The Hippo pathway polarizes the actin cytoskeleton during collective migration of Drosophila border cells

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Introduction

Migration of cells is one of the most dramatic events that underlies the development of animal tissues and the progression of tumors (Condeelis et al., 2005; Sahai, 2005; Montell, 2008). Most of our knowledge of the mechanisms of cell migration comes from the study of single cells migrating in culture (Van Haastert and Devreotes, 2004; Ridley, 2011). However, in vivo, cells often migrate not as individuals but as groups that move collectively (Friedl and Gilmour, 2009; Rørth, 2009; Weijer, 2009).

Drosophila border cell migration is a genetically tractable model system for the study of collective cell movement (Starz-Gaiano and Montell, 2004; Rørth, 2009). Border cells arise in the follicular epithelium that surrounds each egg chamber in the Drosophila ovary (Fig. 1 A). At the anterior pole of the egg chamber, a pair of polar cells recruits a small group (4–8) of neighboring follicle cells into the border cell cluster. At stage 9 of oogenesis, this cluster delaminates from the epithelium and invades the underlying germ line, migrating across the egg chamber between the large nurse cells to reach the oocyte at the posterior pole by stage 10 of oogenesis (Fig. 1, A–C).

A series of important discoveries has revealed many key mechanisms by which border cells are first specified (Montell et al., 1992; Bai et al., 2000; Silver and Montell, 2001; Beccari et al., 2002; Xi et al., 2003; Borghese et al., 2006; Jang et al., 2009), begin their invasive movement (Fulga and Rørth, 2002), detach from the epithelium (McDonald et al., 2008), are guided toward the oocyte (Duchek and Rørth, 2001; Duchek et al., 2001; McDonald et al., 2003; Bianco et al., 2007; Poukkula et al., 2011), sense tension (Somogyi and Rørth, 2004), maintain adhesion (Niewiadomska et al., 1999; Pacquelet and Rørth, 2005; Cobreros-Reguera et al., 2010), and organize their polarity (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; McDonald et al., 2008). Yet, how border cells control the dynamic organization of the actomyosin cytoskeleton to drive cell locomotion is still not fully understood.

Determinants of cell polarity are required to polarize the border cell cytoskeleton to organize cluster architecture and promote collective migration (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; McDonald et al., 2008). Loss of polarity determinants delays migration and can cause the cluster...
can be regulated by determinants of cell polarity, such as Crumbs and aPKC, can respond to changes in the actin cytoskeleton, and can influence the level of F-actin in epithelial cells (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Fernández et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011).

However, the physiological roles for the Hippo pathway as a to disintegrate (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004). The polarity determinants Crumbs, Baz, and the aPKC–Par6 complex localize to membranes where border cells form contacts with one another (Niewiadomska et al., 1999; Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; McDonald et al., 2008). These determinants do not localize to regions of the membrane where border cells are actively migrating across their nurse cell substrate (Niewiadomska et al., 1999; Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; McDonald et al., 2008). Thus, by polarizing the cytoskeleton, polarity determinants promote cohesion between border cells and collective migration of the cluster as a whole. Reduced cytoskeletal dynamics at sites of contact between collectively migrating cells is also evident in several other contexts, including invasive human cancer cells, and may be related to the phenomenon of contact inhibition of cell migration in cell culture (Carmona-Fontaine et al., 2008; Hidalgo-Carcedo et al., 2011).

However, the molecular mechanisms by which border cell polarity determinants organize cluster architecture to promote migration remain unknown.

The Hippo pathway inhibits cell proliferation in growing epithelial tissues of both Drosophila and mammals (Grusche et al., 2010; Oh and Irvine, 2010; Pan, 2010; Badouel and McNeill, 2011; Halder and Johnson, 2011). Hippo signaling is also activated upon contact inhibition in cell culture, where it contributes to the repression of cell proliferation (Zhao et al., 2007; Kim et al., 2011). Recent work indicates that Hippo signaling can be regulated by determinants of cell polarity, such as Crumbs and aPKC, can respond to changes in the actin cytoskeleton, and can influence the level of F-actin in epithelial cells (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Fernández et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011).

