The Hippo Pathway Controls Border Cell Migration Through Distinct Mechanisms in Outer Border Cells and Polar Cells of the *Drosophila* Ovary

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ABSTRACT The Hippo pathway is a key signaling cascade in controlling organ size. The core components of this pathway are two kinases, Hippo (Hpo) and Warts (Wts), and a transcriptional coactivator, Yorkie (Yki). Yes-associated protein (YAP, a Yki homolog in mammals) promotes epithelial–mesenchymal transition and cell migration *in vitro*. Here, we use border cells in the *Drosophila* ovary as a model to study Hippo pathway functions in cell migration *in vivo*. During oogenesis, polar cells secrete Unpaired (Upd), which activates JAK/STAT signaling of neighboring cells and specifies them into outer border cells. The outer border cells form a cluster with polar cells and undergo migration. We find that *hpo* and *wts* are required for migration of the border cell cluster. In outer border cells, overexpression of *hpo* disrupts polarization of the actin cytoskeleton and attenuates migration. In polar cells, knockdown of *hpo* and *wts* or overexpression of *yki* impairs border cell induction and disrupts migration. These manipulations in polar cells reduce JAK/STAT activity in outer border cells. Expression of *upd-lacZ* is increased and decreased in *yki* and *hpo* mutant polar cells, respectively. Furthermore, forced expression of *upd* in polar cells rescues defects of border cell induction and migration caused by *wts* knockdown. These results suggest that Yki negatively regulates border cell induction by inhibiting JAK/STAT signaling. Together, our data elucidate two distinct mechanisms of the Hippo pathway in controlling border cell migration: (1) in outer border cells, it regulates polarized distribution of the actin cytoskeleton; (2) in polar cells, it regulates *upd* expression to control border cell induction and migration.

EPITHELIAL–mesenchymal transition (EMT) and cell migration are fundamental for pattern formation during embryonic development (Thiery *et al.* 2009). In addition, these two cellular processes are critical steps of metastasis, a key event of cancer progression. Therefore, genes and signaling pathways involved in EMT or cell migration are of great interest for both basic and clinical research. To identify genes that are crucial for epithelial cells to become migratory, border cells in the *Drosophila* ovary provide an eligible *in vivo* model.

Border cells are a group of specialized follicle cells. During oogenesis, germline stem cells and follicle stem cells continue to divide and give rise to egg chambers, which are 16-cell germline cysts enwrapped by a single layer of follicle cells. The egg chamber buds off from the germarium and develops gradually until it becomes a mature egg. Polar cells located at the anterior and posterior ends of an egg chamber are specialized follicle cells important for patterning of the follicular epithelium. Based on polyploidization of germline cells, mitotic division of follicle cells, and the size of egg chambers, developmental egg chambers are categorized into different stages. At stage 8, anterior polar cells secrete Unpaired (Upd), a ligand of the JAK/STAT pathway. Upd activates JAK/STAT signaling of neighboring cells, leading to border cell induction (Silver and Montell 2001; Beccari *et al.* 2002). Activation of JAK/STAT signaling in outer border cells induces expression of *slow border cells* (*slbo*), which encodes a C/EBP transcription factor (Montell *et al.* 1992). Slbo induces expression of *focal adhesion kinase* (*Fak*), *singed* (*sn*, a homolog of mammalian *Fascin*), *DE-cadherin*, *armadillo* (*arm*, a *Drosophila* homolog of β-catenin), *stathmin*, and other genes involved in cell migration (Borghese *et al.* 2006; Wang *et al.* 2006). After being specified, outer border cells undergo partial EMT and form a cluster surrounding

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two polar cells. They detach from the follicular epithelium together and migrate toward the oocyte at stage 9 (Figure 1I). By stage 10, the border cell cluster arrives at the oocyte-nurse-cell border. Importantly, activation of JAK/STAT signaling is required throughout the migratory process, suggesting that JAK/STAT signaling is critical for both border cell induction and migration (Silver *et al.* 2005). As in all migratory cells, actin organization regulated by members of the Rho family GTPases, such as Rac, is crucial for border cell migration (Wang *et al.* 2010). Border cell migration is guided by Gurken (a *Drosophila* homolog of EGF) and PDGF/VEGF-related factor 1 (PVF1) secreted from the oocyte. In border cells, signaling through the PDGF/VEGF-related receptor (PVR) and the EGF receptor (EGFR) function together to control their migratory speed and direction (Duchek and Rorth 2001; Duchek *et al.* 2001; McDonald *et al.* 2003, 2006). Other signaling cascades, such as steroid hormones and the Notch pathway, also affect border cell migration (Bai *et al.* 2000; Wang *et al.* 2007; Jang *et al.* 2009). Importantly, homologs of these genes and the same signaling cascades in mammals play roles in regulating cell migration and cancer metastasis (Montell 2003; Naora and Montell 2005; Jang *et al.* 2007), demonstrating the relevance of studies of the *Drosophila* border cells to cancer biology. With powerful genetic tools, it is efficient to use border cells as a model to identify genes or signaling pathways involved in cell migration *in vivo*.

Recently, the Hippo pathway has been demonstrated to be crucial in multiple aspects of tumorigenesis, including cell migration. YAP, a mammalian homolog of *Drosophila* Yorkie), a key effector of this pathway, promotes EMT and cell motility *in vitro* (Overholtzer *et al.* 2006; Zhao *et al.* 2008a; Zhang *et al.* 2009). Furthermore, abnormal YAP activity associates with poor survival of ovarian cancer patients significantly (Hall *et al.* 2010; Zhang *et al.* 2011b). These results drew our attention to investigate roles of the Hippo pathway in cell migration. The Hippo pathway has recently emerged as a critical signaling cascade in controlling organ size by regulating cell proliferation and apoptosis (Lian *et al.* 2010; Lu *et al.* 2010; Oh and Irvine 2010; Pan 2010; Halder and Johnson 2011). The core components of this pathway in *Drosophila* are Hippo (Hpo), Warts (Wts), Salvador (Sav), Mob as a tumor suppressor (Mats), and Yorkie (Yki). Hpo is an Ste20-like kinase that forms a complex with the adaptor protein Sav and phosphorylates Wts, a nuclear Dbf2-related family kinase (Justice *et al.* 1995; Watson 1995; Harvey *et al.* 2003; Pantalacci *et al.* 2003; Wu *et al.* 2003). In association with the adapter protein Mats (Lai *et al.* 2005), Wts phosphorylates Yki, a transcriptional coactivator (Huang *et al.* 2005; Wei *et al.* 2007). Phosphorylated Yki interacts with 14-3-3 phosphopeptide-binding protein, resulting in cytoplasmic retention and repression of its transcriptional activity (Huang *et al.* 2005; Dong *et al.* 2007; Oh and Irvine 2008, 2009; Ren *et al.* 2010b). When Yki is not phosphorylated, it is localized to the nucleus and interacts with transcription factors such as Scalloped (Sd) to induce target gene expression (Goulev *et al.* 2008; Zhang *et al.* 2008; Zhao *et al.* 2008a). Most target genes of Yki, such as *cyclin E*, dm (dMyc), Diap1, and *bantam*, promote cell proliferation or survival (Wu *et al.* 2008; Ziosi *et al.* 2010). These core components are conserved from *Drosophila* to mammals, suggesting that Hippo pathway functions are essential (Harvey and Tapon 2007; Zeng and Hong 2008; Badouel *et al.* 2009; Pan 2010). During oogenesis in *Drosophila*, the Hippo pathway has also been shown to control proliferation of the follicle cell lineage (Zhao *et al.* 2008b; Huang and Kalderon 2014).

Although YAP has been shown to induce EMT and cell migration in cultured cells (Zhao *et al.* 2008a; Zhang *et al.* 2009; Xu *et al.* 2011), whether YAP/Yki or the Hippo pathway regulates cell migration *in vivo* remains unclear. In *Drosophila*, it has been demonstrated that Wts regulates invasion of follicle cells into egg chambers in coordination with basolateral junctional components, such as Fasciclin 2 and Discs large 1 (Zhao *et al.* 2008b). Wts has also been shown to be required for border cell migration (Zhao *et al.* 2008b). In addition, when we were conducting this study, Lucas *et al.* (2013) showed that Hpo and Wts controlled border cell migration through regulating localization of actin polymerization (see *Discussion* for details). In this study, we find that Hpo in outer border cells controls border cell migration and affects the actin cytoskeleton. In polar cells, the Hippo pathway controls the expression of *upd*, which activates JAK/STAT-signaling activity to promote border cell induction and migration. Together, our data show distinct mechanisms of the Hippo pathway in outer border cells and polar cells to regulate migration of the border cell cluster.

Materials and Methods

Fly stocks

Fly lines used for overexpression and knockdown experiments were the following: *y w*; *UAS-hpo/CyO*; *MKRS/TM2*, *y w*; *Sp/CyO*; *UAS-hpo/TM2* (gift from Jin Jiang), *P{UAS-yki.V5.O}attP2* (Bloomington Stock Center BLM28819), *P{UAS-yki.S168A.V5}attP2* (BLM28818), *P{UAS-yki.S111A.S168A.S250A.V5}attP2* (BLM28817), *P{UAS-sd.S}V1* (BLM9373), *UAS-H2B.RFP*, *UAS-GFP*, *P{KK101704}VIE-260B* (Vienna Drosophila Resource Center, V104169), w^{1118}; *P{GD1570}v7823* (V7823), y^1 *sc* v*^{1}; *P{TRiP.GL00046}attP2* (BLM35176), w^{1118}; *P{GD1563}v9928* (V9982), *P{KK101055}VIE-260B* (V106174), y^1 v^1; *P{TRiP.HMS00026}attP2* (BLM34064), y^1 v^1; *P{TRiP.JF02741}attP2* (BLM27662), *P{KK109756}VIE-260B* (V104523), w^{1118}; *P{GD11187}v40497/TM3* (V40497), y^1 v^1; *P{TRiP.JF03119}attP2/TM3*, *Sb*^{1} (BLM31965), y^1 v^1; *P{TRiP.HMS00041}attP2* (BLM34067), *P{KK104232}VIE-260B* (V104197), *upd-Gal4*, *upd-Gal4*; *tub-Gal80*^{ts}*/CyO*, *slbo-Gal4* (gift from G. J. Liaw). Fly lines used for clonal analysis were the following: *ey-flp*; *FRT42D*, *FRT42D yki*^{B5},w^{hs}*/CyO*, w^-; *FRT42D hpo*$^{42\text{-}47}$,w^+*/CyO*, *hs-flp*; *FRT42D ubi-GFP/CyO*, *FRT82B wts*^{x1}*/TM3*,*Sb*, *hs-flp*;; *FRT82B ubi-GFP/TM3*,*Sb*, *y w hs-flp*; *FRT42D arm-LacZ/CyO*. *upd-lacZ/FM6b* and *10Xstat*::*GFP/TM3* were used for examining JAK/STAT-signaling activities. *PZ80-LacZ* and *A101-LacZ* were used for the identification of polar cells.

