

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

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Collective migration of *Drosophila* border cells depends on a dynamic actin cytoskeleton that is highly polarized such that it concentrates around the outer rim of the migrating cluster of cells. How the actin cytoskeleton becomes polarized in these cells to enable collective movement remains unknown. Here we show that the Hippo signaling pathway links determinants of cell polarity to polarization of the actin cytoskeleton in border cells. Upstream Hippo pathway components localize to contacts between border cells inside the cluster and

signal through the Hippo and Warts kinases to polarize actin and promote border cell migration. Phosphorylation of the transcriptional coactivator Yorkie (Yki)/YAP by Warts does not mediate the function of this pathway in promoting border cell migration, but rather provides negative feedback to limit the speed of migration. Instead, Warts phosphorylates and inhibits the actin regulator Ena to activate F-actin Capping protein activity on inner membranes and thereby restricts F-actin polymerization mainly to the outer rim of the migrating cluster.

## 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.

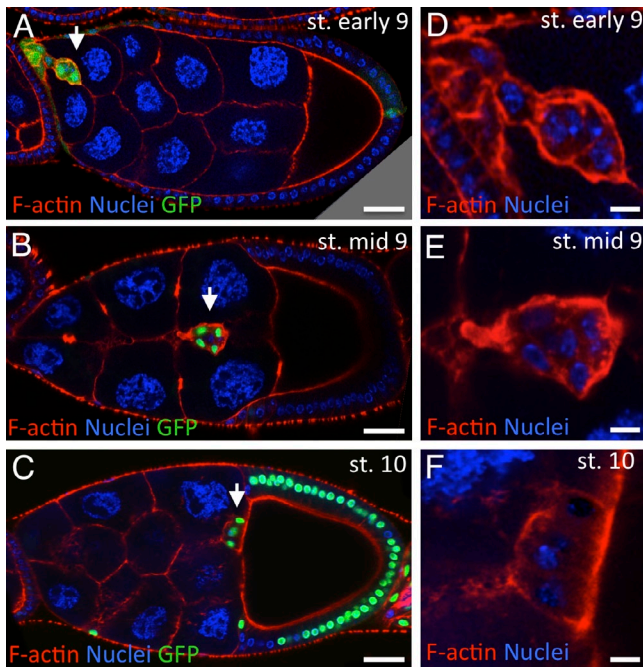
Determinants of cell polarity are required to polarize the border cell cytoskeleton to organize cluster architecture and promote collective migration (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; McDonald et al., 2008). Loss of polarity determinants delays migration and can cause the cluster

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Abbreviations used in this paper: Ena, Enabled; Ex, Expanded; Hpo, Hippo; Kib, Kibra; Mer, Merlin; Wts, Warts; Yki, Yorkie.

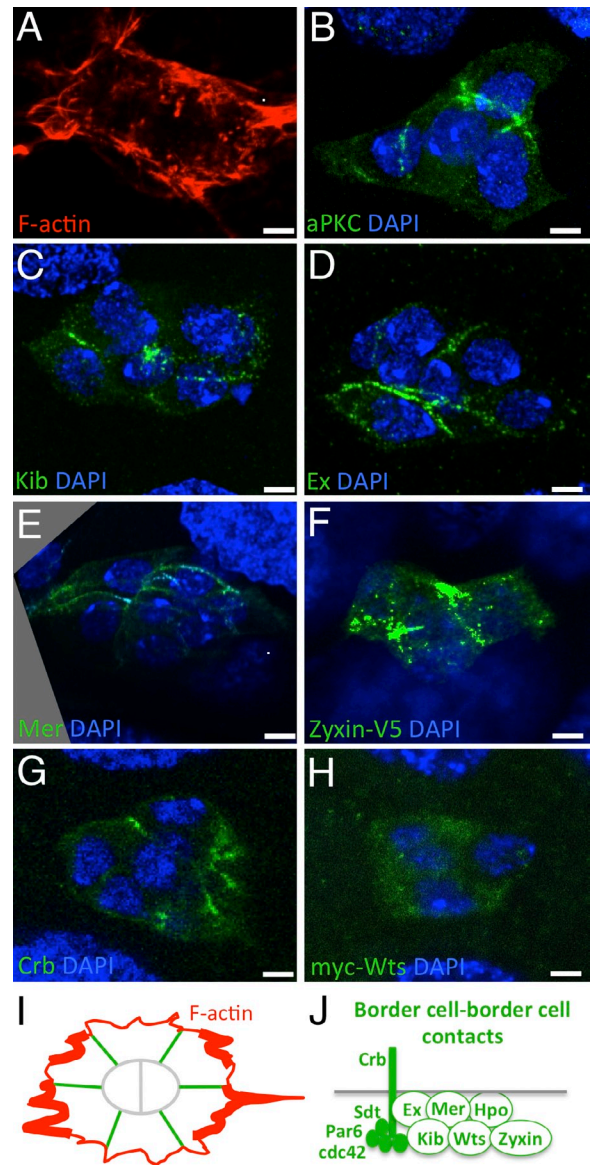
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**Figure 1. Polarization of the actin cytoskeleton to the outer rim of migrating border cell clusters.** (A–C) Border cell clusters visualized with phalloidin (red) and DAPI (blue) form from a small group of anterior follicle cells that invade the germ line nurse cells at early stage 9 (A), migrate through the egg chamber during stage 9 (B), and reach the oocyte by stage 10 (C). MARCM clones expressing GFP-labeled border cells as well as some follicle cells. (D–F) High magnification views of phalloidin (red) and DAPI (blue) staining in migrating border cell clusters at the indicated stages. Note that F-actin accumulates strongly around the outer rim of the cluster and less so in internal membranes. Bars: (A–C) 50  $\mu$ m; (D–F) 5  $\mu$ m.

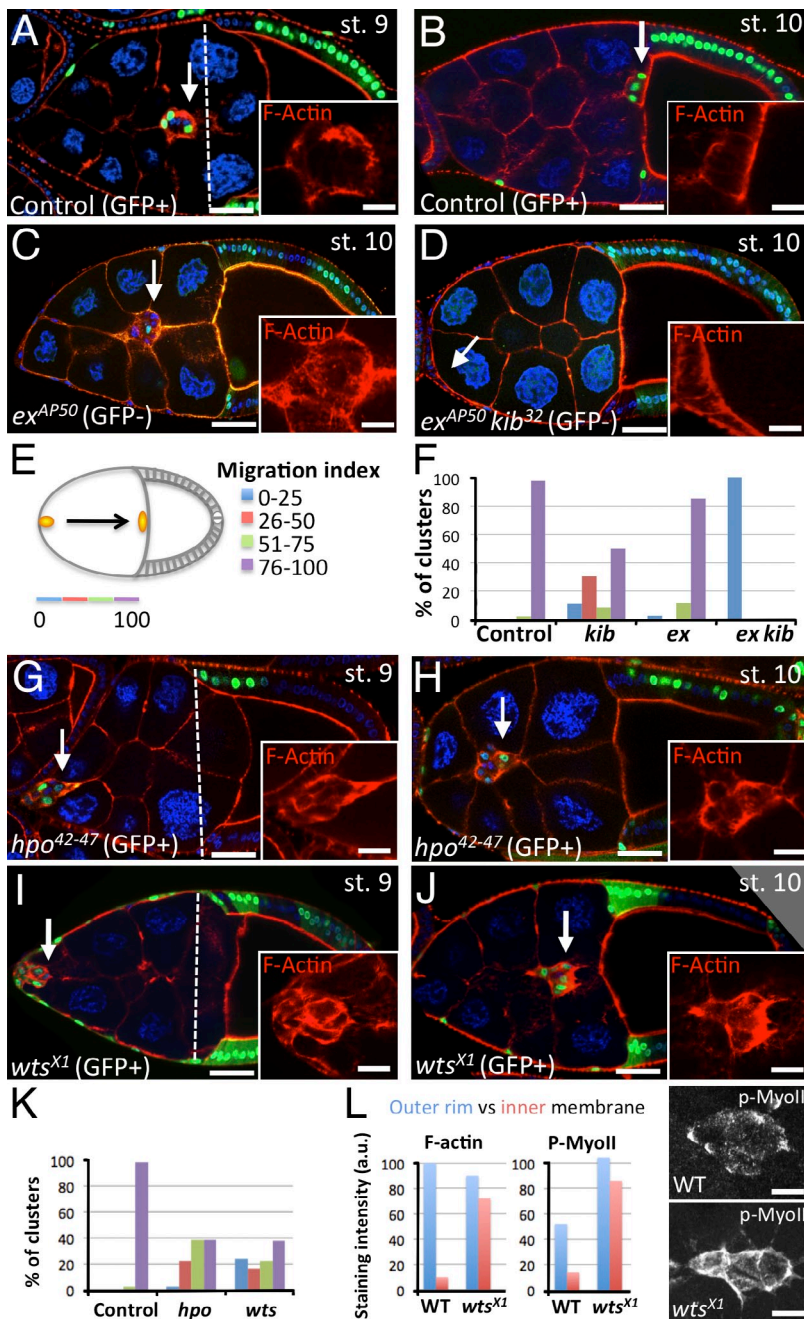
to disintegrate (Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004). The polarity determinants Crumbs, Baz, and the aPKC–Par6 complex localize to membranes where border cells form contacts with one another (Niewiadomska et al., 1999; Abdelilah-Seyfried et al., 2003; Pinheiro and Montell, 2004; 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