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staining—accumulates around the outer rim of the migrating cluster (Fig. 1, A–F). Live imaging of Utrophin-GFP, which labels the actin cytoskeleton, GFP (green) to mark the mutant clones of cells induced with the MARCM technique, and DAPI (blue) to stain all nuclei. Insets show F-actin staining of clusters at high magnification. (C) Stage 10 egg chamber with a cluster (arrow) composed entirely of exAP50 mutant border cells (FRT/FLP, GFP negative). Inset shows F-actin accumulating inside the cluster. (D) Stage 10 egg chamber with a cluster (arrow) composed entirely of exAP50 kib32 double-mutant border cells (FRT/FLP, GFP negative). Inset shows the cluster architecture completely fails to form. (E) Stage 10 migration index for quantitation of border cell migration. (F) Quantification of the stage 10 migration index for the following genotypes: control (n > 100), kib32 (n = 36), exAP50 (n = 34), and exAP50 kib32 (n = 8). (G and H) Stage 9 (G) and stage 10 (H) egg chambers with clusters (arrows) composed entirely of hpo42-47 mutant border cells (GFP positive). (I and J) Stage 9 (I) and stage 10 (J) egg chambers with clusters (arrows) composed entirely of wtsX1 mutant border cells (GFP positive). hpo42-47 and wtsX1 mutant border cell clusters both show delayed migration and F-actin polarization defects (insets). The hpo42-47 and hpo42-47 alleles display the same border cell migration delay phenotype. (K) Quantification of the stage 10 migration index of the following genotypes: control (n > 100), hpo42-47 (n = 94), and wtsX1 (n = 93). In each case, only clusters in which all border cells were mutant for a given allele (GFP positive) were analyzed; n, number of egg chambers examined. (L) Quantification of average F-actin and P-MyoII staining intensity levels at the outer rim versus inner membranes in control and wts mutant clusters (WT clusters, n = 5; wts clusters n = 12). p-MyoII staining in control and wts mutant clusters is also shown. Bars, 50 µm (5 µm for insets).

Figure 3. The Hippo pathway is required to polarize actin and promote migration. (A and B) Confocal micrograph of a control egg chamber at stage 9 (A) or 10 (B) labeled with phalloidin (red) to visualize the actin cytoskeleton, GFP (green) to mark the mutant clones of cells induced with the MARCM technique, and DAPI (blue) to stain all nuclei. Insets show F-actin staining of clusters at high magnification. (C) Stage 10 egg chamber with a cluster (arrow) composed entirely of exAP50 mutant border cells (FRT/FLP, GFP negative). Inset shows F-actin accumulating inside the cluster. (D) Stage 10 egg chamber with a cluster (arrow) composed entirely of exAP50 kib32 double-mutant border cells (FRT/FLP, GFP negative). Inset shows the cluster architecture completely fails to form. (E) Stage 10 migration index for quantitation of border cell migration. (F) Quantification of the stage 10 migration index for the following genotypes: control (n > 100), kib32 (n = 36), exAP50 (n = 34), and exAP50 kib32 (n = 8). (G and H) Stage 9 (G) and stage 10 (H) egg chambers with clusters (arrows) composed entirely of hpo42-47 mutant border cells (GFP positive). (I and J) Stage 9 (I) and stage 10 (J) egg chambers with clusters (arrows) composed entirely of wtsX1 mutant border cells (GFP positive). hpo42-47 and wtsX1 mutant border cell clusters both show delayed migration and F-actin polarization defects (insets). The hpo42-47 and hpo42-47 alleles display the same border cell migration delay phenotype. (K) Quantification of the stage 10 migration index of the following genotypes: control (n > 100), hpo42-47 (n = 94), and wtsX1 (n = 93). In each case, only clusters in which all border cells were mutant for a given allele (GFP positive) were analyzed; n, number of egg chambers examined. (L) Quantification of average F-actin and P-MyoII staining intensity levels at the outer rim versus inner membranes in control and wts mutant clusters (WT clusters, n = 5; wts clusters n = 12). p-MyoII staining in control and wts mutant clusters is also shown. Bars, 50 µm (5 µm for insets).