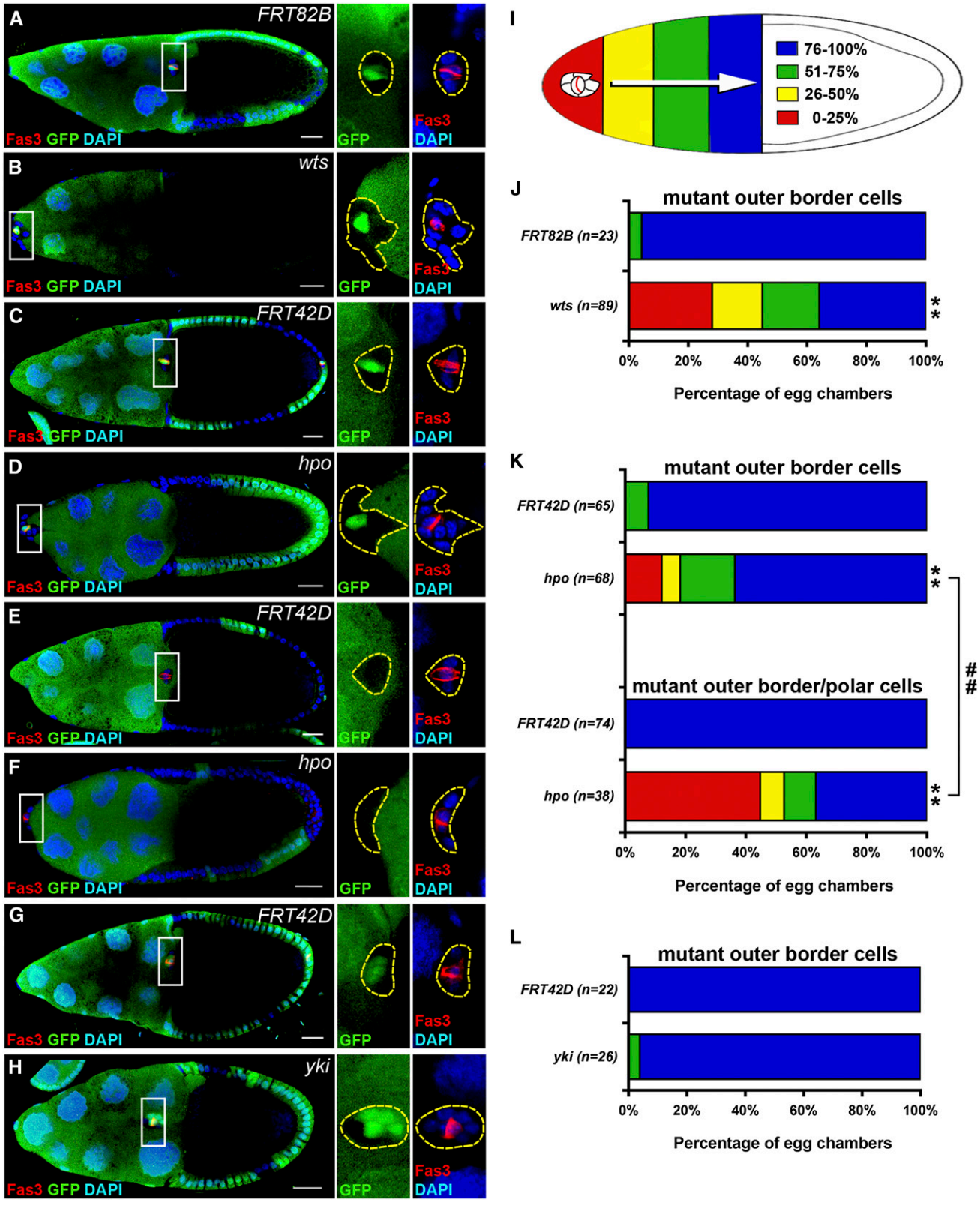

Figure 1 *hpo* and *wts* are required for border cell migration. GFP-negative mitotic clones were generated in *FRT82B* (A), *FRT82B* wts^{X1} (B), *FRT42D* (C and E), and *FRT42D* $hpo^{42\text{-}47}$ (D and F) and examined 6 days after clone induction. Mitotic clones of *FRT42D* (G) and *FRT42D* yki^{B5} (H) were examined 3 days after clone induction. The ovaries were immunostained with anti-Fas3 and anti-GFP antibodies. Cell nuclei were stained with DAPI. Stage-10 egg chambers were selected and oriented as anterior toward the left. The border cell cluster is composed of two Fas3-positive polar cells in the center surrounded by four to six outer border cells. High magnification views of border cell clusters are shown in the panels on the right in A–H. (A) A border cell cluster containing

Antibodies and reagents

The following antibodies were used at the indicated dilutions: mouse anti-Fasciclin III 1:200 [7G10, Developmental Studies Hybridoma Bank (DSHB)], mouse anti-β-galactosidase 1:200 (40-1A, DSHB), mouse anti-Arm 1:200 (N2 7A1, DSHB), mouse anti-Eya 1:200 (eya10H6, DSHB), mouse anti-cyclin B 1:200 (F2F4, DSHB), rabbit anti-GFP 1:1000 (Invitrogen), rat anti-Slbo 1:500 from Pernille Rorth (Borghese *et al.* 2006), Dylight-488 goat anti-rabbit IgG(H+L) 1:1000, Dylight-549 goat anti-mouse IgG(H+L) 1:1000 (Jackson ImmunoResearch Laboratories), Alexa-546 phalloidin 1:50 (Invitrogen).

Overexpression and RNAi knockdown

Offspring from crosses of *UAS* lines and *slbo-Gal4*; *UAS-GFP*, *upd-Gal4*; *UAS-GFP*, or *upd-Gal4*; *tub-Gal80^{ts}/CyO* lines were cultured at 18° until eclosion. Newly eclosed adult flies were collected and grown at 29° for 40 or 54 hr (Gal80^{ts} experiments) or 5–6 days before dissection.

Generation of mitotic clones

Mitotic clones were generated by using the FLP/FRT system (Xu and Rubin 1993). Adult flies eclosed in 3 days were collected and heat-shocked at 37° four times over the next 2 days. On the first day, they were heat-shocked twice for 30 min each time with at least a 3-hr interval. On the second day, they were heat-shocked once for 30 min and once for an hour with at least a 3-hr interval. Flies were incubated at 25° for 3 or 6 days before dissection.

Immunostaining and fluorescence microscopy

Flies were dissected in phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde in PBS 20 min. After fixation, ovaries were washed with PBT (1× PBS with 0.2% Triton X-100) three times and then incubated with PBTB blocking solution (1× PBS, 0.5% Triton X-100, 5% goat serum, 2.5 mg/ml BSA, and 0.05% sodium azide) for an hour. Ovaries were incubated with primary antibodies in PBTB overnight at 4° and then with secondary antibodies in PBTB overnight at 4°. Ovaries were further stained with DAPI in PBT (1 μg/ml; Sigma) and then mounted with mounting solution [85% glycerol, 1× PBS, 3% propyl gallate (Sigma-Aldrich), and ProLong Gold Antifade reagents (Invitrogen)]. All images were taken by Zeiss LSM700 confocal microscope (Carl Zeiss AG) and processed with Photoshop CS3 (Adobe). For quantification of signal intensity, images of the brightest β-galactosidase optical slice were quantified by drawing an elliptic field of identical dimensions for each cell, and reading of the average intensity in the field was done by using ImageJ (National Institutes of Health, Bethesda, MD). A paired Student's *t*-test was used to compare the signal intensities of the mutant and adjacent control cells.

Analysis of border cell migration

Stage-10 egg chambers were selected, and the position of border cell clusters was analyzed. We defined stage 10 as when the oocyte spanned the posterior half of the egg chamber. As an index for migration, these stage-10 egg chambers were categorized based on the location of the border cell cluster as depicted in Figure 1I. The Wilcoxon rank-sum test was used for analyzing the data of border cell migration. All graphs were plotted with Excel (Microsoft).

Results

hpo and wts are required for migration of the border cell cluster

To examine functions of the Hippo pathway in border cell migration, we generated homozygous *wts^{X1}*, *hpo$^{42\text{-}47}$*, or *yki^{B5}* mutant clones in border cell clusters using the FLP/FRT system (Xu and Rubin 1993). *FRT82B* or *FRT42D* clones were generated as controls. The border cell cluster usually arrives at the oocyte-nurse-cell border by stage 10, so we selected egg chambers at stage 10 and examined whether border cells migrated normally. Instead of using arrival of border cells at the oocyte-nurse-cell border as an indication of stage 10, we defined stage 10 as when the oocyte spanned the posterior half of the egg chamber. As an index for migration, these stage 10 egg chambers were further categorized based on the location of the border cell cluster (Figure 1I). Polar cells were identified based on the staining pattern of Fasciclin III (Fas3) (Ruohola *et al.* 1991). In comparison with the *FRT82B* control, migration of border cell clusters with *wts* mutant outer border cells was attenuated 6 days after clone induction (Figure 1, A, B, and J), suggesting

GFP-positive polar cells and GFP-negative *FRT82B* control outer border cells migrated normally and reached the oocyte-nurse-cell border. (B) A border cell cluster containing GFP-positive polar cells and GFP-negative *wts* mutant outer border cells failed to migrate. (C) A border cell cluster containing GFP-positive polar cells and GFP-negative *FRT42D* control outer border cells reached the oocyte-nurse-cell border. (D) A border cell cluster containing GFP-positive polar cells and GFP-negative *hpo* mutant outer border cells failed to migrate. (E) A border cell cluster containing GFP-negative *FRT42D* control polar cells and outer border cells reached the oocyte-nurse-cell border. (F) A border cell cluster containing GFP-negative *hpo* mutant polar cells and border cells failed to migrate. (G) A border cell cluster containing GFP-positive polar cells and GFP-negative *FRT42D* control outer border cells reached the oocyte-nurse-cell border. (H) A border cell cluster containing GFP-positive polar cells and some GFP-negative *yki* mutant outer border cells migrated >75%. (I) A diagram demonstrates colors representing the distance of border cell migration. (J) Quantification and percentage distribution of border cell migration. Only border cell clusters with GFP-positive polar cells and GFP-negative outer border cells were counted. *wts* mutation in outer border cells severely impaired migration. (K) Border cell clusters were categorized into two groups. The group with mutant outer border cells contained GFP-positive polar cells and GFP-negative mutant outer border cells; the group with mutant outer border/polar cells contained one or two GFP-negative mutant polar cells and GFP-negative mutant outer border cells. The migratory defect was more severe in the group with mutant outer border/polar cells than it was in the group with mutant outer border cells. (L) Only border cell clusters with GFP-positive polar cells and some GFP-negative outer polar cells were counted. *yki* mutation did not affect migration 3 days after clone induction. Wilcoxon rank-sum test, $^{**}P < 0.01$ comparing with control; $^{\#\#}P < 0.01$. Bar, 20 μm in A–H.

that *wts* is required for border cell migration. No border cell cluster containing both *wts* mutant outer border cells and polar cells was observed (see *Discussion*). The migration of border cell clusters with *hpo* mutant outer border cells was attenuated compared with that in the *FRT42D* control (Figure 1, C, D, and K), suggesting that *hpo* is also required for border cell migration. Importantly, when both outer polar cells and border cells were mutant for *hpo*, the defect was more severe than it was when only outer border cells were mutant for *hpo* (Figure 1, E, F, and K). This result suggests that Hpo plays roles in both outer border cells and polar cells for migration of the border cell cluster. Although YAP has been demonstrated to induce EMT and cell migration in cultured cells (Zhao *et al.* 2008a; Zhang *et al.* 2009; Xu *et al.* 2011), migration of border cell clusters containing yki^{B5} mutant outer border cells was normal compared with that in the *FRT42D* control 3 days after clone induction (Figure 1, G, H, and L), indicating that *yki* in outer border cells is not required for border cell migration. We did not examine *yki* mutant clones for border cell migration at day 6 after clone induction because most *yki* mutant cells differentiated into polar cells at day 6 as reported previously (Chen *et al.* 2011).