**Figure 2. Upstream Hippo pathway components localize to border cell-border cell contacts inside migrating clusters.** (A) Migrating cluster at stage 9 labeled for F-actin with phalloidin staining, which localizes to the outer rim of the cluster. (B) Migrating cluster at stage 9 stained for aPKC. (C) Migrating cluster at stage 9 stained for Kib. (D) Migrating cluster at stage 9 stained for Ex. (E) Migrating cluster at stage 9 stained for Mer. (F) Migrating cluster at stage 9 stained for V5-tagged Zyxin. (G) Migrating cluster at stage 9 stained for Crb. (H) Migrating cluster at stage 9 stained for myc-Wts. Note that Crb, aPKC, and upstream Hippo pathway components localize to membranes inside the cluster, whereas Wts is cytoplasmic. *slbo-GAL4* was used to express *UAS-Ex* (D), *UAS-Mer* (E), *UAS.Zyxin-V5* (F), or *UAS.myc-Wts* (H). (I) Schematic diagram of F-actin polarization in a migrating border cell cluster. (J) Schematic diagram of Hippo pathway components localizing to the sites of contact between border cells (i.e., inner membranes). The active, phosphorylated forms of Hpo and Wts are thought to be localized in a complex with upstream Hippo pathway components. Bars, 5  $\mu$ m.

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



**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  $ex^{AP50}$  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  $ex^{AP50} kib^{32}$  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 ( $n > 100$ ),  $kib^{32}$  ( $n = 36$ ),  $ex^{AP50}$  ( $n = 34$ ), and  $ex^{AP50} kib^{32}$  ( $n = 8$ ). (G and H) Stage 9 (G) and stage 10 (H) egg chambers with clusters (arrows) composed entirely of  $hpo^{42-47}$  mutant border cells (GFP positive). (I and J) Stage 9 (I) and stage 10 (J) egg chambers with clusters (arrows) composed entirely of  $wts^{X1}$  mutant border cells (GFP positive).  $hpo^{42-47}$  and  $wts^{X1}$  mutant border cell clusters both show delayed migration and F-actin polarization defects (insets). The  $hpo^{42-47}$  and  $hpo^{M1}$  alleles display the same border cell migration delay phenotype. (K) Quantification of the stage 10 migration index of the following genotypes: control ( $n > 100$ ),  $hpo^{M1}$  ( $n = 94$ ), and  $wts^{X1}$  ( $n = 93$ ). 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  $\mu m$  (5  $\mu m$  for insets).

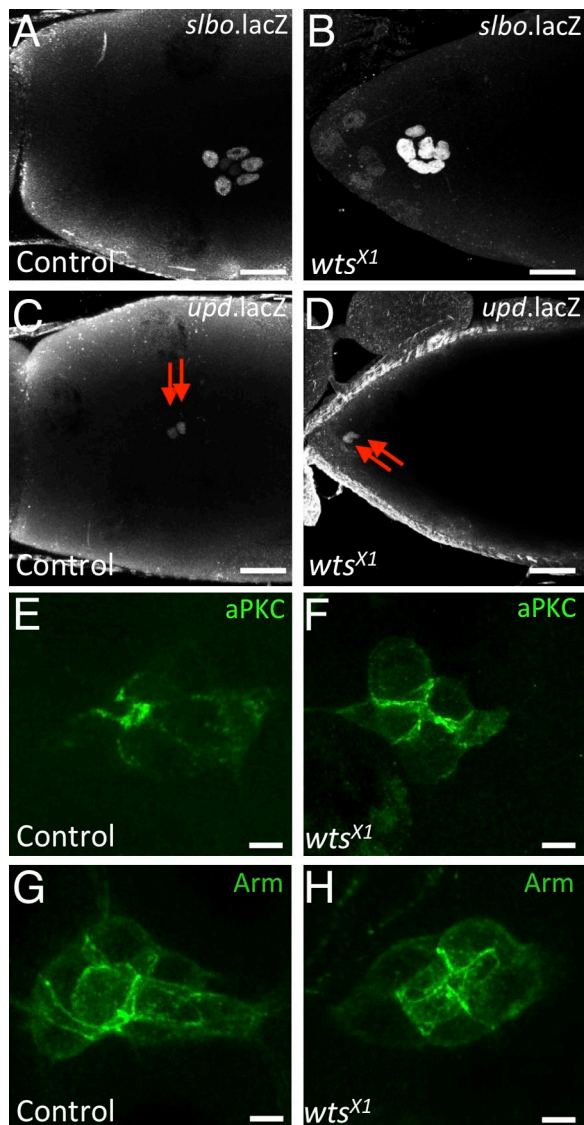
sensor and regulator of cell polarity and F-actin remain unclear, as are the mechanisms by which the Hippo pathway can execute these functions. Because cell polarity and the actin cytoskeleton are of fundamental importance to collective cell migration, yet the role of the Hippo pathway in collective migration has not been explored in any detail, we examined the role of the Hippo pathway in border cells.

## 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;



**Figure 4. Wts is not required for border cell specification or membrane polarity.** (A) Control egg chamber at mid stage 9 showing the *slow border cells* expression in the migrating border cell cluster, as revealed by the *slbo-lacZ* reporter. (B) Stage 10 egg chambers with clusters composed entirely of *wts<sup>X1</sup>* mutant border cells (MARCM, GFP positive, not depicted) display normal *slbo-lacZ* expression. (C) Control egg chamber at mid stage 9 showing the *unpaired* expression in the migrating border cell cluster, as revealed by the *upd-lacZ* reporter. (D) Stage 10 egg chambers with clusters composed entirely of *wts<sup>X1</sup>* mutant border cells (MARCM, GFP positive, not depicted). In both wild-type and mutant clusters, the *upd* expression is restricted to the two polar cells (red arrows). In addition, the expression of the polar cell-specific marker FasIII was unaffected in *wts<sup>X1</sup>* mutant clusters (not depicted), indicating that Wts is not required to specify border cells or to discriminate between polar cells and outer border cells. Anterior is to the left in all panels. (E) Control cluster at mid stage 9 showing normal aPKC localization to inner membranes. (F) *wts<sup>X1</sup>* mutant border cell clusters show normal localization of aPKC. (G) Control cluster at mid stage 9 showing normal Armadillo localization to inner membranes. (H) *wts<sup>X1</sup>* mutant border cell clusters show normal localization of Armadillo. Bars: (A–D) 50  $\mu$ m; (E–H) 5  $\mu$ m.

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; Sudol and Harvey, 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).