Results

Polarization of Hippo pathway components and the actin cytoskeleton in migrating border cell clusters

We began by investigating the actin cytoskeleton during border cell migration. We find that F-actin—detected by phalloidin staining—accumulates around the outer rim of the migrating cluster (Fig. 1, A–F). Live imaging of Utrophin-GFP, which labels the actin cytoskeleton, confirms that actin filaments concentrate and are most dynamic around the outer rim of the cluster (Video 1). Unlike F-actin, the key upstream components of the Hippo pathway Kibra (Kib), Expanded (Ex), and Merlin (Mer) (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010), as well as the recently identified component Zyxin (Rauskolb et al., 2011), localize with the polarity determinants aPKC and Crb to membranes inside the border cell cluster at sites of border cell–border cell contact (Fig. 2, A–G). Note that the bulk Hippo (Hpo) and Warts (Wts) proteins are not localized specifically to any region of the cell, but are well known to be active only in the presence of the upstream components, with which they physically interact;
thus, Hpo and Wts are likely to be most highly active at membranes inside the border cell cluster (Fig. 2, H–J; Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010; Boggiano and Fehon, 2012; Tepass, 2012; Deng et al., 2013). Because Hippo signaling is regulated by determinants of cell polarity in the context of imaginal disc growth control (Chen et al., 2010; Grzeschik et al., 2010; Ling et al., 2010; Fernández et al., 2011; Sansores-Garcia et al., 2011; Wada et al., 2011), our results suggest that Hippo pathway components are ideally positioned to act as effectors of cell polarity determinants to polarize the actin cytoskeleton in migrating border cell clusters (Fig. 2, I and J).

**The Hippo pathway is required to polarize the actin cytoskeleton and promote migration in border cells**

We next tested the requirement for Hippo signaling in border cells. In imaginal disc epithelia, the upstream Hippo pathway components Kib, Ex, and Mer are partially redundant in that they each tend to have weaker loss-of-function phenotypes than hpo or wts mutants, whereas ex, kib or ex, mer double mutants cause very strong phenotypes (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Wild-type border cell clusters normally reach the oocyte by stage 10 of oogenesis, whereas inactivation of ex or kib individually delays border cell migration and the double-mutant combination ex, kib causes very strongly delayed migration, with clusters rarely even initiating migration (Fig. 3, A–F). Polarization of F-actin is abnormal in ex mutant clusters and formation of clusters is completely prevented in ex, kib double mutants (Fig. 3, C and D). These results show that upstream Hippo pathway components are essential for organizing the architecture and motility of border cell clusters.

In epithelia, Kib, Ex, and Mer are known to function by activating the Hpo and Wts kinases at the apical membrane (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010), but can also act independently of Hpo and Wts to help polarize apical determinants (Fletcher et al., 2012). To identify the specific role of signaling through Hpo and Wts in border cell migration, we examined hpo and wts mutant clusters. Approximately 60% of hpo and wts mutant border cell clusters are delayed at stage 10 of oogenesis (Fig. 3, G–K). Unlike control clusters, F-actin fails to polarize to the outer rim of hpo and wts mutant clusters and instead tends to accumulate throughout the cluster (Fig. 3, A–L). Similar results were obtained for phosphorylated myosin II (Fig. 3 L). Live imaging reveals that wts mutant clusters, or clusters expressing RNAi against the Wts cofactor Mats, tend to tumble rather than move directionally and sometimes disintegrate (Fig. S1; Videos 2–5). These results show that signaling through Hpo and Wts is essential to polarize the actin cytoskeleton and promote collective migration in border cells.