Overexpression of hpo in outer border cells disrupts border cell migration

To examine roles of the Hippo pathway in outer border cells, we used *slbo-Gal4* driver to overexpress or knockdown Hippo pathway components in these cells specifically (Rorth *et al.* 1998; Pinheiro and Montell 2004; Inaki *et al.* 2012). RNA interference (RNAi) lines obtained from the Bloomington Drosophila Stock Center or Vienna Drosophila Resource Center have been tested in previous studies (Das Thakur *et al.* 2010; Genevet *et al.* 2010; Boggiano *et al.* 2011; Reddy and Irvine 2011; Chen and Verheyen 2012; Nagaraj *et al.* 2012; Poernbacher *et al.* 2012; Jukam *et al.* 2013). *slbo-Gal4*-driven *UAS-GFP* flies were used as controls. Unexpectedly, knockdown of *hpo* or *wts* in outer border cells by RNAi lines did not affect border cell migration significantly (Figure 2A). It is possible that knockdown of *hpo* and *wts* with *slbo-Gal4* driver was not efficient enough to cause migratory defects in border cells compared with *hpo* and *wts* mutations (Figure 1). Phosphorylation of Yki on Ser residues at positions 111, 168, and 250 by Wts prevents nuclear localization of Yki. Yki with Ser-to-Ala mutation at residues 111/168/250 has been shown to stay in the nucleus to induce downstream targets constitutively (Oh and Irvine 2008, 2009). Neither overexpression of wild-type *yki*, *yki-S168A*, and *yki-S111A, -S168A, -S250A* (*yki-3SA*) nor knockdown of *yki* by various RNAi lines in outer border cells affected border cell migration (Figure 2A). Together with our result from *yki* mutant clones in Figure 1L, these data suggest that *yki* is dispensable in outer border cells for migration of the border cell cluster. Although knockdown of *hpo*, *wts*, and *yki* or overexpression of *yki-3SA* with *slbo-Gal4* did not affect border cell migration, overexpression of *hpo* with *slbo-Gal4* dramatically disrupted migration (Figure 2A). Canonically, Hpo acts by inhibiting Yki activity. However, overexpression of *yki-3SA* did not rescue the migratory defect caused by overexpression of *hpo* (Figure 2A), suggesting that *hpo* controls border cell migration in a *yki*-independent manner. The Hippo pathway has been demonstrated to control actin organization in the wing imaginal disc independently of Yki activity (Fernandez *et al.* 2011). Since rearrangement of the actin cytoskeleton is crucial for cell migration, we tested whether Hpo affected actin structures. In the control group, filamentous actin (F-actin) was distributed at the front edge of the border cell cluster and very little F-actin was localized to the boundary between border cells at stages 9 and 10 (Figure 2, B and D). Overexpression of *hpo* led to mis-localization of F-actin to the boundary between border cells and between outer border and polar cells (Figure 2, C and E), suggesting that *hpo* regulates localization of F-actin at the front edge during cell migration. While we were conducting this study, Lucas *et al.* (2013) showed that polarized distribution of F-actin was disrupted in *hpo* and *wts* mutant border cells, which is consistent with our finding.

The Hippo pathway is required in polar cells for migration of the border cell cluster

Because the migratory defect of border cell clusters was more severe when *hpo* was mutant in both polar and outer border cells than it was when *hpo* was mutant in outer border cells alone (Figure 1K), we hypothesized that the Hippo pathway also played a role in polar cells to control the migration of border cell clusters. To examine this hypothesis, we used *upd-Gal4* to overexpress or knockdown Hippo pathway components in polar cells specifically (Bai and Montell 2002). Because the *10Xstat*::*gfp* (*stat-GFP*) reporter line was used for some experiments (see below), either *UAS-GFP* or *UAS-RFP* was used to visualize *upd-Gal4* expression in polar cells and as a control (Figure 3, A and I). Knockdown of *hpo* or *wts* in polar cells severely disrupted border cell migration (Figure 3, B–D and H), suggesting that *hpo* and *wts* are required in polar cells for the migration of border cell clusters. In the canonical understanding of the Hippo pathway, Hpo and Wts phosphorylate and inactivate Yki. Consistently, overexpression of *yki-3SA* in polar cells showed a similar migratory defect as *hpo* or *wts* knockdown (Figure 3, J, K, and O). Knockdown of *yki* or overexpression of *hpo* in polar cells did not affect border cell migration (Figure 3, E, G, and H). Although Yki predominantly forms a transcription complex with Sd (Goulev *et al.* 2008; Ota and Sasaki 2008; Wu *et al.* 2008; Zhang *et al.* 2008; Zhao *et al.* 2008a), it may also interact with other transcription factors (Alarcon *et al.* 2009; Zhang *et al.* 2011a). We tested whether Sd is required for Yki function in polar cells. Knockdown or overexpression of *sd* alone in polar cells did not affect border cell migration (Figure 3, F, H, L, and O). Knockdown of *sd* in polar cells did alleviate the migratory defect caused by overexpression of *yki-3SA* (Figure 3, M and O), suggesting that *sd* is required for Yki in polar cells to regulate the migration of border cell clusters.

The Hippo pathway controls the number of polar cells

Hippo signaling is a tumor-suppressing pathway inhibiting cell proliferation. When *wts* was knocked down or *yki* was overexpressed with *upd-Gal4*, 5–15 Fas3-positive cells were

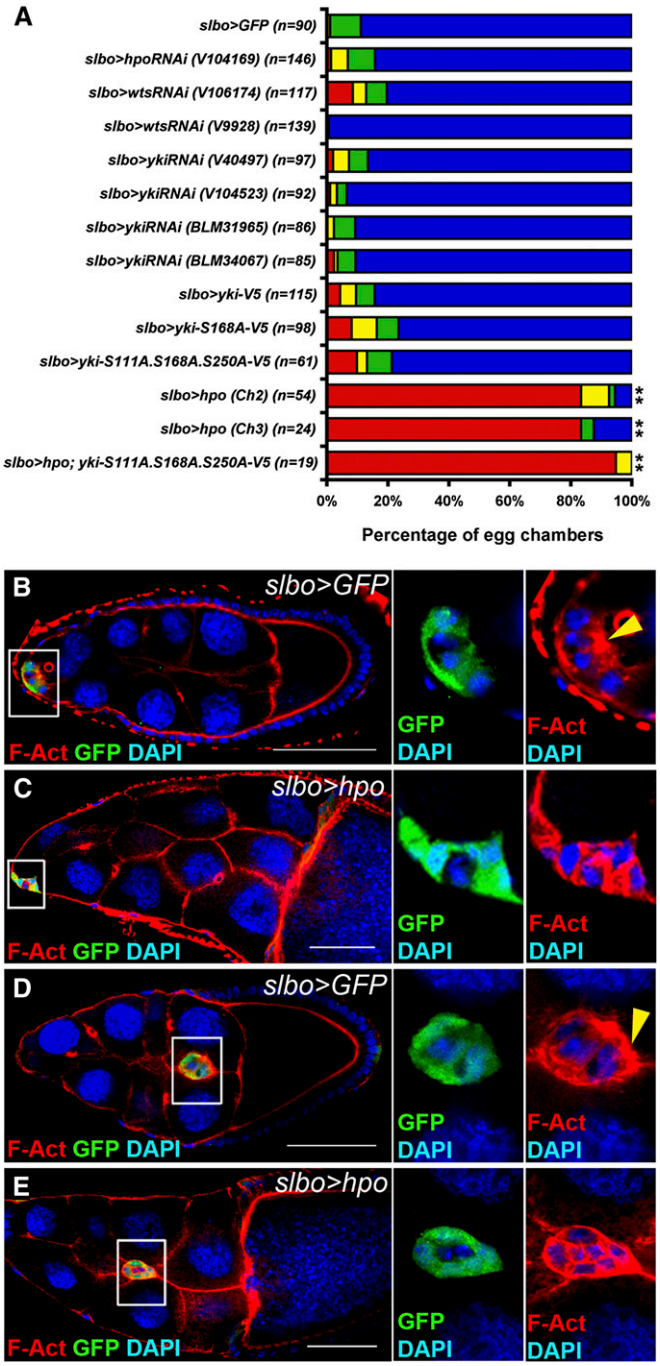

Figure 2 Overexpression of *hpo* with *slbo-Gal4* increases filamentous actin between border cells and disrupts border cell migration. *slbo-Gal4* was used to overexpress or knock down genes specifically in outer border cells. *UAS-GFP* driven by *slbo-Gal4* was used as a control. The flies were dissected after being grown at 29° for 6 days. (A) Stage-10 egg chambers were selected for quantification. Colors of the graph represent the migratory distance depicted in Figure 1I. Numbers of egg chambers examined are indicated. Inhibition of *hpo*, *wts*, or *yki* in outer border cells did not affect border cell migration in comparison with that in the control. Overexpression of *yki* or constitutively active forms of *yki*, *yki-S168A*, and *yki-S111A.S168A.S250A* with *slbo-Gal4* driver did not affect border cell migration. Overexpression of *hpo* with two different *UAS-hpo* lines severely disrupted border cell migration. The migratory defect was not alleviated when *yki-S111A.S168A.S250A* were overexpressed. Wilcoxon rank-sum test, $^{**}P < 0.01$. (B–E) The ovaries were stained with anti-GFP, DAPI, and phalloidin for filamentous actin (F-actin). Border cell clusters were delaminating from the follicular epithelium (B and C) or migrating toward the oocyte (D and E). High magnification of border cell clusters is shown on the right. (B) In the *UAS-GFP* control, F-actin was enriched in the apical region of a border cell cluster indicated by a yellow arrowhead. (C) When *hpo* was overexpressed, F-actin was enriched between border cells. (D) In the *UAS-GFP* control, F-actin was enriched in the outer rim, especially in the front edge, of a migrating border cell cluster indicated by a yellow arrowhead. (E) When *hpo* was overexpressed, F-actin was enriched in boundaries between border cells as well as the outer rim. Bar, 20 μm in B–E.

observed (Figure 3, C, J, K, and M). Since our previous study has demonstrated that the Hippo pathway controls polar cell fate during early oogenesis (Chen *et al.* 2011), it is crucial to examine whether modulation of the Hippo pathway disrupts polar cell fate determination, which in turn attenuates border cell migration. While there were usually two polar cells at the anterior end of an egg chamber (Figure 4, A, D, and F), knockdown of *wts* with *upd-Gal4* led to more than two cells expressing polar cells markers *PZ80-lacZ* (a *lacZ* insertion in *Fas3*) and *A101-lacZ* (a *lacZ* insertion in *neur*) (Figure 4, A–E). In addition, elevated levels of Armadillo (Arm) at the apical side were observed in *wts* knockdown cells, which was similar to the Arm pattern of polar cells in the *UAS-GFP* control (Figure 4, F and G) (Peifer *et al.* 1993). These results suggest that knockdown of *wts* with *upd-Gal4* increases polar cells instead of disrupting polar cell fate. Furthermore, polar cells usually withdraw from cell cycle by stage 2, but we observed Cyclin B (CycB)-positive polar cells when we knocked down *wts* with *upd-Gal4* after stage 7 (Figure 4, H and I). This result suggests that *wts*-knockdown polar cells remain proliferating during mid-oogenesis. Interestingly, extra polar cells may not interfere with the migration of border cell clusters since many of them migrated normally (Figure 3M). To further confirm that the Hippo pathway is required for border migration in addition to its roles in polar cell fate determination during early oogenesis, we used the temperature-sensitive Gal80 system (Mcguire *et al.* 2003). Attenuation of border cell migration was observed after the flies were incubated at 29° for as few as 40 hr (Supporting Information, Figure S1). The effect of the 40-hr incubation was weaker than that of the 54-hr incubation at 29°, which could be a result of either continuous requirement of the Hippo signaling in polar cells or protein perdurance of Wts. Since polar cells are formed before stage 2 and it takes ~36 hr for egg chambers to develop from stage 2 to stage 9 (Horne-Badovinac and Bilder 2005; Ma *et al.* 2014), this result demonstrates a role of the Hippo pathway in border cell migration after determination of polar cell fate during early oogenesis.