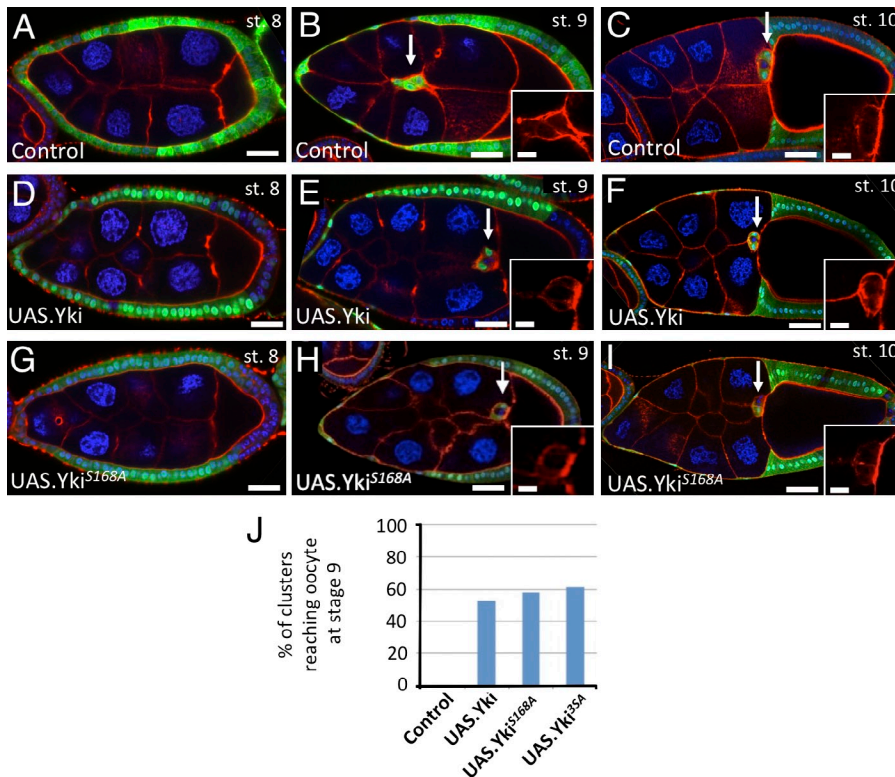
#### The Hippo pathway is required to polarize the actin cytoskeleton and promote migration in border cells

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



**Figure 5. Repression of Yki does not mediate the function of Wts but rather provides negative feedback.** (A–C) Control egg chambers at stage 8 (A), 9 (B), or 10 (C) labeled with phalloidin (red) to visualize the actin cytoskeleton, GFP (green) to mark the clones of cells generated using *actin flipout GAL4*, and DAPI (blue) to stain all nuclei. (D–I) Egg chambers with border cell clusters (arrows) that overexpress wild-type UAS-Yki at stage 8 (D), 9 (E), or 10 (F), or constitutively active UAS-Yki<sup>S168A</sup> at stage 8 (G), 9 (H), or 10 (I) (all clones GFP positive). In all these Yki-expressing genotypes, border cell migration is accelerated and all the clusters migrate posteriorly toward the oocyte prematurely. (J) Quantification of the percentage of stage 9 border cell clusters that prematurely reach the oocyte ( $n > 20$  for each genotype). Anterior is to the left in all panels. Insets show F-actin staining in border cell clusters. Bars, 50  $\mu\text{m}$  (5  $\mu\text{m}$  for insets).

junctions. We find that the polarity determinant aPKC is normally localized in *wts* mutant border cell clusters, as is the adherens junctions protein Armadillo/ $\beta$ -catenin (Fig. 4, E–H).

#### 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 (Yki<sup>S168A</sup>) 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.

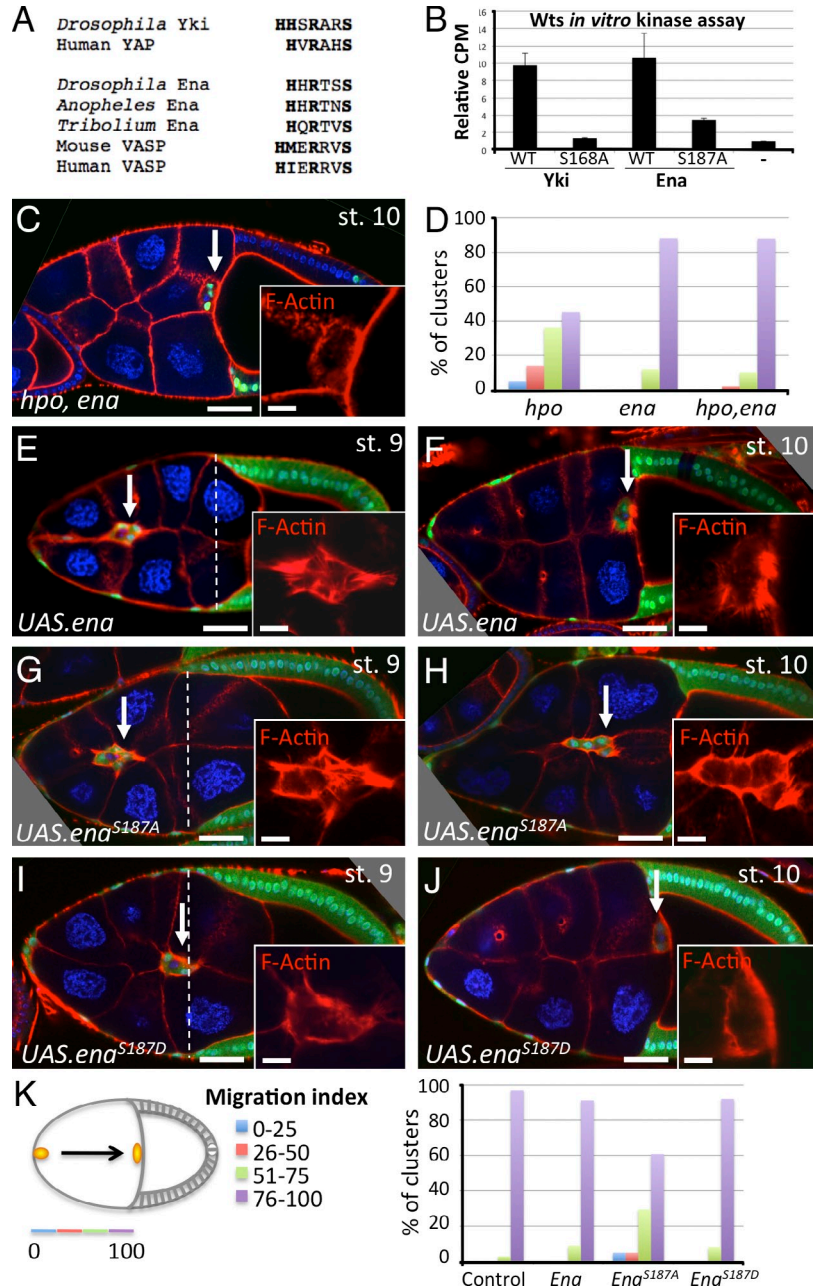
#### The Hippo pathway regulates the activity of the Ena/Capping protein system to organize cluster architecture and motility

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  $\alpha$  and  $\beta$  (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.