To rule out an indirect effect of Wts on border cell migration via misregulation of border cell specification, we tested the effect of wts mutants on markers of border cell fate. We find that expression of slbo.lacZ and upd.lacZ is not affected in wts mutants (Fig. 4, A–D). These results show that border cell specification was not affected by inactivation of Hpo or Wts and support the notion that the Hippo pathway acts directly at the cell cortex to control cluster architecture and motility.

We also sought to rule out the possibility that Wts might regulate polarization of polarity determinants or adherens
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Phosphorylation of Yki by Wts does not mediate the effect of Hippo signaling in polarizing the actin cytoskeleton

In many tissues, Hippo signal transduction proceeds by the Wts kinase phosphorylating and inhibiting the transcriptional coactivator Yorkie (Yki; YAP/TAZ in mammals; Huang et al., 2005; Dong et al., 2007). In Drosophila, most known phenotypes of hpo and wts mutants can be phenocopied by ectopic expression of Yki (Huang et al., 2005; Shaw et al., 2010; Staley and Irvine, 2010). We therefore expected ectopic expression of Yki to inhibit border cell migration. In contrast, we find that expression of wild-type Yki or a constitutively active form of Yki lacking the major Wts phosphorylation site (YkiS168A) does not inhibit border cell migration and instead accelerates it (Fig. 5, A–J). This surprising result indicates that Hpo and Wts act directly to promote border cell migration, rather than by signaling through Yki to the nucleus, and that repression of Yki by the Hippo pathway provides negative feedback to limit migration.

The Hippo pathway regulates the activity of the Ena/Capping protein system to organize cluster architecture and motility

To explore how the Hippo pathway regulates the actomyosin cytoskeleton, we considered the role of the actin regulator Enabled (Ena; VASP in mammals). At the leading edge of migrating cells in culture, Ena/VASP proteins are known to drive actin polymerization and cortical protrusions by inhibiting the activity of F-actin Capping proteins, which normally limit actin polymerization (Bear and Gertler, 2009). Several lines of evidence suggest potential links between the Hippo pathway and Ena. First, at focal adhesions in cultured cells, Zyxin binds to Ena/VASP proteins and modulates their activity to produce a stable cortex. Second, loss of Capping proteins α and β (Cpa and Cpb) has been shown to induce Hippo signaling in the context of growth control (Fernández et al., 2011; Sansores-Garcia et al., 2011). Third, Ena and Capping proteins have been implicated as regulators of border cell migration (Gates et al., 2009). Fourth, we identify a conserved Wts consensus phosphorylation site in Ena that is highly similar to the site in Yki whose phosphorylation inhibits Yki (Fig. 6 A). We find that this site in Ena can be directly phosphorylated by Wts in vitro, similar to the site in Yki (Fig. 6 B). These results suggest that Hippo signaling may act by phosphorylating and inactivating Ena to polarize the actin cytoskeleton.
clusters revealed a tumbling motion highly reminiscent of \textit{wts} mutant clusters (compare Video 5 with Videos 2–4). These results indicate that \textit{Ena} is a key target of Hippo pathway in polarizing the actin cytoskeleton during border cell migration.

\textit{Ena} is thought to antagonize the action of Capping proteins, which compete with \textit{Ena} for binding to F-actin barbed ends (Bear and Gertler, 2009). \textit{Ena} promotes F-actin polymerization, whereas Capping proteins inhibit polymerization. In border cells, mutation of \textit{cpb} caused clusters to accumulate F-actin inside the cluster and to exhibit delayed migration at stage 9 and 10 (Fig. 7, A–D). Around 10\% of \textit{cpb} mutant clusters disintegrated, highly similar to \textit{wts} or \textit{hpo} mutants (Fig. S1; Video 3 and Video 6). Finally, overexpression of \textit{Cpb} was able to fully rescue the migration defect and F-actin polarization defects of \textit{wts} mutant border cell clusters (Fig. 7, E–G; Video 7). These results indicate that Hippo signaling promotes border cell migration by inhibiting \textit{Ena} and thus promoting \textit{Cpb} activity inside the cluster to help restrict F-actin to the outer rim of migrating clusters.