The Hippo pathway is required in polar cells for the induction of outer border cells

In addition to the migratory defect, border cell clusters usually failed to form when *wts* was knocked down or *yki-3SA* was overexpressed in polar cells (Figure 3, C, J, and K).

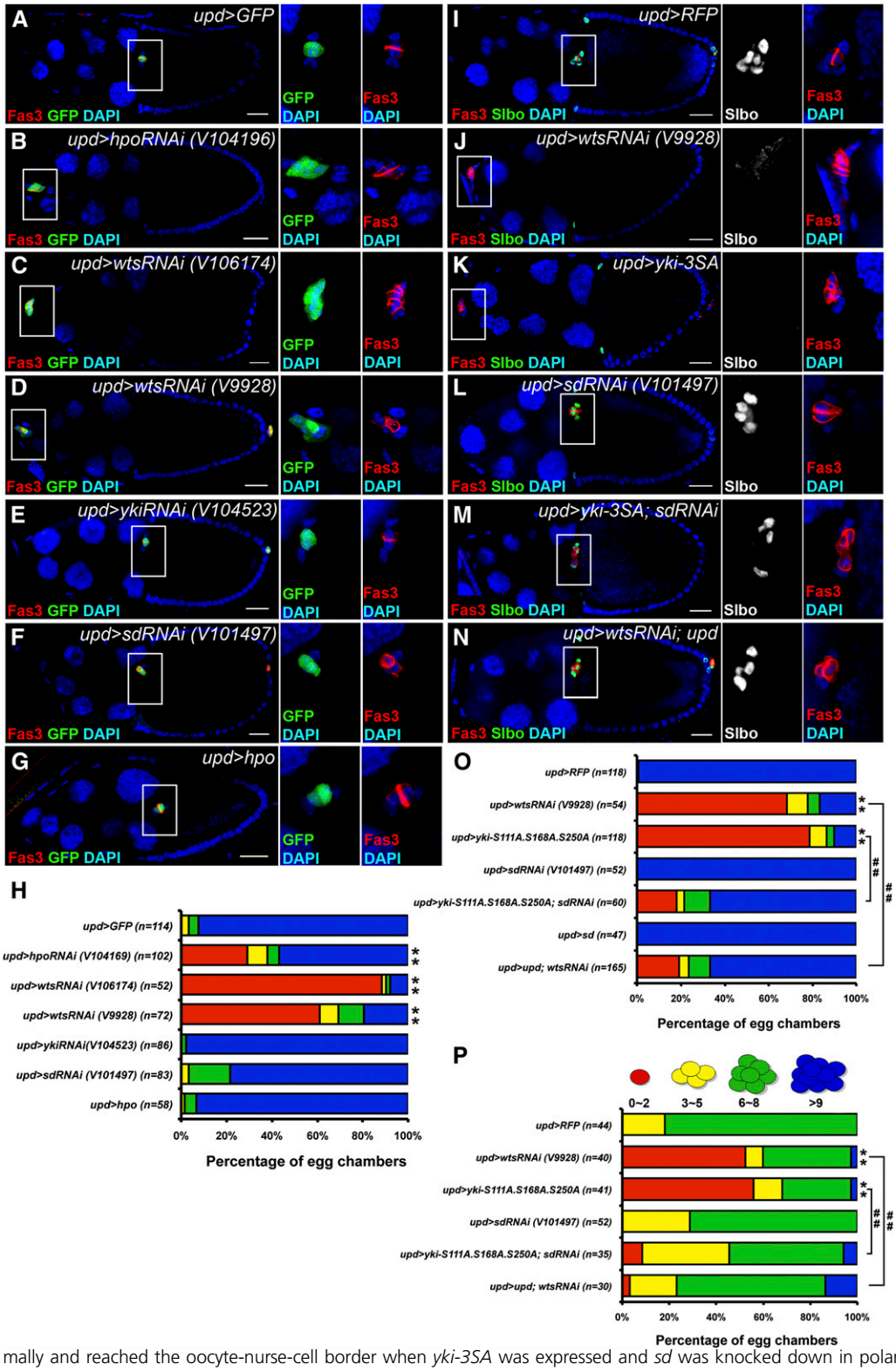

Figure 3 *hpo* and *wts* are required in polar cells for the migration of border cell clusters and the induction of outer border cells. *upd-Gal4* was used to overexpress or knock down genes specifically in polar cells. *UAS-GFP* or *UAS-RFP* driven by *upd-Gal4* was used as a control (A and I). *UAS-GFP* was included in B–G. The flies were dissected after being grown at 29° for 5–6 days. The ovaries were immunostained with anti-Fas3 and anti-GFP (A–G) or anti-Slbo (I–N). Stage-10 egg chambers were selected and oriented as anterior toward the left (A–G and I–N). High magnification views of border cell clusters are shown on the right. (A) In the *UAS-GFP* control, a border cell cluster migrated normally and reached the oocyte-nurse-cell border. (B) Knockdown of *hpo* impaired border cell migration. (C and D) Knockdown of *wts* with two RNAi lines impaired border cell migration. Extra Fas3-positive cells were observed. (E) Knockdown of *yki* did not impair border cell migration. (F) Knockdown of *sd* did not impair border cell migration. (G) Overexpression of *hpo* did not impair border cell migration. (H) Quantification and percentage distribution of border cell migration. Wilcoxon rank-sum test, $^{**}P < 0.01$. (I) In the *UAS-RFP* control, a border cell cluster with at least five Slbo-positive cells migrated normally and reached the oocyte-nurse-cell border. (J) No Slbo-positive cell was observed, and the border cell cluster did not migrate when *wts* was knocked down in polar cells. (K) No Slbo-positive cell was observed, and the border cell cluster did not migrate when *yki-S111A.S168A.S250A* (*yki-3SA*) was expressed in polar cells. (L) A border cell cluster with at least four Slbo-positive cells migrated normally when *sd* was knocked down in polar cells. (M) A border cell cluster with at least four Slbo-positive cells migrated normally and reached the oocyte-nurse-cell border when *yki-3SA* was expressed and *sd* was knocked down in polar cells. (N) A border cell cluster with at least four Slbo-positive cells migrated normally and reached the oocyte-nurse-cell border when *wts* was knocked down and *upd* was overexpressed in polar cells. (O) Quantification and percentage distribution of border cell migration. (P) Quantification and percentage distribution of Slbo-positive cell numbers in stage-10 egg chambers. Wilcoxon rank-sum test, $^{**}P < 0.01$ comparing with control; $^{\#\#}P < 0.01$. Bar, 20 μm in A–G and I–N.

We hypothesized that the Hippo pathway in polar cells might be essential for the induction of outer border cells. To test this hypothesis, we stained ovaries with an antibody against Slbo, a marker for border cells (Rorth *et al.* 2000), and we counted Slbo-positive cells. Stage 10 egg chambers were categorized based on the number of Slbo-positive

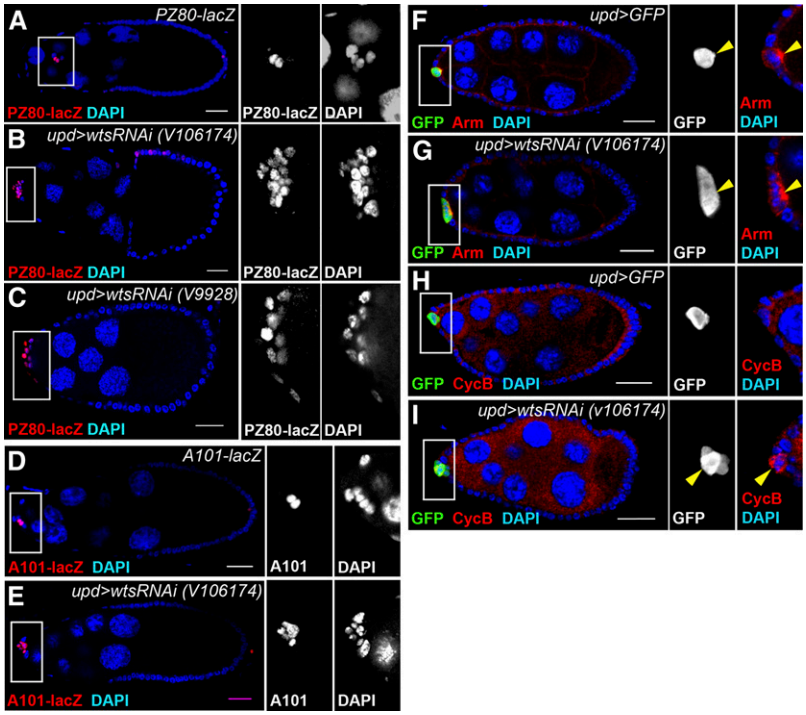

Figure 4 The Hippo pathway controls the number of polar cells. *wts* was knocked down in polar cells by using *upd-Gal4*. The ovaries were immunostained with anti-Fas3, anti-β-Gal (A–E), anti-Arm (F and G), and anti-CycB (H and I). Stage-9 or stage-10 egg chambers (A–E) or stage 7–8 egg chambers (F–I) were selected and oriented as anterior toward the left. High magnification views of border cell clusters are shown in the panels on the right. (A) In the control, exactly two polar cells in the border cell cluster expressing *PZ80-lacZ* were observed. (B and C) Knockdown of *wts* with two different RNAi lines increased the number of cells expressing *PZ80-lacZ*. (D) In the control, exactly two polar cells in the border cell cluster expressing *A101-lacZ* were observed. (E) Knockdown of *wts* increased the number of cells expressing *A101-lacZ*. (F) In the control, Arm was enriched in the apical region of two polar cells indicated by yellow arrowheads. (G) Knockdown of *wts* increased the number of cells with Arm enriched in the apical region indicated by yellow arrowheads. (H) In the control, polar cells were not positive for CycB. (I) Knockdown of *wts* led to ectopic CycB-positive cells indicated by yellow arrowheads. Bar, 20 μm in A–I.

cells in border cell clusters (Figure 3P). Most control egg chambers contained six to eight Slbo-positive cells in each border cell cluster (Figure 3, I and P). Knockdown of *wts* or overexpression of *yki-3SA* in polar cells strongly reduced the number of cells positive for Slbo (Figure 3, J, K, and P), demonstrating that the Hippo pathway in polar cells is crucial for the induction of outer border cells. Knockdown of *sd* alone in polar cells did not affect outer border cell induction (Figure 3, L and P). In contrast, knockdown of *sd* in *yki-3SA* overexpressing polar cells increased the cell number of Slbo-positive cells (Figure 3, M and P), suggesting that the function of Yki in outer border cell induction requires *sd*.

The Hippo pathway in polar cells is required for the activation of JAK/STAT signaling in outer border cells

Slbo acts downstream of JAK/STAT signaling in regulating border cell fate and migration (Beccari *et al.* 2002; Silver *et al.* 2005). Since the Hippo pathway in polar cells controls the number of Slbo-positive cells as well as the migration of border cell clusters, it is possible that it regulates JAK/STAT signaling activity in outer border cells. We examined JAK/STAT signaling activity using a reporter line *10Xstat*::*gfp* (*stat-GFP*). In the control group, GFP was detected in outer border cells throughout the process of border cell induction and migration (Figure 5, A and B). Knockdown of *wts* or overexpression of *yki-3SA* in polar cells significantly reduced the level of *stat*-GFP in outer border cells (Figure 5, C and D). Overexpression of *sd* in polar cells did not affect the level of *stat*-GFP in outer border cells (Figure 5E). This result suggests that the Hippo pathway in polar cells is required for the activation of JAK/STAT signaling in outer border cells.