If Hippo signaling acts by inhibiting Ena, then the phenotype of *hpo* mutant border cell clusters should be caused by excessive Ena activity and rescued in *hpo ena* double mutants. Accordingly, we find that *hpo ena* double-mutant clusters migrate normally and exhibit a normally polarized actin cytoskeleton (Fig. 6, C and D). Furthermore, overexpression of Ena is sufficient to mimic a mild Hippo pathway loss-of-function phenotype, with F-actin accumulating throughout the Ena-expressing clusters and delayed migration during stage 9 (Fig. 6 E). However, Ena-expressing clusters recover and are not delayed by stage 10 (Fig. 6 F). Expression of phospho-mutant EnaS187A has a stronger effect, with clusters delayed at both stage 9 and 10, whereas expression of an Ena S187D phosphomimic mutant does not delay migration (Fig. 6, G–K). Live imaging of Ena-expressing

**Figure 6. Wts phosphorylates Ena and represses its activity to polarize F-actin and promote border cell migration.** (A) Conserved Wts phosphorylation sites in Yki/YAP and Ena/VASP proteins from different species. (B) Recombinant Wts kinase can directly phosphorylate Ena on its conserved Wts site *in vitro*. (C) *hpo*<sup>42,47</sup>, *ena*<sup>210</sup> double mutant clusters migrate almost normally. (D) Quantification of *hpo*<sup>42,47</sup> single mutant (*n* = 62), *hpo*<sup>42,47</sup>, *ena*<sup>210</sup> double mutant (*n* = 50), and *ena*<sup>210</sup> single mutant (*n* = 35) cluster migration at stage 10. The double mutant clusters migrate as well as the *ena*<sup>210</sup> single mutant and do not show the strong delays observed in a *hpo*<sup>42,47</sup> mutant (see Fig 1). (E) Overexpression of Ena causes delayed border cell migration during stage 9, with prominent accumulation of F-actin throughout the border cell cluster (inset). (F) Ena-expressing clusters recover to achieve normal migration at stage 10. Inset shows increased levels of F-actin, but the cytoskeleton is still polarized. (G) Overexpression of EnaS187A phosphomutant causes delayed migration at stage 9 with F-actin accumulation throughout the cluster. (H) EnaS187A-expressing clusters can still be delayed at stage 10. (I) Overexpression of EnaS187D phosphomimic does not delay migration or depolarize F-actin at stage 9. (J) Overexpression of EnaS187D phosphomimic does not delay migration or depolarize F-actin at stage 10. (K) Quantification of migration delay at stage 10 for control (*n* = 69), UAS.Ena (*n* = 80), UAS.EnaS187A (*n* = 102), and UAS.EnaS187D (*n* = 63). Note all three transgenes are inserted in the same second-chromosomal attP landing site. Bars, 50  $\mu$ m (5  $\mu$ m for insets).



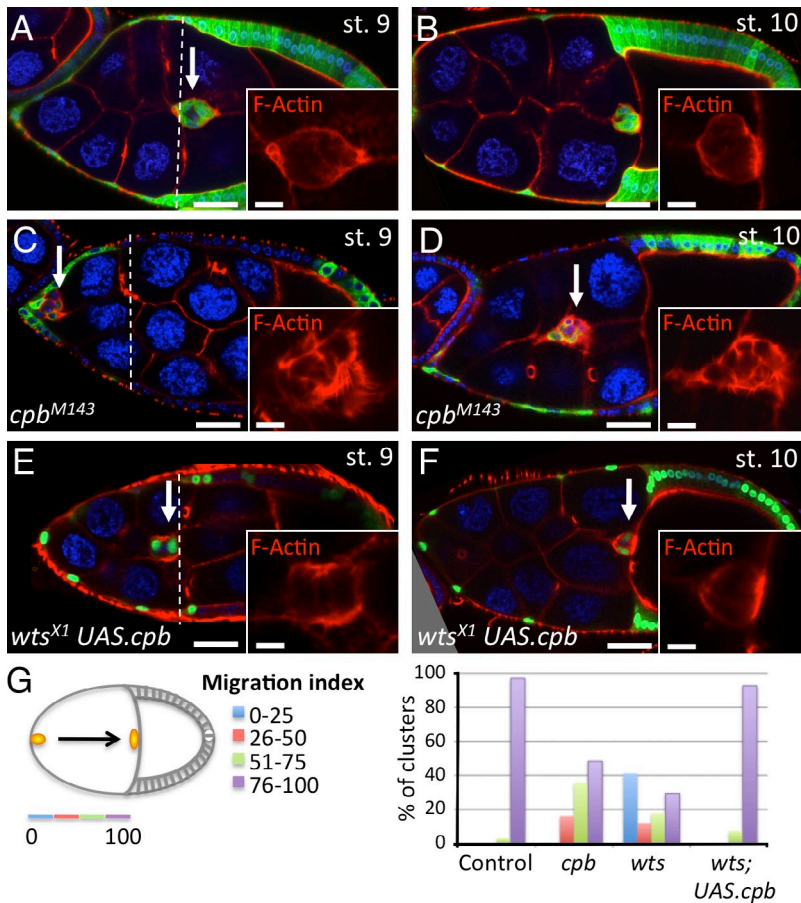
clusters revealed a tumbling motion highly reminiscent of *wts* mutant clusters (compare Video 5 with Videos 2–4). These results indicate that Ena is a key target of Hippo pathway in polarizing the actin cytoskeleton during border cell migration.

Ena is thought to antagonize the action of Capping proteins, which compete with Ena for binding to F-actin barbed ends (Bear and Gertler, 2009). Ena promotes F-actin polymerization, whereas Capping proteins inhibit polymerization. In border cells, mutation of *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 *cpb* mutant clusters disintegrated, highly similar to *wts* or *hpo* mutants (Fig. S1; Video 3 and Video 6). Finally, overexpression of Cpb was able to fully rescue the migration defect and F-actin polarization defects of *wts* mutant border cell clusters (Fig. 7, E–G;

Video 7). These results indicate that Hippo signaling promotes border cell migration by inhibiting Ena and thus promoting Cpb activity inside the cluster to help restrict F-actin to the outer rim of migrating clusters.

## 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



**Figure 7. Capping protein activity mediates the function of Wts in polarizing F-actin and promoting border cell migration.** (A) Control egg chamber at stage 9. (B) Control egg chamber at stage 10. (C and D) *Capping protein β* (*cpb*<sup>M143</sup>) mutant border cell clusters are strongly delayed at stage 9 (C) and 10 (D), with strong accumulation of F-actin throughout the border cell cluster (inset), similar to a *wts*<sup>X1</sup> mutant (see Fig 3). (E and F) *wts*<sup>X1</sup> mutant clusters rescued by expression of *UAS.cpb* at stage 9 (E) or 10 (F) migrate normally and show normal F-actin polarization and levels (insets). (G) Quantification of migration index in stage 10 *wts*<sup>X1</sup> (*n* = 17) and *cpb*<sup>M143</sup> mutants (*n* = 31) and of the rescue of *wts*<sup>X1</sup> mutants by overexpression of *cpb*<sup>M143</sup> (*n* = 41). Bars, 50 μm (5 μm for insets).

