (Zelhof et al., 2001). Amph is expressed at higher levels in trailing BCs (not in contact with PC Fas2) than in leading BCs (Fig. 7B-C′). Amph polarity develops around the time of BC differentiation at stage 8 of oogenesis (Fig. 7A-B′), supporting hypothesis of its functional significance for cluster motility.

Discussion

As outlined by Kolega (Kolega, 1981), several functions are thought to be essential for movement as a cluster, as opposed to single cells: (1) intercellular interactions that modulate movement, (2) directional mass motion between cells individually capable of motion in any direction, (3) determination of locomotive-active regions of individual cells, and (4) integration of these processes through organization of cluster polarity. In vertebrates, expression of cell-adhesion molecules in a subset of cells within a migrating cluster has suggested that they may have the requisite properties to execute these functions (Hegerfeldt et al., 2002; Nakagawa and Takeichi, 1995; Toba et al., 2002). Our genetic and cell biological analysis provides the first direct evidence that a cell adhesion molecule, Fas2, organizes the activities outlined by Kolega (Kolega, 1981). Below, we propose a model that explains how Fas2 organizes epithelial clusters to control delamination and migration.

A Fas2 morphogenetic switch

Just preceding cluster migration, Fas2 is polarized in PCs with an orientation that predicts the direction of BC movement (Fig. 1E′-F). Polarization precedes delamination and migration by 4-6 hours. Furthermore, Fas2 polarity is not perturbed in clusters that fail to migrate (*slbo*^{1310}; data not shown). We conclude that Fas2 polarization is not a consequence of delamination or migration. The timing of Fas2 polarization is determined by the temporal specificity of developmentally programmed loss of Fas2 in surrounding BCs at stage 8. As loss of Fas2 in anterior follicle cells is controlled transcriptionally (Fig. 1H,I), and includes stretch cells, not just BCs, it is unlikely that BC specific transcription factors such as Slbo, Jing or Stat (reviewed by Montell, 2003) control developmentally programmed loss of Fas2 expression. Other factors, such as Eyeless, are expressed in precisely the cells in which Fas2 is lost, while being lost in PCs (S.G., unpublished). Eyeless thus may be part of a transcriptional regulatory network that developmentally programs loss of Fas2 expression, as well as programs expression of other genes specifically involved in morphogenesis of anterior follicle cell motility.

How does developmentally programmed loss of Fas2 expression in BCs permit Fas2 polarization in PCs? Our data indicate that this is a multistep process. First, Fas2 homophilic interactions between BCs and PCs are lost, and several of our experiments indicate that they are replaced by Fas2 heterophilic interactions with a putative BC receptor. These interactions are essential for maintaining Fas2 in PC membranes contacting BCs (Fig. 2F,G). Second, loss of Fas2 from BCs causes relocation of the majority of PC Fas2 to the interface between PCs, where it is maintained because of homophilic interactions with Fas2 from the adjacent PC. In support of this interpretation, misexpression of Fas2 in PCs appears to oversaturate Fas2 between PCs, causing its circumferential accumulation at the contact sites with BCs (Fig. 6H). We conclude that the accumulation of Fas2 between PCs ensures that Fas2 is kept at sufficiently low level at the sites of contact with BCs to allow its polarization to the leading half of PCs. Third, Fas2 polarization is directed by PC Dlg and Lgl, as loss of function of either protein causes loss of Fas2 polarity (Fig. 6E,G). However, Fas2 can also polarize Dlg and Lgl, as loss of Fas2 causes loss of Dlg and Lgl polarity (Fig. 6C′,C″), while ectopic Fas2 redirects Dlg and Lgl localization (Fig. 6H′,H″). Thus, Fas2 is in a positive feedback loop with Dlg and Lgl that ensures the build up of a PC signaling and adhesion complex at the leading half of the PCs.