The Hippo pathway controls the expression of upd in polar cells

During border cell induction and migration, JAK/STAT signaling in outer border cells is activated by Upd secreted from polar cells (Silver and Montell 2001; Beccari *et al.* 2002; Ghiglione *et al.* 2002). Since the Hippo pathway is required in polar cells for border cell induction and migration, it is possible that the Hippo pathway controls *upd* expression cell-autonomously in polar cells. To test this, we generated mitotic clones mutant for *yki* or *hpo* in an *upd-lacZ* background. *FRT42D* clones were generated as controls. We specifically analyzed those pairs of polar cells with a GFP-negative *yki* or *hpo* mutant polar cell and a GFP-positive control polar cell (Figure 6, A–C). In *FRT42D* clones, expression of *upd-lacZ* was comparable between GFP-negative *FRT42D* and the adjacent GFP-positive polar cells (Figure 6, A and D). *yki* mutant polar cells expressed more *upd-lacZ* in comparison with the adjacent control polar cells; *hpo* mutant polar cells expressed less *upd-lacZ* in comparison with the adjacent control polar cells (Figure 6, B–D). This result shows that the Hippo pathway controls *upd* expression in polar cells. In addition, some *yki* mutant cells expressed ectopic *upd-lacZ* and induced ectopic border cell migration (Figure S2). Importantly, the levels of *upd*-lacZ in polar cells and *stat*-GFP in outer border cells were not affected when outer border cells were mutant for *hpo* (Figure S3, A and B). Outer border cells mutant for *hpo* remained positive for Slbo (Figure S3C). These results exclude the possibility that the Hippo pathway regulates *upd* expression in a non-cell-autonomous manner. Together, our data demonstrate that the Hippo pathway promotes *upd* expression in polar cells.

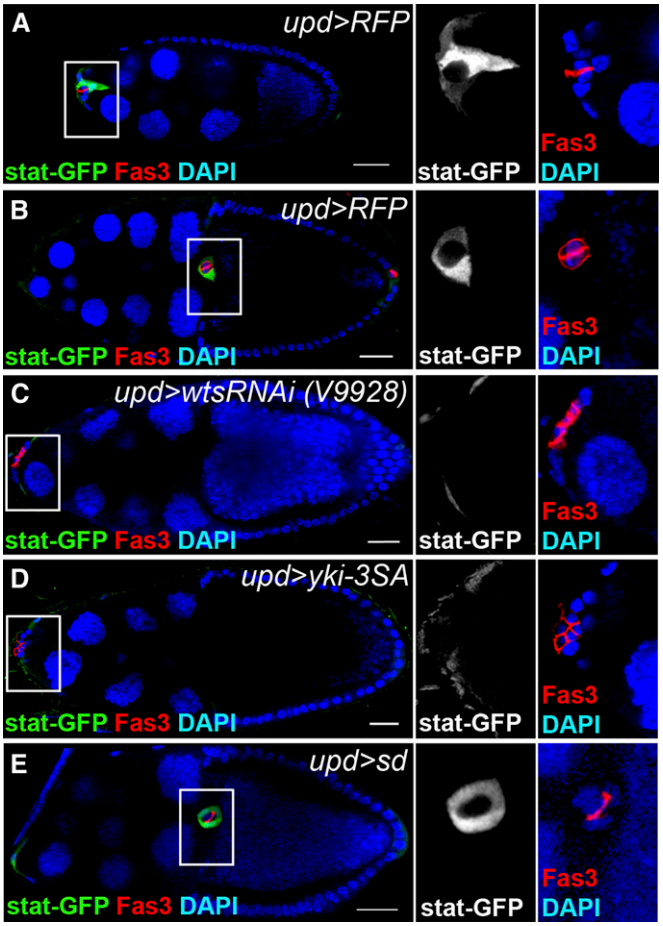

Figure 5 The Hippo pathway in polar cells is required for the activation of the JAK/STAT signaling in outer border cells. *upd-Gal4* was used to knockdown or overexpress genes in a *10Xstat::gfp* (stat-GFP) reporter background (A–E). *UAS-RFP* driven by *upd-Gal4* was used as a control (A and B). The flies were incubated at 29° for 5–6 days before dissection. The ovaries were immunostained with anti-GFP and anti-Fas3. Egg chambers at stage 9 (A) or stage 10 (B–E) were selected and oriented as anterior toward the left. High magnification views of border cell clusters are shown in the panels on the right. (A) In the *UAS-RFP* control, a border cell cluster with *stat*-GFP-positive outer border cells started to migrate at stage 9. (B) A border cell cluster with *stat*-GFP-positive outer border cells migrated normally and reached the oocyte-nurse-cell border at stage 10. (C) The *stat*-GFP level was reduced, and the border cell cluster did not migrate when *wts* was knocked down in polar cells. (D) The *stat*-GFP level was reduced, and the border cell cluster did not migrate when *yki-3SA* was overexpressed in polar cells. (E) A border cell cluster with *stat*-GFP-positive outer border cells migrated normally and reached the oocyte-nurse-cell border when *sd* was overexpressed in polar cells. Bar, 20 μm in A–E.

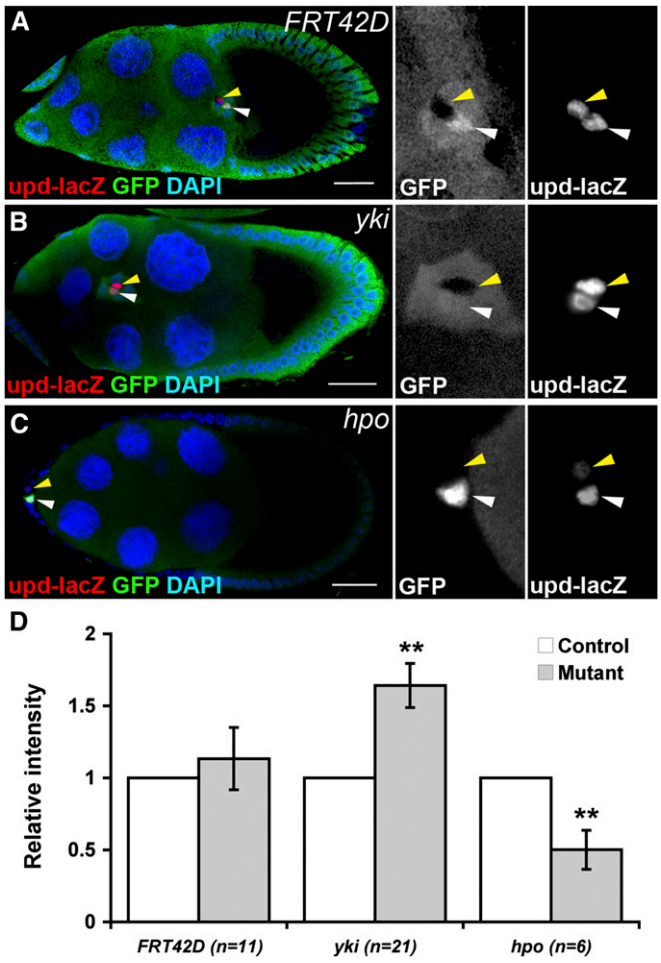

Figure 6 The Hippo pathway controls the expression of *upd* in polar cells. GFP-negative mitotic clones were generated in *FRT42D* (A), *FRT42D* $hpo^{42\text{-}47}$ (B), and *FRT42D* yki^{B5} (C) in an *upd-lacZ* background. The ovaries were immunostained with anti-Fas3 and anti-β-galactosidase (β-Gal). Stage-9 and stage-10 egg chambers were selected and oriented as anterior toward the left. High magnification views of border cell clusters are shown on the right. (A) An *FRT42D* control polar cell (yellow arrowheads) was positive for β-Gal as the neighboring GFP-positive polar cell (white arrowheads). (B) A *yki* mutant polar cell (yellow arrowheads) showed a higher β-Gal level than that of the neighboring GFP-positive polar cell (white arrowheads). (C) A *hpo* mutant polar cell (yellow arrowheads) showed a lower β-Gal level than that of the neighboring GFP-positive polar cell (white arrowheads). (D) Quantification of β-Gal levels in *FRT42D*, *yki*, and *hpo* mutant polar cells in an *upd-lacZ* background. The bar graph is shown as mean ± SEM. Paired Student's *t*-test, $^{**}P < 0.01$. Bar, 20 μm in A–C.

The Hippo pathway in polar cells controls border cell induction and migration through regulating upd expression

If Upd functions downstream of the Hippo pathway in polar cells to promote outer border cell induction and migration, overexpression of *upd* in polar cells should rescue the defects caused by *wts* knockdown. Indeed, overexpression of *upd* in polar cells promoted border cell migration and increased the number of Slbo-positive cells in *wts* knockdown cells (Figure 3, N, O, and P), suggesting that Upd functionally acts downstream of the Hippo pathway in polar cells to induce border cell formation and promote migration.

Discussion

In this study, we identified two roles of the Hippo pathway in border cell migration: (1) in outer border cells, the Hippo pathway regulates localization of the actin cytoskeleton; (2)

in polar cells, the Hippo pathway promotes *upd* expression to regulate the induction of outer border cells and border cell migration (Figure 7). A recent study reports that some Hippo pathway components regulate polarization of actin cytoskeleton of the border cell cluster (Lucas *et al.* 2013). They show that Wts is activated and localized to the interface between border cells, where Wts phosphorylates and inhibits Enabled, a crucial actin regulator. Thus actin polymerization is restricted to the outer rim of the migrating cluster instead of to the boundaries between border cells. In our study, overexpression of *hpo* led to abnormal actin polymerization between border cells and attenuated cell migration (Figure 2, B–E). Since asymmetric distribution and activation of Wts is crucial (Lucas *et al.* 2013), either gain- or loss-of-function of the Hippo pathway components may disrupt the distribution or activation of Wts, thereby attenuating cell migration. Both Lucas *et al.* (2013) and we show that Yki does not act downstream of the Hippo pathway in regulating actin polymerization as described in a previous study using the *Drosophila* wing imaginal disc as a model (Fernandez *et al.* 2011). While Lucas *et al.* did not dissociate roles of the Hippo pathway in polar cells and outer border cells during border cell migration, we additionally demonstrated here that the Hippo pathway controls JAK/STAT signaling through promoting *upd* expression in polar cells. Thus, we have identified a new role of the Hippo pathway in regulating border cell migration.

We did not observe any migratory defect when *hpo* or *wts* was knocked down in outer border cells with *slbo-Gal4* (Figure 2A). On the contrary, border cell clusters with *hpo* or *wts* mutant border cells showed severe migratory defects (Figure 1). We did find strong migratory defects when we overexpressed *hpo* with *slbo-Gal4*, suggesting that the *slbo-Gal4* driver indeed promoted transgene expression efficiently in outer border cells. Therefore, it is likely that driving RNAi with *slbo-Gal4* may not efficiently knock down genes prior to or during border cell migration in outer border cells when the target protein is stable (Yang *et al.* 2012).

In our experimental conditions, border cell clusters of entirely *wts* mutant cells have never been observed. Furthermore, border cell clusters containing *hpo* or *wts* mutant polar cells and wild-type outer border cells have never been obtained (Figure 1). This phenomenon can be explained by our previous findings that the Hippo pathway is involved in polar cell determination (Chen *et al.* 2011). The Hippo pathway is critical to promote polar cell fate by suppressing Notch signaling during early oogenesis. Because mitotic mutant clones of *hpo* or *wts* were usually induced at early stages, we could not observe *hpo* or *wts* mutant polar cells. In addition to polar cell fate determination, we find that the Hippo pathway controls polar cell numbers by regulating cell proliferation. During cell fate determination in early oogenesis, the Hippo pathway promotes polar cell fate. Therefore, mutation of *hpo* or *wts* suppresses polar cell formation (Chen *et al.* 2011). Once polar cells are determined, the Hippo pathway suppresses proliferation of polar cells.