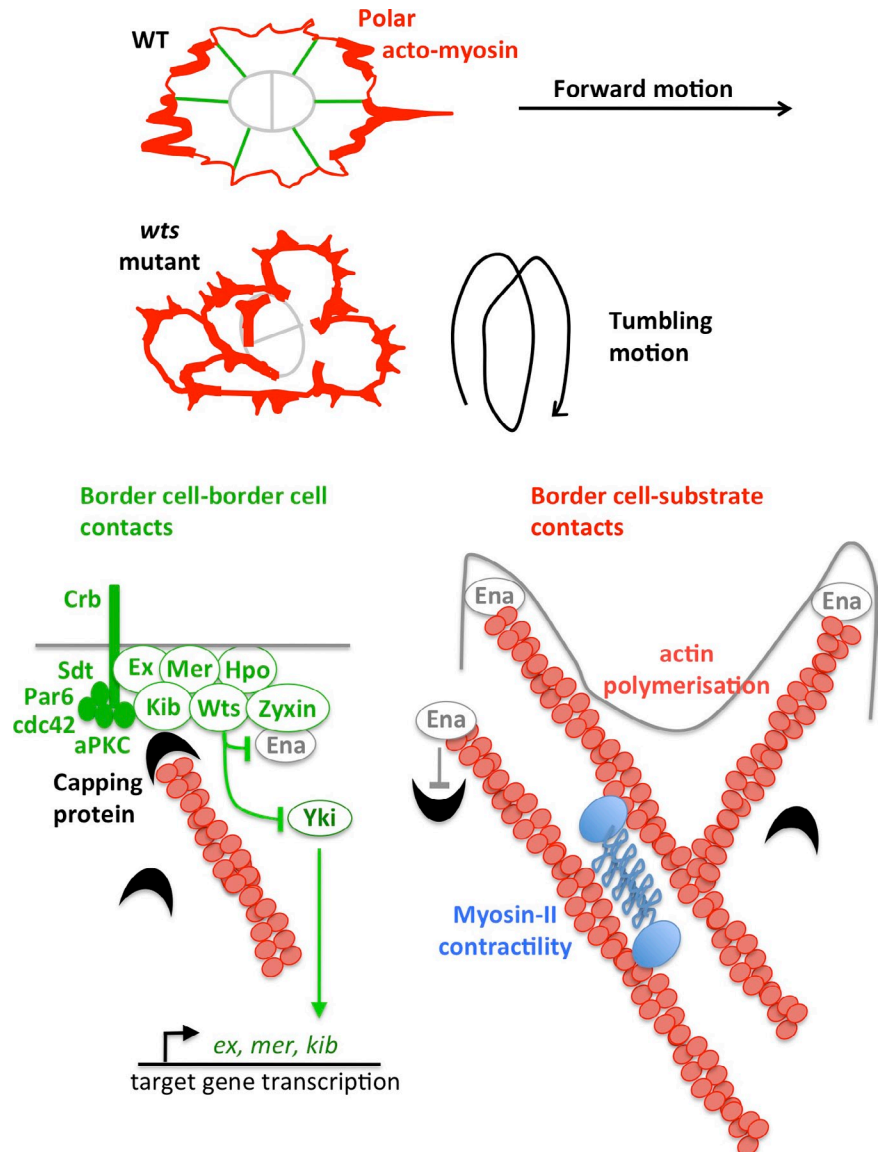
localize to sites of contact between border cells. Our results show that upstream components of the Hippo pathway—Kib, Ex, and Mer—are recruited to border cell contacts and signal through Hpo and Wts to polarize the actin cytoskeleton. Double mutants for *ex*, *kib* have an even stronger phenotype than 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,

Figure 8. **Model.** The role of the Hippo pathway in apico-basal polarization of border cells is shown schematically. At sites of contact between border cells inside the cluster (green), the Hippo pathway acts to suppress actin polymerization via regulation of the Ena/Capping protein system. Consequently, actin polymerization and motility occur primarily at the “basal” outer rim of the border cell cluster.



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 *hpo*<sup>42,47</sup>/FRT42D *tubGal80*; *tubGal4*/+ (Wu et al., 2003); *yw* *hsFLP* UAS-*nucGFPmyc*; FRT42D *hpo*<sup>JM1</sup>/FRT42D *tubGal80*; *tubGal4*/+ (Jia et al., 2003); *yw* *hsFLP*, *tubGal4*, UAS-*nucGFPmyc*; FRT82B *wts*<sup>X1</sup>/FRT82B *tubGal80* (Xu et al., 1995); *yw* *hsFLP*, *tubGal4*, UAS-*nucGFPmyc*; FRT82B *kib*<sup>32</sup>/FRT82B *tubGal80* (Genevet et al., 2010); *yw* *hsFLP*; *ex*<sup>AP50</sup> FRT40A/FRT40A *ubi-GFP* (Hamaratoglu et al., 2006); *yw* *hsFLP*; *ex*<sup>AP50</sup> FRT40A/FRT40A *ubi-GFP*; FRT82B *kib*<sup>32</sup>/FRT82B *ubi-GFP*; *yw* *hsFLP*, *tubGal4*, UAS-*nucGFPmyc*; FRT82B *cpb*<sup>M143</sup>/FRT82B *tubGal80* (Fernández et al., 2011); *yw* *hsFLP*, *tubGal4*, UAS-*nucGFPmyc*; UAS-*cpb*/+; FRT82B *wts*<sup>X1</sup>/FRT82B *tubGal80*; *yw* *hsFLP* UAS-*nucGFPmyc*; FRT42D *hpo*<sup>42,47</sup>, *ena*<sup>210</sup>/FRT42D *tubGal80*; *tubGal4*/+.

The *slbo-lacZ* enhancer trap line was obtained from the Bloomington Stock Center (Bloomington, IN) and the *upd-lacZ* enhancer trap line has been described previously (Shaw et al., 2010). Information on these and other transgenes is available at <http://www.flybase.org>.

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.Yki<sup>WT</sup>GFP, and UAS.Yki<sup>S168A</sup>GFP (Oh and Irvine, 2008), as well as UAS.Yki<sup>WT</sup>V5, UAS.Yki<sup>S168A</sup>V5, and UAS.Yki<sup>S111AS168AS250A</sup>V5 (Oh and Irvine, 2009). UAS.Ena (Bloomington) was also expressed with the Flip-out Gal4 driver. UAS.Ena<sup>S187A</sup> and



*UAS.EnaS187D* 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.Mer*, and *UAS.Ex* (Udan et al., 2003) transgenes were expressed in border cells using the *slbo GAL4* driver (Rørth 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

**MARCM stocks.** The Mosaic Analysis with a Repressible Cell Marker (MARCM) 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 (FRT) 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<sup>1</sup> (y<sup>1</sup>)*, FBgn0004034; *white<sup>1118</sup> (w<sup>1118</sup>)*, FBal0018186; *hsFLP*, FBti0000785; *FRT40A*, FBti0002071; *FRT42D*, FBti0141188; *FRT80B*, FBti0002073; *FRT82B*, FBti0002074; *tubGal80*, FBtp0002650; *tubGal4*, FBtp0002651; *UAS.nucGFPmyc* (a gift of G. Struhl; not yet described in Flybase); *hpo<sup>42,47</sup>*, FBal0151857; *hpo<sup>JM1</sup>*, FBal0151851; *wts<sup>X1</sup>*, FBal0044527; *ex<sup>AP50</sup>*, FBal0217810; *kib<sup>32</sup>*, FBal0244965; *cpb<sup>M143</sup>*, FBal0103870; *UAS.cpb*, FBal0180369; *ena<sup>210</sup>*, FBal0031206; *slbo-lacZ*, FBti0025849; *upd-lacZ*, FBti0003758; *actin.FRT.CD2.FRT.Gal4*, FBtp0001640; *UAS.Yki*, FBal0191232; *UAS.Yki<sup>WT</sup>GFP*, FBal0239730; *UAS.Yki<sup>S168A</sup>GFP*, FBal0265566; *UAS.Yki<sup>WT</sup>V5*, FBal0218166; *UAS.Yki<sup>S168A</sup>V5*, FBal0239740; *UAS.Yki<sup>S11AS168AS250A</sup>V5*, FBal0239743; *UAS.Ena*, FBal0241978; *UAS.Ena*, this paper (attP 28E7 Landing Site Second Chromosome); *UAS.EnaS187A*, this paper (attP 28E7 Landing Site Second Chromosome); *UAS.EnaS187D*, this paper (attP 28E7 Landing Site Second Chromosome); *UAS.Kib*, FBal0244966; *UAS.Mer*, FBal0091705; *UAS.Ex*, FBal0062857; *slboGAL4*, FBal0044834; *UAS.mycWts*, FBal0151322; *UAS.ZyxinV5*, FBtp0068055; *UAS.HA-Cpa*, FBal0193689; and *c306.Gal4*, FBti0003935.

#### Immunofluorescence microscopy

Ovary dissection was performed in PBS, followed by fixation in 4% PFA in PBS for 20 min. After fixation, ovaries were rinsed with PBT (1× PBS and 0.1% Triton X-100) and then incubated in the blocking solution (1× PBS, 0.1% Triton X-100, and 5% goat serum) for at least 30 min. Ovaries were then incubated with the primary antibodies diluted in PBT and 5% goat serum overnight at 4°C, washed with PBT, followed by secondary antibody staining. For P-MyoII staining, ovaries were fixed in 8% PFA for 10 min, blocked for 1 h in PBT with 5% BSA, and then incubated with the primary antibody at 4°C overnight in PBT, 5% BSA.