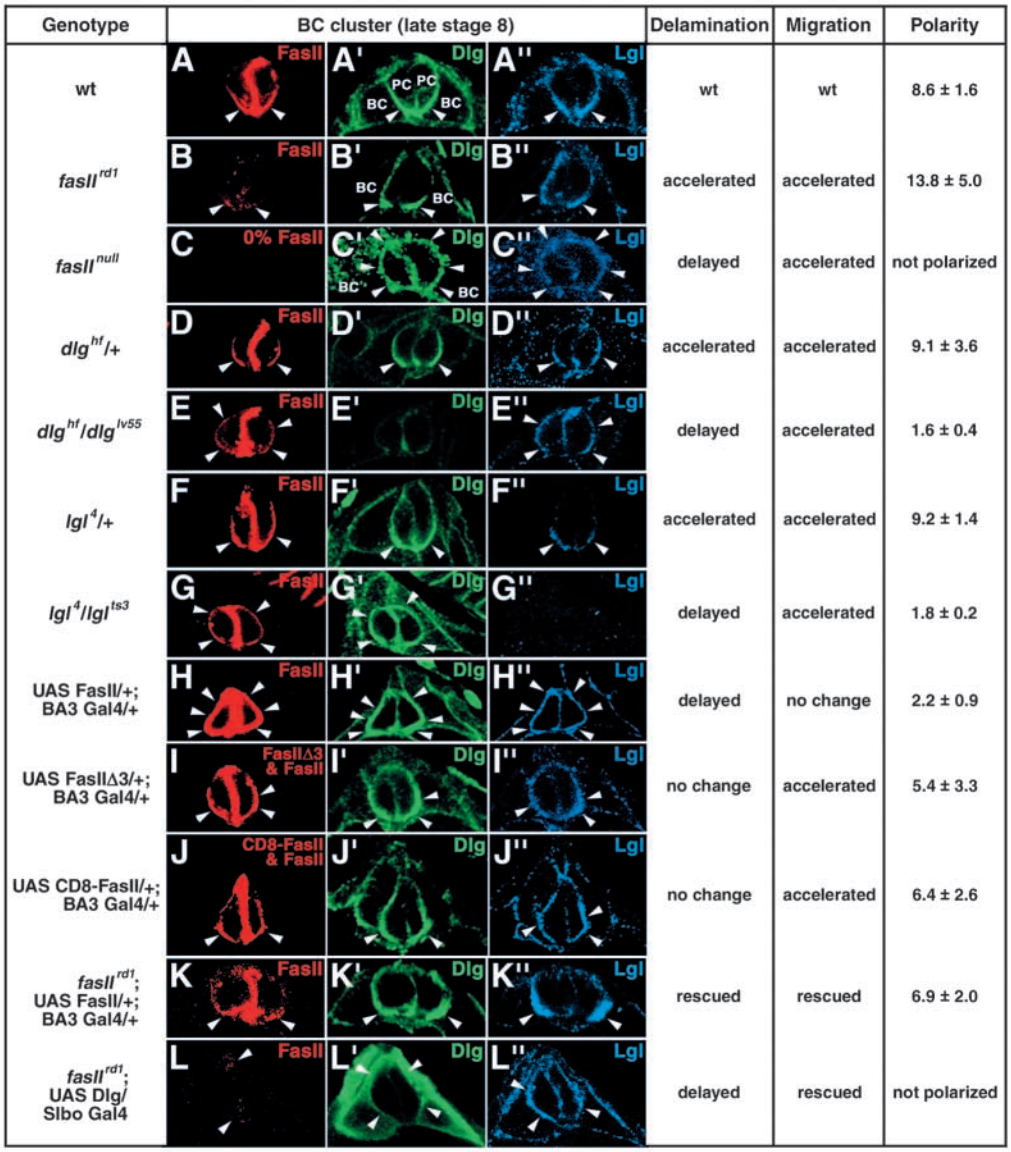

Fig. 6. Localization of Fas2, Dlg and Lgl in mutant, misexpression and rescue clusters. The delamination and migration times are shown in Fig. 4. Polarity values are ratios of Fas2 at the leading half of PC to Fas2 at the trailing half of PC, and are mean±s.d. from on average 10 measurements. (A-A″) Fas2 colocalizes with Dlg and Lgl at the leading half of the PCs. Dlg and Lgl colocalize in the cortex of BCs. (B-B″) In *Fas2*^{rd1} clusters, Dlg and Lgl are mislocalized in BCs. In PCs, Fas2 remains polarized, and Dlg and Lgl colocalize in a similar pattern (arrowheads). (C-C″) Complete loss of Fas2 in *Fas2*^{null} PCs causes Dlg and Lgl fragmentation and loss of polarity (arrowheads). As in *Fas2*^{rd1} clusters, Dlg and Lgl are mislocalized in BCs. Fas2 is polarized in *dlg*^{hf}/+ and *lgl*^{4}/+ clusters that contain functional Dlg or Lgl reduced by half (D,F). Further reduction of Dlg in *dlg*^{hf}/*dlg*^{lv55} clusters (E′), and Lgl in *lgl*^{4}/*lgl*^{ts3} clusters (G″) results in the loss of Fas2 polarity (E,G). (H-H″) Fas2, Dlg and Lgl colocalize in a circumferential pattern in PCs in UAS-Fas2/+; BA3-Gal4/+ clusters. Fas2, Dlg and Lgl colocalize in polarized pattern in PCs in UAS-Fas2Δ3/+; BA3-Gal4/+ clusters (I-I″), and in UAS CD8-Fas2/+; BA3 Gal4/+ clusters (J-J″). (K) Fas2 polarity in *Fas2*^{rd1} PCs is restored when Fas2 is targeted to PCs. (L) Polarity of *Fas2*^{rd1} PCs is abolished when Dlg is targeted to BCs. In all cases, decrease of Fas2 polarity correlates with the delay of BC delamination. Arrowheads indicate the accumulation of Fas2, Dlg or Lgl.

These data indicate that Fas2 is involved in intercellular interactions crucial for organizing polarity, an important criterion proposed by Kolega (Kolega, 1981) for a function specifically involved in regulation of motility in multicellular clusters.