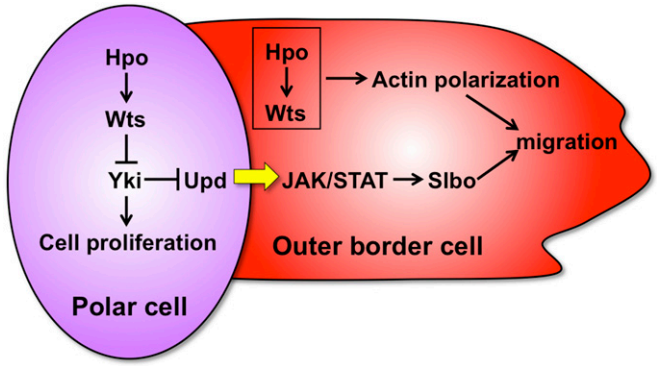

Figure 7 The Hippo pathway controls the migration of border cell clusters through two mechanisms. In outer border cells, the Hippo pathway regulates polarized distribution of F-actin and controls border cell migration. In polar cells, the Hippo pathway induces *upd* expression. Upd from polar cells activates JAK/STAT signaling in outer border cells. Activation of JAK/STAT signaling induces *slbo* expression, which promotes the formation and migration of border cell clusters. The Hippo pathway also controls polar cell proliferation.

The *upd-Gal4* is expressed after polar cell fate is determined, so knockdown of *wts* or overexpression of *yki-3SA* with *upd-Gal4* should increase polar cell numbers through promoting cell proliferation. Although these *wts*-knockdown cells resembled polar cells based on their expression of *Fas3*, *PZ80-lacZ*, and *A101-lacZ* and an increased level of Arm at the apical region (Figure 3 and Figure 4), they were positive for Eya after stage 7 (Figure S4). Eya should be detected only in follicle cell precursors and main-body follicle cells, but not in polar cells (Bai and Montell 2002). It is possible that the Hippo pathway controls *eya* expression because ectopic Eya is also detected in posterior follicle cells mutant for *hpo* or *wts* after stage 8 (Meignin *et al.* 2007; Polesello and Tapon 2007; Yu *et al.* 2008). Although knockdown of *wts* or overexpression of *yki-3SA* increased polar cells, these polar cells did not induce *stat-GFP* in neighboring cells (Figure 5, C and D). This result suggests that polar cell fate can be dissociated from *upd* expression.

In addition to polar cell fate determination and cell proliferation (Chen *et al.* 2011), the Hippo pathway plays roles in multiple steps of follicle cell development by interacting with various signaling cascades. In follicle stem cells, the Hippo pathway acts in parallel with Hedgehog signaling in regulating follicle stem cell proliferation (Huang and Kalderon 2014). In posterior follicle cells, the Hippo pathway is required for inducing their Gurken- and Notch-dependent differentiation (Meignin *et al.* 2007; Polesello and Tapon 2007; Yu *et al.* 2008). In this study, we find that the Hippo pathway controls JAK/STAT signaling through regulation of *upd* expression during border cell migration. All together, integration with other signaling cascades to control various cellular functions appears to be a particularly prominent feature of the Hippo pathway. In the *Drosophila* eye, the Hippo pathway has also been shown to regulate cell proliferation and apoptosis during early development as well as

cell fate determination in postmitotic photoreceptors (Jukam *et al.* 2013). As to the regulation of actin cytoskeleton, the Hippo pathway has been demonstrated to respond to mechanical force and cell contact by acting downstream of cytoskeletal rearrangement (Sansores-Garcia *et al.* 2011). Here during cell migration, the Hippo pathway regulates actin polymerization (Figure 2) (Lucas *et al.* 2013). It will be interesting to examine whether cytoskeletal rearrangement also regulates the Hippo pathway to control cell migration.

Previous studies have shown that inactivation of the Hippo pathway or activation of Yki induces *upd* expression in midgut and certain regions of the wing imaginal discs where *upd* is endogenously expressed (Karpowicz *et al.* 2010; Ren *et al.* 2010a; Shaw *et al.* 2010; Staley and Irvine 2010). In our study, we found that *upd* expression was increased in *yki* mutant clones (Figure 6), suggesting that activation of the Hippo pathway or inactivation of Yki induces *upd* expression in polar cells. This difference may be caused by cell type- or tissue-specific functions or interactions of Yki with other proteins. Our results show that Yki requires Sd to function in polar cells (Figure 3, M, O, and P), so it is less likely that Yki directly represses *upd* expression through interacting with other transcription factors. It requires further analysis to demonstrate whether Yki and Sd directly or indirectly suppress *upd* expression in polar cells.

In conclusion, our data show that the Hippo pathway regulates border cell migration through controlling polarized distribution of F-actin and interacting with the JAK/STAT-signaling pathway. Both Hippo and JAK/STAT pathways are involved in various aspects of tumorigenesis, such as tumor growth, EMT, and metastasis of tumor cells (Hou *et al.* 2002; Pan 2010; Wang and Huang 2010; Harvey *et al.* 2013). It will be interesting to study the interplay of these two pathways in cancer formation.

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Literature Cited

Alarcon, C., A. I. Zaromytidou, Q. Xi, S. Gao, J. Yu *et al.*, 2009 Nuclear CDKs drive Smad transcriptional activation and turnover in BMP and TGF-beta pathways. Cell 139: 757–769.

Badouel, C., A. Garg, and H. McNeill, 2009 Herding Hippos: regulating growth in flies and man. Curr. Opin. Cell Biol. 21: 837–843.

Bai, J., and D. Montell, 2002 Eyes absent, a key repressor of polar cell fate during Drosophila oogenesis. Development 129: 5377–5388.

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 coactivator amplified in breast cancer. Cell 103: 1047–1058.

Beccari, S., L. Teixeira, and P. Rorth, 2002 The JAK/STAT pathway is required for border cell migration during Drosophila oogenesis. Mech. Dev. 111: 115–123.

Boggiano, J. C., P. J. Vanderzalm, and R. G. Fehon, 2011 Tao-1 phosphorylates Hippo/MST kinases to regulate the Hippo-Salvador-Warts tumor suppressor pathway. Dev. Cell 21: 888–895.

Borghese, L., G. Fletcher, J. Mathieu, A. Atzberger, W. C. Eades *et al.*, 2006 Systematic analysis of the transcriptional switch inducing migration of border cells. Dev. Cell 10: 497–508.

Chen, H. J., C. M. Wang, T. W. Wang, G. J. Liaw, T. H. Hsu *et al.*, 2011 The Hippo pathway controls polar cell fate through Notch signaling during Drosophila oogenesis. Dev. Biol. 357: 370–379.

Chen, J., and E. M. Verheyen, 2012 Homeodomain-interacting protein kinase regulates Yorkie activity to promote tissue growth. Curr. Biol. 22: 1582–1586.

Das Thakur, M., Y. Feng, R. Jagannathan, M. J. Seppa, J. B. Skeath *et al.*, 2010 Ajuba LIM proteins are negative regulators of the Hippo signaling pathway. Curr. Biol. 20: 657–662.

Dong, J., G. Feldmann, J. Huang, S. Wu, N. Zhang *et al.*, 2007 Elucidation of a universal size-control mechanism in Drosophila and mammals. Cell 130: 1120–1133.

Duchek, P., and P. Rorth, 2001 Guidance of cell migration by EGF receptor signaling during Drosophila oogenesis. Science 291: 131–133.

Duchek, P., K. Somogyi, G. Jekely, S. Beccari, and P. Rorth, 2001 Guidance of cell migration by the Drosophila PDGF/VEGF receptor. Cell 107: 17–26.

Fernandez, B. G., P. Gaspar, C. Bras-Pereira, B. Jezowska, S. R. Rebelo *et al.*, 2011 Actin-Capping Protein and the Hippo pathway regulate F-actin and tissue growth in Drosophila. Development 138: 2337–2346.

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.

Ghiglione, C., O. Devergne, E. Georgenthum, F. Carballes, C. Medioni *et al.*, 2002 The Drosophila cytokine receptor Domeless controls border cell migration and epithelial polarization during oogenesis. Development 129: 5437–5447.

Goulev, Y., J. D. Fauny, B. Gonzalez-Marti, D. Flagiello, J. Silber *et al.*, 2008 SCALLOPED interacts with YORKIE, the nuclear effector of the hippo tumor-suppressor pathway in Drosophila. Curr. Biol. 18: 435–441.

Halder, G., and R. L. Johnson, 2011 Hippo signaling: growth control and beyond. Development 138: 9–22.

Hall, C. A., R. Wang, J. Miao, E. Oliva, X. Shen *et al.*, 2010 Hippo pathway effector Yap is an ovarian cancer oncogene. Cancer Res. 70: 8517–8525.

Harvey, K., and N. Tapon, 2007 The Salvador-Warts-Hippo pathway: an emerging tumour-suppressor network. Nat. Rev. Cancer 7: 182–191.

Harvey, K. F., C. M. Pfleger, and I. K. Hariharan, 2003 The Drosophila Mst ortholog, hippo, restricts growth and cell proliferation and promotes apoptosis. Cell 114: 457–467.

Harvey, K. F., X. Zhang, and D. M. Thomas, 2013 The Hippo pathway and human cancer. Nat. Rev. Cancer 13: 246–257.

Horne-Badovinac, S., and D. Bilder, 2005 Mass transit: epithelial morphogenesis in the Drosophila egg chamber. Dev. Dyn. 232: 559–574.

Hou, S. X., Z. Zheng, X. Chen, and N. Perrimon, 2002 The Jak/STAT pathway in model organisms: emerging roles in cell movement. Dev. Cell 3: 765–778.