The following primary antibodies were used: rabbit anti-αPKC (C20, 1:250; Santa Cruz Biotechnology, Inc.), rat anti-DE-cadherin (DCAD2, 1:100; Developmental Studies Hybridoma Bank [DSHB]), mouse anti-Armadillo (N27A1, 1:500; DSHB), mouse anti-α-Spectrin (3A9, 1:10; DSHB), mouse anti-β<sub>5</sub>-Integrin (CF 6G11, 1:5; DSHB), rabbit anti-phosphomyosin LC2 (1:50; Cell Signaling Technology), mouse anti-Dlg (4F3, 1:250; DSHB), rabbit anti-Ex (1:500; gift from A. Laughon, University of Wisconsin-Madison, Madison, WI), rabbit anti-Kibra (1:200; Genevet et al., 2010), rabbit or mouse anti-V5 (1:100; Abcam), rat anti-HA (1:100; Roche), mouse anti-Fasciclin III (7G10, 1:200; DSHB), rabbit anti-GFP (1:400; Torrey Pines) and rabbit anti-βgal (1:200; Cappel). Goat secondary antibodies conjugated to Alexa Fluor 488, 546, or 647 (Invitrogen) were used at a dilution of 1:200 in PBT and incubated for 2–4 h at room temperature. Phalloidin-TRITC (Sigma-Aldrich) was used to stain F-actin. Ovaries were further stained with DAPI (Sigma-Aldrich) in PBT to visualize nuclei before mounting in Vectashield (Vector Laboratories).

Images were acquired on a confocal microscope (SP5 [Leica]; or LSM710 [Carl Zeiss]) using 40× or 63× oil immersion objectives at room temperature, and were processed with Adobe Photoshop CS3. 3D reconstructions of image stacks acquired at 1-μm intervals were generated using Imaris 7.3.1 software (Bitplane).

#### Live imaging

Ovaries were dissected in Schneider's *Drosophila* media (Gibco) with insulin and FBS. Individual egg chambers were carefully removed and transferred

to poly-D-lysine-coated imaging chambers containing Schneider's media, insulin, FBS, trehalose, adenosine deaminase, methoprene, ecdysone, and FM4-64 dye (Bianco et al., 2007). Videos were acquired on an inverted confocal microscope (LSM780; Carl Zeiss) using 40× water immersion objectives; 15 sections were taken 1.6 μm apart with a 3-min interval period between stacks. 3–5 egg chambers were simultaneously imaged using multi-position imaging. Sections covering the migrating cluster were projected for each time point using LSM Image Examiner software (Carl Zeiss) and the videos were processed into a montage using MetaMorph software (Molecular Devices).

#### In vitro kinase assay

Peptides used in this study (LRI peptide synthesis) were: Yki S168 HSRAL-HHSRRSSPASLQQNY (molecular weight 2,516.8 D); Yki S168A HSR-LAIHHSRRARASPASLQQNY (molecular weight 2,500.8 D); Ena S187 SPPTPQGGHRTSAPPAPQPQQQ (molecular weight 2,431.6 D); Ena S187A SPPTPQGGHRTSAPPAPQPQQQ (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 (SignalChem) was measured in a kinase assay with 8,000 ng of peptide (Yki S168, Yki S168A, Ena S187, Ena S187A) and 350 ng of Lats1 kinase diluted in kinase dilution buffer III (SignalChem). The kinase reaction mixture consisted of 2 μl of 5× kinase assay buffer I (SignalChem), 10 μl of ATP cocktail (9.4 μl of kinase dilution buffer III, 10 μM cold ATP, and 3 μCi of γ-[<sup>32</sup>P]ATP [PerkinElmer]). The kinase assay was incubated for 30 min at 30°C. Samples were blotted on P81 phosphocellulose squares (EMD Millipore) and washed 3× in 0.1% phosphoric acid and then in acetone. Incorporation of γ-[<sup>32</sup>P] 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 *wts<sup>x1</sup>* or *mats-IR* clusters versus a control. Video 3 shows disintegration of *wts<sup>x1</sup>* or *mats-IR* clusters versus a control. Video 4 shows failure of detachment of *wts<sup>x1</sup>* or *mats-IR* clusters versus a control. Video 5 shows tumbling migration of a *UAS-ena* cluster versus a control. Video 6 shows disintegration of a *cpb<sup>M143</sup>* cluster versus a control. Video 7 shows rescue of *wts<sup>x1</sup>* mutant migration by expression of *Cpb*. 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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## References

- Abdelilah-Seyfried, S., D.N. Cox, and Y.N. Jan. 2003. Bazooka is a permissive factor for the invasive behavior of discs large tumor cells in *Drosophila* ovarian follicular epithelia. *Development*. 130:1927–1935. <http://dx.doi.org/10.1242/dev.00420>
- Badouel, C., and H. McNeill. 2011. SnapShot: The hippo signaling pathway. *Cell*. 145:484–484. e1. <http://dx.doi.org/10.1016/j.cell.2011.04.009>
- Bai, J., Y. Uehara, and D.J. Montell. 2000. Regulation of invasive cell behavior by taiman, a *Drosophila* protein related to AIB1, a steroid receptor co-activator amplified in breast cancer. *Cell*. 103:1047–1058. [http://dx.doi.org/10.1016/S0092-8674\(00\)00208-7](http://dx.doi.org/10.1016/S0092-8674(00)00208-7)
- Baumgartner, R., I. Poernbacher, N. Buser, E. Hafen, and H. Stocker. 2010. The WW domain protein Kibra acts upstream of Hippo in *Drosophila*. *Dev. Cell*. 18:309–316. <http://dx.doi.org/10.1016/j.devcel.2009.12.013>
- Bear, J.E., and F.B. Gertler. 2009. Ena/VASP: towards resolving a pointed controversy at the barbed end. *J. Cell Sci.* 122:1947–1953. <http://dx.doi.org/10.1242/jcs.038125>
- Beccari, S., L. Teixeira, and P. Rørth. 2002. The JAK/STAT pathway is required for border cell migration during *Drosophila* oogenesis. *Mech. Dev.* 111:115–123. [http://dx.doi.org/10.1016/S0925-4773\(01\)00615-3](http://dx.doi.org/10.1016/S0925-4773(01)00615-3)