Significantly, our results indicate that molecules used for polarizing epithelial cells are reorganized to polarize a motile cell cluster. The timing of the reorganization of epithelial polarity is crucial for timing delamination (Fig. 4, Fig. 6, Fig. 7D). Fas2 therefore plays a direct role in mediating intercellular interactions that modulate movement, a second property proposed by Kolega (Kolega, 1981) for a function specifically involved in regulating cluster motility as opposed to single cells. We conclude that the Fas2 morphogentic switch facilitates development of motile polarity essential for timely BC delamination. A similar switch mechanism may be important in other processes that crucially depend on timing of Fas2 activity, such as axon pathfinding (Goodman et al., 1997), and learning and memory (Cheng et al., 2001).

A model for organization of cell cluster motility

Fas2 polarity appears to compartmentalize PCs into distinct functional domains in order to control functionally distinct intercellular communication with leading versus trailing BCs (see model, Fig. 7D). Leading BCs play a functionally distinct role by pioneering invasion between germ cells while simultaneously detaching from the epithelium. Trailing BCs are likely to play a less active role in invasion, but must mediate precisely timed detachment from the epithelium. Fas2 polarization is thus likely to be crucial for facilitating coordination of the distinct functional requirements of leading versus trailing BCs, by establishing distinct sets of intercellular contact and communication between the PCs and leading versus trailing BCs. In support of this hypothesis, previous