Huang, J., and D. Kalderon, 2014 Coupling of Hedgehog and Hippo pathways promotes stem cell maintenance by stimulating proliferation. J. Cell Biol. 205: 325–338.
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.
Inaki, M., S. Vishnu, A. Cliffe, and P. Rorth, 2012 Effective guidance of collective migration based on differences in cell states. Proc. Natl. Acad. Sci. USA 109: 2027–2032.
Jang, A. C., M. Starz-Gaiano, and D. J. Montell, 2007 Modeling migration and metastasis in Drosophila. J. Mammary Gland Biol. Neoplasia 12: 103–114.
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.
Jukam, D., B. Xie, J. Rister, D. Terrell, M. Charlton-Perkins *et al.*, 2013 Opposite feedbacks in the Hippo pathway for growth control and neural fate. Science 342: 1238016.
Justice, R. W., O. Zilian, D. F. Woods, M. Noll, and P. J. Bryant, 1995 The Drosophila tumor suppressor gene warts encodes a homolog of human myotonic dystrophy kinase and is required for the control of cell shape and proliferation. Genes Dev. 9: 534–546.
Karpowicz, P., J. Perez, and N. Perrimon, 2010 The Hippo tumor suppressor pathway regulates intestinal stem cell regeneration. Development 137: 4135–4145.
Lai, Z. C., X. Wei, T. Shimizu, E. Ramos, M. Rohrbaugh *et al.*, 2005 Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. Cell 120: 675–685.
Lian, I., J. Kim, H. Okazawa, J. Zhao, B. Zhao *et al.*, 2010 The role of YAP transcription coactivator in regulating stem cell self-renewal and differentiation. Genes Dev. 24: 1106–1118.
Lu, L., Y. Li, S. M. Kim, W. Bossuyt, P. Liu *et al.*, 2010 Hippo signaling is a potent in vivo growth and tumor suppressor pathway in the mammalian liver. Proc. Natl. Acad. Sci. USA 107: 1437–1442.
Lucas, E. P., I. Khanal, P. Gaspar, G. C. Fletcher, C. Polesello *et al.*, 2013 The Hippo pathway polarizes the actin cytoskeleton during collective migration of Drosophila border cells. J. Cell Biol. 201: 875–885.
Ma, H., H. Xu, and P. H. O'Farrell, 2014 Transmission of mitochondrial mutations and action of purifying selection in Drosophila melanogaster. Nat. Genet. 46: 393–397.
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.
McDonald, J. A., E. M. Pinheiro, L. Kadlec, T. Schupbach, and D. J. Montell, 2006 Multiple EGFR ligands participate in guiding migrating border cells. Dev. Biol. 296: 94–103.
McGuire, S. E., P. T. Le, A. J. Osborn, K. Matsumoto, and R. L. Davis, 2003 Spatiotemporal rescue of memory dysfunction in Drosophila. Science 302: 1765–1768.
Meignin, C., I. Alvarez-Garcia, I. Davis, and I. M. Palacios, 2007 The salvador-warts-hippo pathway is required for epithelial proliferation and axis specification in Drosophila. Curr. Biol. 17: 1871–1878.
Montell, D. J., 2003 Border-cell migration: the race is on. Nat. Rev. Mol. Cell Biol. 4: 13–24.
Montell, D. J., P. Rorth, 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.
Nagaraj, R., S. Gururaja-Rao, K. T. Jones, M. Slattery, N. Negre *et al.*, 2012 Control of mitochondrial structure and function by the Yorkie/YAP oncogenic pathway. Genes Dev. 26: 2027–2037.
Naora, H., and D. J. Montell, 2005 Ovarian cancer metastasis: integrating insights from disparate model organisms. Nat. Rev. Cancer 5: 355–366.
Oh, H., and K. D. Irvine, 2008 In vivo regulation of Yorkie phosphorylation and localization. Development 135: 1081–1088.
Oh, H., and K. D. Irvine, 2009 In vivo analysis of Yorkie phosphorylation sites. Oncogene 28: 1916–1927.
Oh, H., and K. D. Irvine, 2010 Yorkie: the final destination of Hippo signaling. Trends Cell Biol. 20: 410–417.
Ota, M., and H. Sasaki, 2008 Mammalian Tead proteins regulate cell proliferation and contact inhibition as transcriptional mediators of Hippo signaling. Development 135: 4059–4069.
Overholtzer, M., J. Zhang, G. A. Smolen, B. Muir, W. Li *et al.*, 2006 Transforming properties of YAP, a candidate oncogene on the chromosome 11q22 amplicon. Proc. Natl. Acad. Sci. USA 103: 12405–12410.
Pan, D., 2010 The hippo signaling pathway in development and cancer. Dev. Cell 19: 491–505.
Pantalacci, S., N. Tapon, and P. Leopold, 2003 The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. Nat. Cell Biol. 5: 921–927.
Peifer, M., S. Orsulic, D. Sweeton, and E. Wieschaus, 1993 A role for the Drosophila segment polarity gene armadillo in cell adhesion and cytoskeletal integrity during oogenesis. Development 118: 1191–1207.
Pinheiro, E. M., and D. J. Montell, 2004 Requirement for Par-6 and Bazooka in Drosophila border cell migration. Development 131: 5243–5251.
Poernbacher, I., R. Baumgartner, S. K. Marada, K. Edwards, and H. Stocker, 2012 Drosophila Pez acts in Hippo signaling to restrict intestinal stem cell proliferation. Curr. Biol. 22: 389–396.
Polesello, C., and N. Tapon, 2007 Salvador-warts-hippo signaling promotes Drosophila posterior follicle cell maturation downstream of notch. Curr. Biol. 17: 1864–1870.
Reddy, B. V., and K. D. Irvine, 2011 Regulation of Drosophila glial cell proliferation by Merlin-Hippo signaling. Development 138: 5201–5212.
Ren, F., B. Wang, T. Yue, E. Y. Yun, Y. T. Ip *et al.*, 2010a Hippo signaling regulates Drosophila intestine stem cell proliferation through multiple pathways. Proc. Natl. Acad. Sci. USA 107: 21064–21069.
Ren, F., L. Zhang, and J. Jiang, 2010b Hippo signaling regulates Yorkie nuclear localization and activity through 14–3-3 dependent and independent mechanisms. Dev. Biol. 337: 303–312.
Rorth, P., K. Szabo, A. Bailey, T. Laverty, J. Rehm *et al.*, 1998 Systematic gain-of-function genetics in Drosophila. Development 125: 1049–1057.
Rorth, P., K. Szabo, and G. Texido, 2000 The level of C/EBP protein is critical for cell migration during Drosophila oogenesis and is tightly controlled by regulated degradation. Mol. Cell 6: 23–30.
Ruohola, H., K. A. Bremer, D. Baker, J. R. Swedlow, L. Y. Jan *et al.*, 1991 Role of neurogenic genes in establishment of follicle cell fate and oocyte polarity during oogenesis in Drosophila. Cell 66: 433–449.
Sansores-Garcia, L., W. Bossuyt, K. Wada, S. Yonemura, C. Tao *et al.*, 2011 Modulating F-actin organization induces organ growth by affecting the Hippo pathway. EMBO J. 30: 2325–2335.
Shaw, R. L., A. Kohlmaier, C. Polesello, C. Veelken, B. A. Edgar *et al.*, 2010 The Hippo pathway regulates intestinal stem cell proliferation during Drosophila adult midgut regeneration. Development 137: 4147–4158.
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.
Silver, D. L., E. R. Geisbrecht, and D. J. Montell, 2005 Requirement for JAK/STAT signaling throughout border cell migration in Drosophila. Development 132: 3483–3492.

Staley, B. K., and K. D. Irvine, 2010 Warts and Yorkie mediate intestinal regeneration by influencing stem cell proliferation. Curr. Biol. 20: 1580–1587.

Thiery, J. P., H. Acloque, R. Y. Huang, and M. A. Nieto, 2009 Epithelial-mesenchymal transitions in development and disease. Cell 139: 871–890.

Wang, X., J. Bo, T. Bridges, K. D. Dugan, T. C. Pan *et al.*, 2006 Analysis of cell migration using whole-genome expression profiling of migratory cells in the Drosophila ovary. Dev. Cell 10: 483–495.

Wang, X., J. C. Adam, and D. Montell, 2007 Spatially localized Kuzbanian required for specific activation of Notch during border cell migration. Dev. Biol. 301: 532–540.

Wang, X., L. He, Y. I. Wu, K. M. Hahn, and D. J. Montell, 2010 Light-mediated activation reveals a key role for Rac in collective guidance of cell movement in vivo. Nat. Cell Biol. 12: 591–597.

Wang, Y. H., and M. L. Huang, 2010 Organogenesis and tumorigenesis: insight from the JAK/STAT pathway in the Drosophila eye. Dev. Dyn. 239: 2522–2533.

Watson, K. L., 1995 Drosophila warts–tumor suppressor and member of the myotonic dystrophy protein kinase family. BioEssays 17: 673–676.

Wei, X., T. Shimizu, and Z. C. Lai, 2007 Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. EMBO J. 26: 1772–1781.

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.

Wu, S., Y. Liu, Y. Zheng, J. Dong, and D. Pan, 2008 The TEAD/TEF family protein Scalloped mediates transcriptional output of the Hippo growth-regulatory pathway. Dev. Cell 14: 388–398.

Xu, M. Z., S. W. Chan, A. M. Liu, K. F. Wong, S. T. Fan *et al.*, 2011 AXL receptor kinase is a mediator of YAP-dependent oncogenic functions in hepatocellular carcinoma. Oncogene 30: 1229–1240.

Xu, T., and G. M. Rubin, 1993 Analysis of genetic mosaics in developing and adult Drosophila tissues. Development 117: 1223–1237.

Yang, N., M. Inaki, A. Cliffe, and P. Rorth, 2012 Microtubules and Lis-1/NudE/dynein regulate invasive cell-on-cell migration in Drosophila. PLoS ONE 7: e40632.

Yu, J., J. Poulton, Y. C. Huang, and W. M. Deng, 2008 The hippo pathway promotes Notch signaling in regulation of cell differentiation, proliferation, and oocyte polarity. PLoS ONE **3:** e1761.

Zeng, Q., and W. Hong, 2008 The emerging role of the hippo pathway in cell contact inhibition, organ size control, and cancer development in mammals. Cancer Cell 13: 188–192.

Zhang, J., J. Y. Ji, M. Yu, M. Overholtzer, G. A. Smolen *et al.*, 2009 YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. Nat. Cell Biol. 11: 1444–1450.

Zhang, L., F. Ren, Q. Zhang, Y. Chen, B. Wang *et al.*, 2008 The TEAD/TEF family of transcription factor Scalloped mediates Hippo signaling in organ size control. Dev. Cell 14: 377–387.

Zhang, T., Q. Zhou, and F. Pignoni, 2011a Yki/YAP, Sd/TEAD and Hth/MEIS control tissue specification in the Drosophila eye disc epithelium. PLoS ONE 6: e22278.

Zhang, X., J. George, S. Deb, J. L. Degoutin, E. A. Takano *et al.*, 2011b The Hippo pathway transcriptional co-activator, YAP, is an ovarian cancer oncogene. Oncogene 30: 2810–2822.

Zhao, B., X. Ye, J. Yu, L. Li, W. Li *et al.*, 2008a TEAD mediates YAP-dependent gene induction and growth control. Genes Dev. 22: 1962–1971.

Zhao, M., P. Szafranski, C. A. Hall, and S. Goode, 2008b Basolateral junctions utilize warts signaling to control epithelial-mesenchymal transition and proliferation crucial for migration and invasion of *Drosophila* ovarian epithelial cells. Genetics 178: 1947–1971.

Ziosi, M., L. A. Baena-Lopez, D. Grifoni, F. Froldi, A. Pession *et al.*, 2010 dMyc functions downstream of Yorkie to promote the supercompetitive behavior of hippo pathway mutant cells. PLoS Genet. 6: e1001140.

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The Hippo Pathway Controls Border Cell Migration Through Distinct Mechanisms in Outer Border Cells and Polar Cells of the *Drosophila* Ovary

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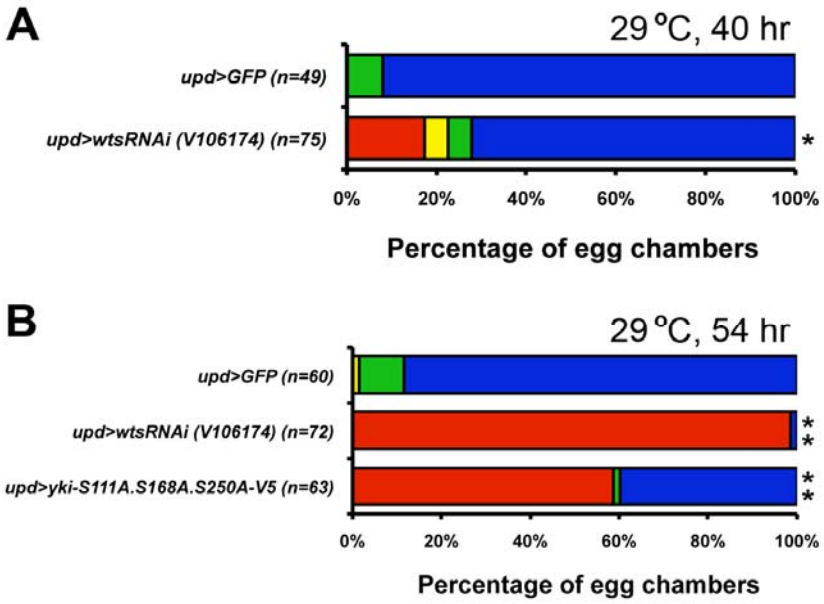

Figure S1 The Hippo pathway regulates border cell migration after polar cell specification.
upd-Gal4 was used to knockdown or over-express genes in polar cells. The temperature-sensitive Gal80 system was adapted to control knockdown or expression of genes temporally. *UAS-GFP* was used as a control. (A) Quantification and percentage distribution of border cell migration after adult flies were incubated at 29°C for 40 hours. Knockdown of *wts* in polar cells attenuated border cell migration. (B) Quantification and percentage distribution of border cell migration after adult flies were incubated at 29°C for 54 hours. Knockdown of *wts* or over-expression of *yki-3SA* in polar cells severely attenuated border cell migration. Wilcoxon rank-sum test, *: $p < 0.05$, **: $p < 0.01$.