\textbf{Discussion}

Our results show that the Hippo pathway provides a mechanism linking determinants of cell polarity with polarization of the actin cytoskeleton—a mechanism that is responsible for organizing the architecture and motility of collectively migrating border cell clusters. Collective migration depends on actomyosin polymerizing and contracting around the outer rim of the cluster, where border cells migrate over their nurse cell substrates, but not in the center of the cluster, where polarity determinants...
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localize to sites of contact between border cells. Our results show that upstream components of the Hippo pathway—Kib, Ex, and Mer—are recruited to border cell contacts and signal through Hpo and Wts to polarize the actin cytoskeleton. Double mutants for ex, kib have an even stronger phenotype than loss of hpo or wts, demonstrating that these upstream components have an additional role aside from activating Hippo signaling that is likely to involve directly assisting polarization of polarity determinants (Fletcher et al., 2012). Nevertheless, the upstream components also signal via Hpo and Wts to polarize the actin cytoskeleton and promote migration.

Our results indicate that Wts acts by regulating the Ena/Capping protein system, which is one system that cells use to control polymerization of actin (see model in Fig. 8). Loss of Wts results in excessive F-actin polymerization inside the cluster. Loss of Capping protein has the same effect, as does overexpression of the Capping protein inhibitor Ena. Ena contains a conserved Wts phosphorylation site located at the start of the proline-rich region (PRR) domain, which mediates binding to Profilin, so phosphorylation might disturb this binding interaction and thus inhibit Ena function. Our results support the notion that Ena is inactivated upon Hippo signaling, so that Capping protein can be active and thereby repress actin polymerization on inner membranes. Hence, in hpo or wts mutants, ectopic Ena activation inhibits Capping protein activity and leads to ectopic F-actin polymerization inside the cluster. In support of this view, hpo or wts mutants can be rescued by loss of Ena or overexpression of Capping protein, respectively. Notably, the rescued clusters show normal polarization of F-actin and can migrate normally, indicating that mechanisms other than polarization of Ena activity must also exist to help polarize the actin cytoskeleton in border cells, consistent with the fact that aside from Ena there are many other regulators of F-actin polymerization. Nevertheless, Hpo–Wts signaling is clearly one important mechanism of F-actin polarization for border cells because its disruption leads to the majority of border cell clusters migrating slowly in a tumbling fashion or even disintegrating.

Our results show that the role of the Hippo pathway in restraining F-actin polymerization at inner membranes is a direct one that is not mediated by the nuclear signaling effector Yki. Instead, our results indicate that repression of Yki by Wts functions solely as a negative feedback loop that is important to limit the speed of migration. Previous work has shown that excessive F-actin levels can cause a loss of Hippo pathway activity, which activates Yki, inducing expression of several key upstream components of the Hippo pathway to bolster pathway activity at the cortex (Fernández et al., 2011; Sansores-Garcia et al., 2011). In the context of border cell migration, such a negative feedback loop mechanism may be important for homeostatic control of F-actin polymerization. Excessive F-actin levels might therefore be expected to feedback to restrain F-actin polymerization via the Yki-mediated negative feedback loop. This phenomenon may explain the unusual behavior of Ena-overexpressing clusters, which strongly up-regulate F-actin and delay migration at stage 9,
but always recover to reach the oocyte by stage 10. In contrast, border cells never recover from loss of Capping protein, which, unlike overexpressed Ena, cannot be ameliorated by Wts phosphorylation. Hence, our results provide a physiological context for understanding the role of Yki as a negative feedback regulator of Hippo signaling.

In conclusion, our findings establish a novel role for Hippo signaling in collective migration and provide a novel mechanism for polarization of the actin cytoskeleton. Our results suggest that examination of the role of the Hippo pathway in human cancer should consider not only its potential to regulate cell proliferation and survival, but also its potential to regulate cell polarity, the actomyosin cytoskeleton, and collective cell invasion.