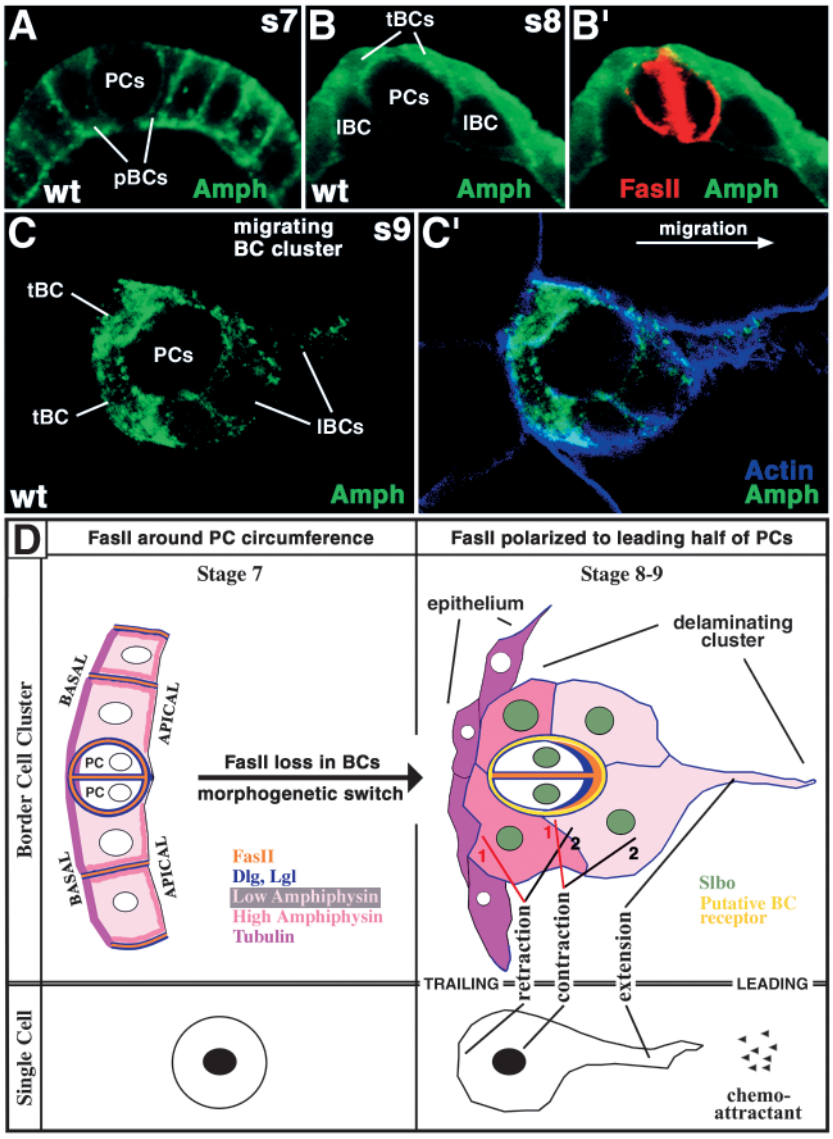

Fig. 7. Polarity of the BC cluster and proposed mechanism by which Fas2, Dlg and Lgl control movement. (A-C′) The polarity of the BC cluster is demonstrated by the high levels of Amph in trailing BCs (tBCs) compared with leading BCs (lBCs). Amph polarity is established just preceding BC differentiation (stage 7-8; pBCs, presumptive BCs). (D) Model of a delaminating BC cluster. At stage 7, Fas2 (orange), and Dlg and Lgl (blue) are localized around the circumference of PCs and on lateral membranes of presumptive BC epithelial cells. At stage 8, the BCs differentiate when Slbo turns on. Fas2 is lost from BCs, and other anterior epithelial cells, except PCs. Fas2 loss from BCs is crucial for Fas2 polarization to the leading half of the PCs (morphogenetic switch, Fig. 2B). Dlg and Lgl become localized around the circumference of the BCs. The PC Fas2-Dlg-Lgl complex acts through a putative BC receptor to maintain cortical organization of Dlg and Lgl in BCs, which is crucial for inhibiting rate of BC movement. Polarized communication between PCs and front BCs assures timely delamination of the BC cluster. The polarized nature of the cluster suggests that the work of the extension-retraction-contraction cycle found in single cells may be distributed between multiple cells in the migrating cluster and coordinated through Fas2 intercellular communication (1). Another possibility is that polarized Fas2 signaling is required to polarize front BCs in an active pattern similar to individual migrating cells, with the back border cells playing a passive role (2).