- Bianco, A., M. Poukkula, A. Cliffe, J. Mathieu, C.M. Luque, T.A. Fulga, and P. Rørth. 2007. Two distinct modes of guidance signalling during collective migration of border cells. *Nature*. 448:362–365. <http://dx.doi.org/10.1038/nature05965>
- Boggiano, J.C., and R.G. Fehon. 2012. Growth control by committee: intercellular junctions, cell polarity, and the cytoskeleton regulate Hippo signaling. *Dev. Cell*. 22:695–702. <http://dx.doi.org/10.1016/j.devcel.2012.03.013>
- Borghese, L., G. Fletcher, J. Mathieu, A. Atzberger, W.C. Eades, R.L. Cagan, and P. Rørth. 2006. Systematic analysis of the transcriptional switch inducing migration of border cells. *Dev. Cell*. 10:497–508. <http://dx.doi.org/10.1016/j.devcel.2006.02.004>
- Carmona-Fontaine, C., H.K. Matthews, S. Kuriyama, M. Moreno, G.A. Dunn, M. Parsons, C.D. Stern, and R. Mayor. 2008. Contact inhibition of locomotion in vivo controls neural crest directional migration. *Nature*. 456:957–961. <http://dx.doi.org/10.1038/nature07441>
- Chen, C.L., K.M. Gajewski, F. Hamaratoglu, W. Bossuyt, L. Sansores-Garcia, C. Tao, and G. Halder. 2010. The apical-basal cell polarity determinant Crumbs regulates Hippo signaling in *Drosophila*. *Proc. Natl. Acad. Sci. USA*. 107:15810–15815. <http://dx.doi.org/10.1073/pnas.1004060107>
- Cobreras-Reguera, L., A. Fernández-Miñán, C.H. Fernández-Espartero, H. López-Schier, A. González-Reyes, and M.D. Martín-Bermudo. 2010. The Ste20 kinase misshapen is essential for the invasive behaviour of ovarian epithelial cells in *Drosophila*. *EMBO Rep*. 11:943–949. <http://dx.doi.org/10.1038/embor.2010.156>
- Condeelis, J., R.H. Singer, and J.E. Segall. 2005. The great escape: when cancer cells hijack the genes for chemotaxis and motility. *Annu. Rev. Cell Dev. Biol*. 21:695–718. <http://dx.doi.org/10.1146/annurev.cellbio.21.122303.120306>
- Deng, Y., Y. Matsui, Y. Zhang, and Z.C. Lai. 2013. Hippo activation through homodimerization and membrane association for growth inhibition and organ size control. *Dev. Biol*. 375:152–159. <http://dx.doi.org/10.1016/j.ydbio.2012.12.017>
- Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang, S.A. Comerford, M.F. Gayyed, R.A. Anders, A. Maitra, and D. Pan. 2007. Elucidation of a universal size-control mechanism in *Drosophila* and mammals. *Cell*. 130:1120–1133. <http://dx.doi.org/10.1016/j.cell.2007.07.019>
- Duchek, P., and P. Rørth. 2001. Guidance of cell migration by EGF receptor signaling during *Drosophila* oogenesis. *Science*. 291:131–133. <http://dx.doi.org/10.1126/science.291.5501.131>
- Duchek, P., K. Somogyi, G. Jékely, S. Beccari, and P. Rørth. 2001. Guidance of cell migration by the *Drosophila* PDGF/VEGF receptor. *Cell*. 107:17–26. [http://dx.doi.org/10.1016/S0092-8674\(01\)00502-5](http://dx.doi.org/10.1016/S0092-8674(01)00502-5)
- Fernández, B.G., P. Gaspar, C. Brás-Pereira, B. Jezowska, S.R. Rebelo, and F. Janody. 2011. Actin-capping protein and the Hippo pathway regulate F-actin and tissue growth in *Drosophila*. *Development*. 138:2337–2346. <http://dx.doi.org/10.1242/dev.063545>
- Fletcher, G., E. Lucas, R. Brain, A. Tournier, and B.J. Thompson. 2012. Positive feedback and mutual antagonism combine to polarise Crumbs in epithelial cells. *Curr. Biol*. 22:1116–1122. <http://dx.doi.org/10.1016/j.cub.2012.04.020>
- Friedl, P., and D. Gilmour. 2009. Collective cell migration in morphogenesis, regeneration and cancer. *Nat. Rev. Mol. Cell Biol*. 10:445–457. <http://dx.doi.org/10.1038/nrm2720>
- Fulga, T.A., and P. Rørth. 2002. Invasive cell migration is initiated by guided growth of long cellular extensions. *Nat. Cell Biol*. 4:715–719. <http://dx.doi.org/10.1038/ncb848>
- Gates, J., S.H. Nowotarski, H. Yin, J.P. Mahaffey, T. Bridges, C. Herrera, C.C. Homem, F. Janody, D.J. Montell, and M. Peifer. 2009. Enabled and Capping protein play important roles in shaping cell behavior during *Drosophila* oogenesis. *Dev. Biol*. 333:90–107. <http://dx.doi.org/10.1016/j.ydbio.2009.06.030>
- Genevet, A., M.C. Wehr, R. Brain, B.J. Thompson, and N. Tapon. 2010. Kibra is a regulator of the Salvador/Warts/Hippo signaling network. *Dev. Cell*. 18:300–308. <http://dx.doi.org/10.1016/j.devcel.2009.12.011>
- Grusche, F.A., H.E. Richardson, and K.F. Harvey. 2010. Upstream regulation of the hippo size control pathway. *Curr. Biol*. 20:R574–R582. <http://dx.doi.org/10.1016/j.cub.2010.05.023>
- Grzeschik, N.A., L.M. Parsons, M.L. Allott, K.F. Harvey, and H.E. Richardson. 2010. Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. *Curr. Biol*. 20:573–581. <http://dx.doi.org/10.1016/j.cub.2010.01.055>
- Halder, G., and R.L. Johnson. 2011. Hippo signaling: growth control and beyond. *Development*. 138:9–22. <http://dx.doi.org/10.1242/dev.045500>
- Hamaratoglu, F., M. Willecke, M. Kango-Singh, R. Nolo, E. Hyun, C. Tao, H. Jafar-Nejad, and G. Halder. 2006. The tumour-suppressor genes NF2/Merlin and Expanded act through Hippo signalling to regulate cell proliferation and apoptosis. *Nat. Cell Biol*. 8:27–36. <http://dx.doi.org/10.1038/ncb1339>
- Hidalgo-Carcedo, C., S. Hooper, S.I. Chaudhry, P. Williamson, K. Harrington, B. Leitinger, and E. Sahai. 2011. Collective cell migration requires suppression of actomyosin at cell-cell contacts mediated by DDR1 and the cell polarity regulators Par3 and Par6. *Nat. Cell Biol*. 13:49–58. <http://dx.doi.org/10.1038/ncb2133>
- Huang, J., S. Wu, J. Barrera, K. Matthews, and D. Pan. 2005. The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the *Drosophila* homolog of YAP. *Cell*. 122:421–434. <http://dx.doi.org/10.1016/j.cell.2005.06.007>
- Jang, A.C., Y.C. Chang, J. Bai, and D. Montell. 2009. Border-cell migration requires integration of spatial and temporal signals by the BTB protein Abrupt. *Nat. Cell Biol*. 11:569–579. <http://dx.doi.org/10.1038/ncb1863>
- Jia, J., W. Zhang, B. Wang, R. Trinko, and J. Jiang. 2003. The *Drosophila* Ste20 family kinase dMST functions as a tumor suppressor by restricting cell proliferation and promoting apoptosis. *Genes Dev*. 17:2514–2519. <http://dx.doi.org/10.1101/gad.1134003>
- Kim, N.G., E. Koh, X. Chen, and B.M. Gumbiner. 2011. E-cadherin mediates contact inhibition of proliferation through Hippo signaling-pathway components. *Proc. Natl. Acad. Sci. USA*. 108:11930–11935. <http://dx.doi.org/10.1073/pnas.1103345108>
- Lee, T., and L. Luo. 1999. Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron*. 22:451–461. [http://dx.doi.org/10.1016/S0896-6273\(00\)80701-1](http://dx.doi.org/10.1016/S0896-6273(00)80701-1)
- Ling, C., Y. Zheng, F. Yin, J. Yu, J. Huang, Y. Hong, S. Wu, and D. Pan. 2010. The apical transmembrane protein Crumbs functions as a tumor suppressor that regulates Hippo signaling by binding to Expanded. *Proc. Natl. Acad. Sci. USA*. 107:10532–10537. <http://dx.doi.org/10.1073/pnas.1004279107>
- McDonald, J.A., E.M. Pinheiro, and D.J. Montell. 2003. PVF1, a PDGF/VEGF homolog, is sufficient to guide border cells and interacts genetically with Taiman. *Development*. 130:3469–3478. <http://dx.doi.org/10.1242/dev.00574>
- McDonald, J.A., A. Khodyakova, G. Aranjuez, C. Dudley, and D.J. Montell. 2008. PAR-1 kinase regulates epithelial detachment and directional protrusion of migrating border cells. *Curr. Biol*. 18:1659–1667. <http://dx.doi.org/10.1016/j.cub.2008.09.041>
- Montell, D.J. 2008. Morphogenetic cell movements: diversity from modular mechanical properties. *Science*. 322:1502–1505. <http://dx.doi.org/10.1126/science.1164073>
- Montell, D.J., P. Rørth, and A.C. Spradling. 1992. slow border cells, a locus required for a developmentally regulated cell migration during oogenesis, encodes *Drosophila* C/EBP. *Cell*. 71:51–62. [http://dx.doi.org/10.1016/0092-8674\(92\)90265-E](http://dx.doi.org/10.1016/0092-8674(92)90265-E)
- Niewiadomska, P., D. Godt, and U. Tepass. 1999. DE-Cadherin is required for intercellular motility during *Drosophila* oogenesis. *J. Cell Biol*. 144:533–547. <http://dx.doi.org/10.1083/jcb.144.3.533>
- Oh, H., and K.D. Irvine. 2008. In vivo regulation of Yorkie phosphorylation and localization. *Development*. 135:1081–1088. <http://dx.doi.org/10.1242/dev.015255>
- Oh, H., and K.D. Irvine. 2009. In vivo analysis of Yorkie phosphorylation sites. *Oncogene*. 28:1916–1927. <http://dx.doi.org/10.1038/onc.2009.43>
- Oh, H., and K.D. Irvine. 2010. Yorkie: the final destination of Hippo signaling. *Trends Cell Biol*. 20:410–417. <http://dx.doi.org/10.1016/j.tcb.2010.04.005>
- Pacquelet, A., and P. Rørth. 2005. Regulatory mechanisms required for DE-cadherin function in cell migration and other types of adhesion. *J. Cell Biol*. 170:803–812. <http://dx.doi.org/10.1083/jcb.200506131>
- Pan, D. 2010. The hippo signaling pathway in development and cancer. *Dev. Cell*. 19:491–505. <http://dx.doi.org/10.1016/j.devcel.2010.09.011>
- Pignoni, F., and S.L. Zipursky. 1997. Induction of *Drosophila* eye development by decapentaplegic. *Development*. 124:271–278.
- Pinheiro, E.M., and D.J. Montell. 2004. Requirement for Par-6 and Bazooka in *Drosophila* border cell migration. *Development*. 131:5243–5251. <http://dx.doi.org/10.1242/dev.01412>
- Poukkula, M., A. Cliffe, R. Changede, and P. Rørth. 2011. Cell behaviors regulated by guidance cues in collective migration of border cells. *J. Cell Biol*. 192:513–524. <http://dx.doi.org/10.1083/jcb.201010003>
- Rauskolb, C., G. Pan, B.V. Reddy, H. Oh, and K.D. Irvine. 2011. Zyxin links fat signaling to the hippo pathway. *PLoS Biol*. 9:e1000624. <http://dx.doi.org/10.1371/journal.pbio.1000624>
- Ridley, A.J. 2011. Life at the leading edge. *Cell*. 145:1012–1022. <http://dx.doi.org/10.1016/j.cell.2011.06.010>
- Rørth, P. 2009. Collective cell migration. *Annu. Rev. Cell Dev. Biol*. 25:407–429. <http://dx.doi.org/10.1146/annurev.cellbio.042308.113231>
- Rørth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm, G.M. Rubin, K. Weigmann, M. Milán, V. Benes, W. Ansorge, and S.M. Cohen. 1998. Systematic gain-of-function genetics in *Drosophila*. *Development*. 125:1049–1057.
- Sahai, E. 2005. Mechanisms of cancer cell invasion. *Curr. Opin. Genet. Dev*. 15:87–96. <http://dx.doi.org/10.1016/j.gde.2004.12.002>