Materials and methods

Drosophila stocks and genetics
Flies were raised and crossed at 25°C according to standard procedures. w or yw flies were used as the wild-type stock. The FLP/FRT site-specific recombination system was used to generate mutant clones with a heat-shock promoter (Xu and Rubin, 1993; Lee and Luo, 1999). Flies of the following genotypes were generated: yw hsFLP UAS-nucGFPmyc; FRT42D hpo42-47/FRT42D tubGal80; tubGal4/+ (Wu et al., 2003); yw hsFLP UAS-nucGFPmyc; FRT42D hpoJM1/FRT42D tubGal80; tubGal4/+ (Jia et al., 2003); yw hsFLP; tubGal4, UAS-nucGFPmyc; FRT82B wtsX1/FRT82B tubGal80 (Xu et al., 1995); yw hsFLP, tubGal4, UAS-nucGFPmyc; FRT82B kib32/FRT82B tubGal80 (Genevet et al., 2010); yw hsFLP; ex AP50 FRT40A/FRT40A ubi-GFP (Hamaratoglu et al., 2006); yw hsFLP; exAP50 FRT40A/FRT40A ubi-GFP; FRT82B kib32/FRT82B ubi-GFP; FRT82B cpa1/FRT82B tubGal80; ubi-GFP; FRT82B ubi-GFP; FRT82B tubGal4, UAS-nucGFPmyc; FRT82B cpa1/FRT82B tubGal80; FRT82B ubi-GFP; FRT82B tubGal80; FRT82B ubi-GFP; yw hsFLP UAS-nucGFPmyc; FRT82D hpo42-47, ena210/FRT42D tubGal80; tubGal4/+.

To obtain mutant border cell clones, 1–3-d-old female progeny was heat-shocked at 37°C for 1 h, twice a day; during 3 d and ovaries were dissected 4–6 d after heat-shock.

The “Flip-out” actin.FRT.CD2.FRT.Gal4/UAS system (Pignoni and Zipursky, 1997) was used to express the following UAS.Yki constructs: UAS.Yki [Huang et al., 2005], UAS.Yki182AGFP, and UAS.Yki168AGFP (Oh and Irvine, 2008), as well as UAS.Yki182AV5, UAS.Yki168AV5, and UAS.Yki111A5164A2250AV5 (Oh and Irvine, 2009). UAS.Ena (Bloomington) was also expressed with the Flip-out Gal4 driver: UAS.EnaS187A and
UAS.YkiS168AV5 transgenes were generated in the course of this work. To express the transgenes, newly eclosed females were heat-shocked at 37°C for 15 min and ovaries were dissected 2 d after heat-shock.

The UAS.Kib (Genevet et al., 2010), UAS.Me, and UAS.Ex (Udan et al., 2003) transgenes were expressed in border cells using the sibo Gal4 driver (Rarth et al., 1998). UAS.mycWts, UAS.ZyxinV5 (Rauskolb et al., 2011), and UAS.HA-Cpa (Fernández et al., 2011) have been previously described and were expressed with the c306 Gal4 driver (Bloomington).

Additional information on Drosophila mutations and transgenes
MARC M stock. The Mosaic Analysis with a Repressible Cell Marker (MARC M) system is used to positively mark homozygous mutant clones by UAS-promoter-driven expression of a full-length GFP protein featuring an N-terminal nuclear localization sequence and a C-terminal myc epitope tag (UAS.nucGFPmyc transgene; gift of G. Struhl, Columbia University, New York, NY). The system is based on a constitutive tubulin-driven Gal4 transgene that is repressed by a constitutive tubulin Gal80. Heat-shock FLP recombinase mediates mitotic recombination at FLP recombinase target (FTT) sites on a chromosome arm that generates a clone of cells homozygous mutant for the gene of interest that also lacks the tubulin-driven Gal80 transgene, hence leading to tubulin driving expression of Gal4 and thus of the UAS.nucGFPmyc transgene and any other UAS transgene present. A list of mutants and transgenes used in MARCM experiments and other experiments in this study with their Flybase ID follows: yellow (y); Fbal0018186; hsFLP, Fbi0000785; FRT40A, Fbi0002071; FRT420, Fbi0141188; FRT808, Fbi0002073; FRT282, Fbi0002074; tubGal80, Fbt0026260; tubGal80, Fbt0026561; UAS.nucGFPmyc (a gift of G. Struhl; not yet described in Flybase); Ato, Fbi0151857; Ato, Fbi0151851; wts, Fbi0044527; ex250, Fbi00127810; kib, Fbt00249465; Fbt00120783; c306, Fbi0039353.