studies have suggested that leading and trailing BCs are functionally distinct. In BC clusters comprising a mixture of wild type and *slbo*, *jing*, *taiman* or *DE-cadherin* mutant cells, wild-type BCs always lead invasion (Liu and Montell, 2001; Niewiadomska et al., 1999; Rørth et al., 2000). Furthermore, we documented additional structural evidence for cluster asymmetry. Amph, a vesicle trafficking protein that regulates Dlg and Lgl localization (Zelhof et al., 2001), is expressed at higher level in trailing BCs compared to leading BCs (Fig. 7B-C′). Amph, Dlg and Lgl, are thus good candidates for proteins that differentially regulate cortical and cell surface activities needed to mediate distinct interactions of leading and trailing BCs with adjacent epithelial cells and germ cells during the delamination process.

As only Dlg and Lgl are mislocalized in *Fas2* clusters, but not Fas3, α-Spec or Crb (P.S. and S.G., unpublished), our data suggest that Fas2 directs localization of specific molecules within distinct regions of different cells of the cluster to control motility. A putative Fas2-binding BC receptor may be another molecule whose polarity is controlled by Fas2 (see previous section). Interaction with this putative receptor appears to facilitate organization of the global polarity of the cluster, as the orientation of delamination, mediated by the BCs, directly correlates with Fas2 polarity in PCs (Fig. 2E,E′). These data thus suggest that Fas2 coordinates directional mass motion between cells that are potentially capable of motion in any direction, and that it helps to determine the locomotive-active regions of these cells, additional criteria proposed by Kolega (Kolega, 1981) for a function specifically involved in regulating cluster motility. Thus, as Fas2 is required for regulation of several activities that distinguish how single cells versus clusters move, our data provide the first molecular model for understanding the organization of epithelial cluster polarity during delamination and movement (Fig. 7D). One argument against this proposal might be that the PCs appear to be highly specialized. However, we think this is likely to be of less significance as PCs express epithelial polarity proteins in a pattern similar to adjacent follicle epithelial cells (Fig. 1E-E‴, Fig. 2A-A″, Fig. 6A-A″).

As we have shown for BC clusters, several vertebrate studies have shown that transmembrane proteins are differently expressed within different cell subpopulations in migrating clusters (Hegerfeld et al., 2002; Nakagawa and Takeichi, 1995; Toba et al., 2002). Furthermore, the structure and functions of Fas2, Dlg and Lgl homologs are conserved across phylogeny (Abbate et al., 1999; Gonzalez-Mariscal et al., 2000; Hoover et al., 1998; Huang et al., 2003; Ito et al., 1995; Matsumine et al., 1996; Roesler et al., 1997; Watson et al., 2002). Thus, the

involvement of Fas2, Dlg and Lgl in organizing cell cluster motility also may be conserved. We conclude that although the precise mechanism of cluster movement may not be conserved in vertebrates, the information that we glean about how BCs regulate epithelial polarity to dynamically organize cluster polarity and movement will be generally useful for understanding how cell cluster motility is organized across phylogeny.

Fas2 intercellular signals inhibit cluster migration

The previous sections discussed the role of Fas2 in delamination; here, we discuss the role of Fas2 in regulating migration. Loss- and gain-of-function experiments demonstrate that PC Fas2 acts as a signal to inhibit the rate of BC migration (Fig. 4). Our work builds on previous studies demonstrating the importance of PCs in determining BC fate (Bai et al., 2000; Beccari et al., 2002). However, our work is the first example of an intercellular signal that specifically organizes cluster movement, rather than determining cell fate. Fas2 clearly has a signaling function, as PCs do not contact the migration substrate. Thus, these data demonstrate for the first time the existence of intercellular communication between cells of a migratory cluster, which is specifically required to modulate migration (Kolega, 1981).