- Sansores-Garcia, L., W. Bossuyt, K. Wada, S. Yonemura, C. Tao, H. Sasaki, and G. Halder. 2011. Modulating F-actin organization induces organ growth by affecting the Hippo pathway. *EMBO J.* 30:2325–2335. <http://dx.doi.org/10.1038/emboj.2011.157>
- Shaw, R.L., A. Kohlmaier, C. Polesello, C. Veelken, B.A. Edgar, and N. Tapon. 2010. The Hippo pathway regulates intestinal stem cell proliferation during *Drosophila* adult midgut regeneration. *Development.* 137:4147–4158. <http://dx.doi.org/10.1242/dev.052506>
- Silver, D.L., and D.J. Montell. 2001. Paracrine signaling through the JAK/STAT pathway activates invasive behavior of ovarian epithelial cells in *Drosophila*. *Cell.* 107:831–841. [http://dx.doi.org/10.1016/S0092-8674\(01\)00607-9](http://dx.doi.org/10.1016/S0092-8674(01)00607-9)
- Somogyi, K., and P. Rørth. 2004. Evidence for tension-based regulation of *Drosophila* MAL and SRF during invasive cell migration. *Dev. Cell.* 7:85–93. <http://dx.doi.org/10.1016/j.devcel.2004.05.020>
- Staley, B.K., and K.D. Irvine. 2010. Warts and Yorkie mediate intestinal regeneration by influencing stem cell proliferation. *Curr. Biol.* 20:1580–1587. <http://dx.doi.org/10.1016/j.cub.2010.07.041>
- Starz-Gaiano, M., and D.J. Montell. 2004. Genes that drive invasion and migration in *Drosophila*. *Curr. Opin. Genet. Dev.* 14:86–91. <http://dx.doi.org/10.1016/j.gde.2003.12.001>
- Sudol, M., and K.F. Harvey. 2010. Modularity in the Hippo signaling pathway. *Trends Biochem. Sci.* 35:627–633. <http://dx.doi.org/10.1016/j.tibs.2010.05.010>
- Tepass, U. 2012. The apical polarity protein network in *Drosophila* epithelial cells: regulation of polarity, junctions, morphogenesis, cell growth, and survival. *Annu. Rev. Cell Dev. Biol.* 28:655–685. <http://dx.doi.org/10.1146/annurev-cellbio-092910-154033>
- Udan, R.S., M. Kango-Singh, R. Nolo, C. Tao, and G. Halder. 2003. Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* 5:914–920. <http://dx.doi.org/10.1038/ncb1050>
- Van Haastert, P.J., and P.N. Devreotes. 2004. Chemotaxis: signalling the way forward. *Nat. Rev. Mol. Cell Biol.* 5:626–634. <http://dx.doi.org/10.1038/nrm1435>
- Wada, K., K. Itoga, T. Okano, S. Yonemura, and H. Sasaki. 2011. Hippo pathway regulation by cell morphology and stress fibers. *Development.* 138:3907–3914. <http://dx.doi.org/10.1242/dev.070987>
- Weijer, C.J. 2009. Collective cell migration in development. *J. Cell Sci.* 122:3215–3223. <http://dx.doi.org/10.1242/jcs.036517>
- Wu, S., J. Huang, J. Dong, and D. Pan. 2003. *hippo* encodes a Ste-20 family protein kinase that restricts cell proliferation and promotes apoptosis in conjunction with *salvador* and *warts*. *Cell.* 114:445–456. [http://dx.doi.org/10.1016/S0092-8674\(03\)00549-X](http://dx.doi.org/10.1016/S0092-8674(03)00549-X)
- Xi, R., J.R. McGregor, and D.A. Harrison. 2003. A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium. *Dev. Cell.* 4:167–177. [http://dx.doi.org/10.1016/S1534-5807\(02\)00412-4](http://dx.doi.org/10.1016/S1534-5807(02)00412-4)
- Xu, T., and G.M. Rubin. 1993. Analysis of genetic mosaics in developing and adult *Drosophila* tissues. *Development.* 117:1223–1237.
- Xu, T., W. Wang, S. Zhang, R.A. Stewart, and W. Yu. 1995. Identifying tumor suppressors in genetic mosaics: the *Drosophila lats* gene encodes a putative protein kinase. *Development.* 121:1053–1063.
- Yu, J., Y. Zheng, J. Dong, S. Klusza, W.M. Deng, and D. Pan. 2010. Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Dev. Cell.* 18:288–299. <http://dx.doi.org/10.1016/j.devcel.2009.12.012>
- Zhao, B., X. Wei, W. Li, R.S. Udan, Q. Yang, J. Kim, J. Xie, T. Ikenoue, J. Yu, L. Li, et al. 2007. Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. *Genes Dev.* 21:2747–2761. <http://dx.doi.org/10.1101/gad.1602907>