Immunofluorescence microscopy
Ovary dissection was performed in PBS, followed by fixation in 4% PFA for 20 min. After fixation, ovaries were rinsed with PBT (1× PBS and 0.1% Triton X-100, and 5% goat serum) for at least 30 min. Ovaries were blocked for 1 h in PBT with 5% BSA, and then incubated with the primary antibody or antibodies of interest. Ovaries were further stained with DAPI (Sigma-Aldrich) in 1:250 PBT and 5% goat serum, and stained with secondary antibodies. Ovaries were mounted on glass slides in Mowiol 4-88 (Calbiochem) and viewed with a Zeiss LSM710 confocal microscope (Carl Zeiss) using 40× or 63× oil immersion objectives at room temperature. Images were acquired on a confocal microscope (LSM710; Carl Zeiss) using 40× or 63× oil immersion objectives at room temperature and the videos were processed into a montage using MetaMorph software (Molecular Devices).

In vitro kinase assay
Peptides used in this study (BR peptide synthesis) were: Yki S168 HSRLAHSLHSSRPASPLQNY (molecular weight 2,516.8 D); Yki S168A HSRLAHSLHSSRPASPLQNY (molecular weight 2,500.8 D); Ena S187 SPPTPGGQRHTSSAPPAQGQQ (molecular weight 2,431.6 D); Ena S187A SPPTPGGQRHTSSAPPAQGQQ (molecular weight 2,415.6 D). HPLC purified peptide substrates were diluted with deionised water to working dilutions (1 mg/ml) and stored at −20°C. The activity of recombinant Lats1 kinase (SigmaChem) was measured in a kinase assay with 8,000 ng of peptide [Yki S168, Yki S168A, Ena S187, Ena S187A] and 350 ng of Lat1 kinase diluted in kinase dilution buffer III (SigmaChem). The kinase reaction mixture consisted of 2 μl of kinase assay buffer I (SigmaChem), 10 μl of ATP cocktail (9.4 μl of kinase dilution buffer III, 10 μM cold ATP, and 3 μCi of [γ-32P]ATP [PerkinElmer]). The kinase assay was incubated for 30 min at 30°C. After incubation, 100 μl of PBT, 30% PFA and phosphoellose squares (EMD Millipore) and washed 3x in 1.0% phosphoric acid and then in aceton. Incorporation of [γ-32P]ATP was quantified in counts per minute (cpm) by liquid scintillation (LS 6500 counter; Beckman Coulter). Relative cpm was determined by dividing the absolute cpm by the cpm in the control sample lacking substrate.

Online supplemental material
Fig S1 shows quantification of disintegration defects in wts mutant clusters. Video 1. Shows polarization of F-actin visualized with Utrophin-GFP in border cells. Video 2 shows tumbling migration of wts1+ or mats-R clusters versus a control. Video 3 shows disintegration of wts1+ or mats-R clusters versus a control. Video 4 shows failure of detachment of wts1+ or mats-R clusters versus a control. Video 5 shows tumbling migration of a UAS-ena cluster versus a control. Video 6 shows disintegration of a cpbM143 cluster versus a control. Video7 shows rescue of wts1+ mutant migration by expression of Cbp. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201210073/DC1. Additional data are available in the JCB DataViewer at http://dx.doi.org/10.1083/jcb.201210073.dv.

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