Contact inhibition of movement

PC Fas2 signaling inhibits the rate of cluster movement by maintaining Dlg and Lgl localization in BCs (Fig. 2E,E′, Fig. 4). The putative BC receptor that Fas2 interacts with (see previous section) may control Dlg and Lgl localization in BCs. As Dlg is localized to the cortex of BCs, Dlg must inhibit the rate of migration through cortical activities in BCs. One cortical activity controlled by Dlg is the recruitment of Lgl to the membrane (Fig. 6C′′,E′′). As *lgl* clusters have very similar migration phenotypes to *dlg* clusters (Fig. 4), our data indicate that Lgl and Dlg cooperate to inhibit BC movement. The importance of Dlg and Lgl in regulating cell movement probably derives from the same scaffolding activities they use to organize and control membrane, cytoskeletal and signaling specialization during the polarization of epithelial and neuronal cells (Bear et al., 2000; Kim et al., 1998; Lehman et al., 1999; Peng et al., 2000; Strand et al., 1994). We propose that Dlg and Lgl scaffolding organizes and integrates transmembrane signaling and adhesion proteins with signaling, trafficking and cytoskeletal effectors in the cortex of BCs to mediate contact-inhibition of cluster movement.

Relevance to tumor cell invasion

BCs resemble *dlg* invasive tumor cells in that they lose epithelial polarity by accumulating Dlg and Lgl around their circumference (Fig. 1F,F′, Fig. 2A′,A′′, Fig. 6A′,A′′) (P.S. and S.G., unpublished), but in contrast to BCs, *dlg* tumor cells migrate between germ cells without temporal or spatial control (Goode and Perrimon, 1997) (S.G., unpublished). Our data demonstrate that Dlg and Lgl not only control polarity and delamination of epithelial clusters, but also actively inhibit movement. Thus, *dlg* tumor invasion is likely to be caused by a combination of loss of epithelial polarity and over-activation of motility pathways. In this context our results appear to be paradoxical in that loss of epithelial polarity is generally considered to be crucial for facilitating acquisition of motility, but we see that loss of polarity in normal migrating clusters delays initiation of movement. Our data resolve this paradox in that during normal development molecules used for polarizing epithelial cells are reorganized to polarize a motile cell cluster. It therefore seems likely that in carcinomas, inappropriate loss of epithelial polarity simultaneously disrupts acquisition of motile polarity, but this phenomenon is not appreciated because ultimately the tumor cells migrate. Thus, we postulate that overactivation of motility pathways, as we see with loss of Dlg and Lgl in BCs, may be especially crucial for achieving carcinoma invasion. Consistent with this hypothesis, some *dlg* mutations that cause loss of epithelial polarity do not lead to tumor invasion (Goode and Perrimon, 1997), suggesting that acquisition of motility is a separate Dlg function.

Gene expression data for human cancers suggests that mutations that promote tumor formation, through loss of epithelial polarity and increased proliferation, may be the same mutations that subsequently cause tumor cell invasion (Couzin, 2003). Based on the observation that Dlg is required to maintain polarity, inhibit proliferation (Woods and Bryant, 1991) and inhibit movement (Goode and Perrimon, 1997) (this study), we propose that tumor suppressors such as Dlg that regulate signaling and adhesion at epithelial junctions may unify human gene expression data by providing an ultrastructural target that controls contact inhibition of both proliferation and movement. Progressive deterioration of epithelial junctions may thus provide a common mechanism through which multiple tumor suppressor pathways impact the cascade from cell proliferation to tumor invasion, either through mutation or mislocalization of critical junctional proteins.

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