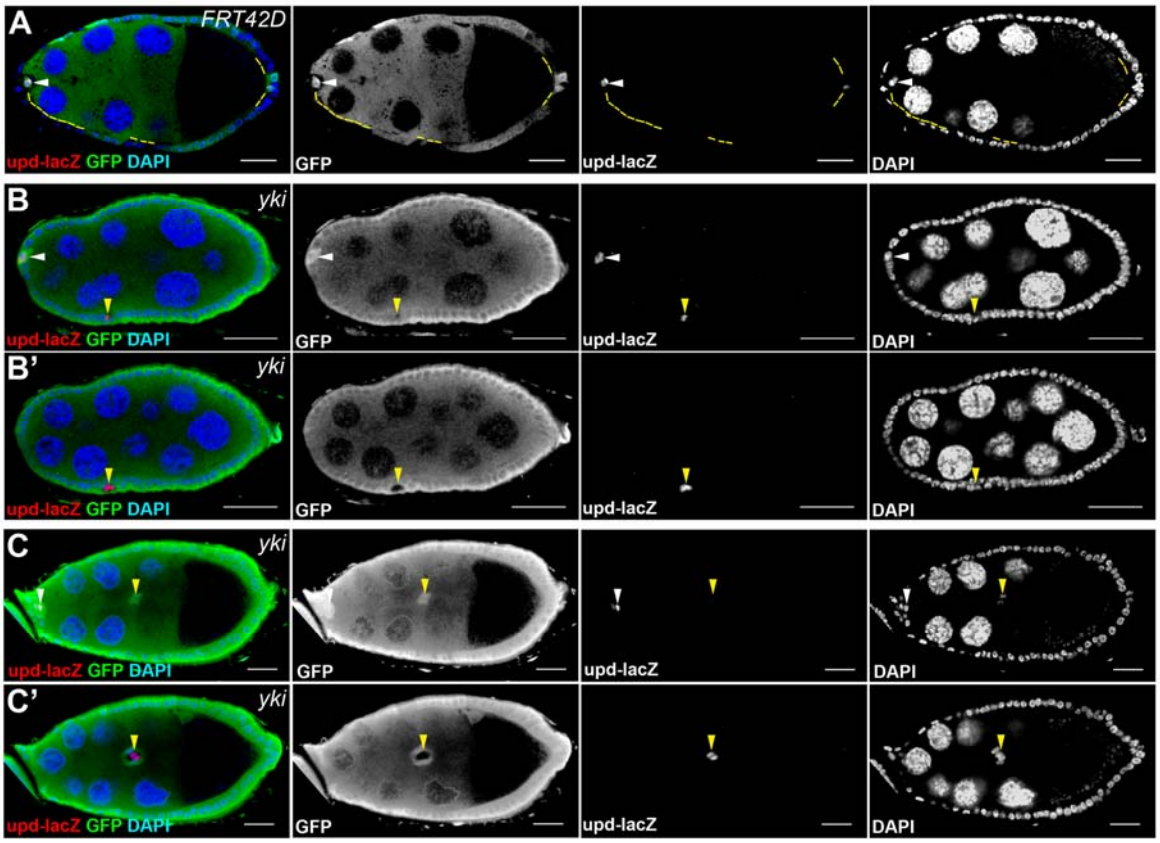

Figure S2 Yki suppresses *upd* expression and controls border cell induction and migration.
Mitotic clones of *FRT42D* (A) or *FRT42D yki*^{B5} (B, C) were generated in an *upd-lacZ* background and examined six days after clone induction. The ovaries were immunostained with anti-β-Gal and anti-GFP. (A) *upd-lacZ* was expressed in polar cells (white arrowheads) but not GFP-negative *FRT42D* cells (marked by yellow dashed lines). (B, B') Two focal planes of an egg chamber. A border cell cluster with GFP-positive polar cells (white arrowheads) expressed *upd-lacZ*. *yki* mutant cells (yellow arrowheads) also expressed *upd-lacZ* ectopically. (C, C') Two border cell clusters on different focal planes of an egg chamber. One cluster with wild-type GFP-positive polar cells expressing *upd-lacZ* remained at the anterior end (white arrowheads). The other cluster with GFP-negative *yki* mutant polar cells expressing *upd-lacZ* was migrating toward the oocyte (yellow arrowheads). Length of scale bar is 20 μm for all panels.

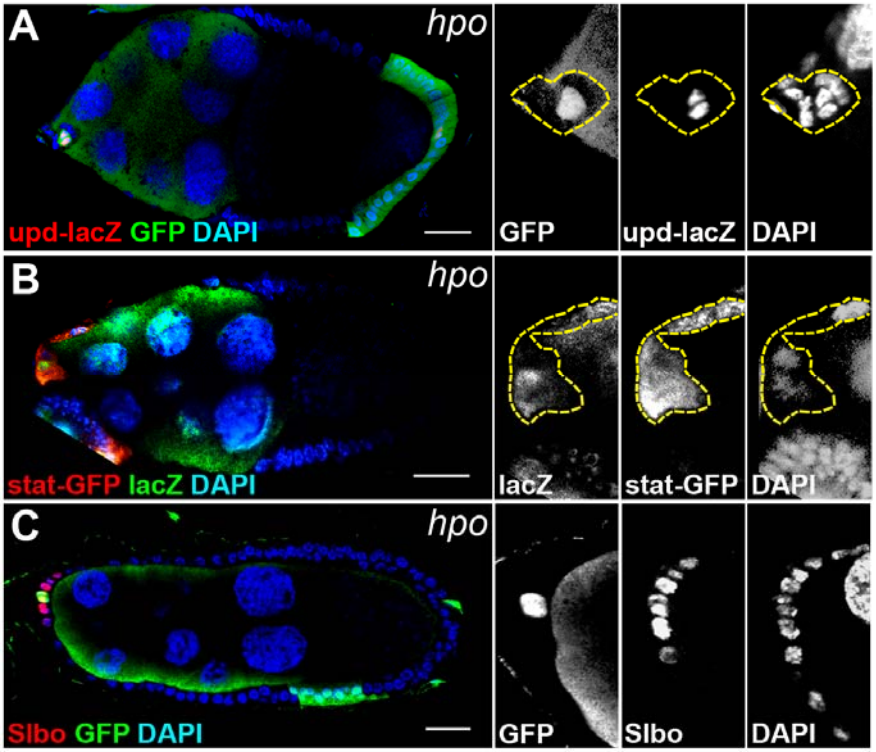

Figure S3 The Hippo pathway in outer border cells does not control JAK/STAT signaling in border cell clusters.
Mitotic clones of *FRT42D hpo*$^{42\text{-}47}$ were generated and examined six days after clone induction. Egg chambers at stages 9 or 10 were selected and oriented as anterior towards the left. High magnification views of the border cell cluster are shown in the panels on the right. The ovaries were immunostained with anti-GFP, anti-β-Gal (A, B), and anti-Slbo (C). (A) *hpo* mutant clones labeled by the absence of GFP were generated in an *upd-lacZ* background. A border cell cluster with *hpo* mutant outer border cells and GFP-positive control polar cells showed normal *upd-lacZ* expression in polar cells. (B) *hpo* mutant clones labeled by the absence of β-Gal were generated in a *stat-GFP* background. A border cell cluster with *hpo* mutant outer border cells and β-Gal-positive control polar cells showed normal *stat-GFP* expression in outer polar cells. (C) *hpo* mutant clone labeled by the absence of GFP were generated. A border cell cluster with *hpo* mutant outer border cells and GFP-positive control polar cells were positive for Slbo. Length of scale bar is 20 μm for all panels.

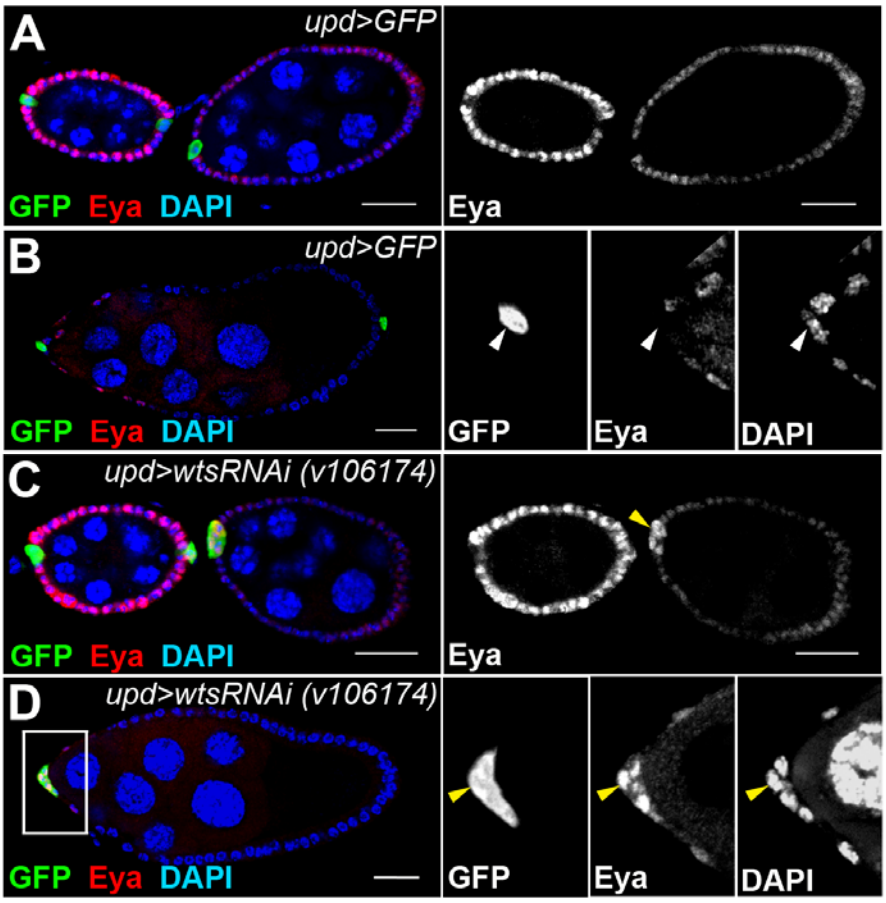

Figure S4 The Hippo pathway decreases the level of Eya in polar cells.
wts was knocked down in polar cells by using *upd-Gal4*. *UAS-GFP* was used as a control (A, C). The ovaries were immunostained with anti-Fas3 and anti-Eya. Egg chambers at stages 5, 7 (A, C), and stage 9 (B, D) were selected and oriented as anterior towards the left. High magnification views of border cell clusters at stage 9 are shown in the panels on the right. (A) In the control, Eya was detected in main-body follicle cells but not polar cells. The level of Eya was reduced when main-body follicle cells entered endoreplication after stage 6. (B) In the control, low level of Eya was detected in outer border cells and stretch cells at stage 9. No Eya was detected in polar cells (white arrowheads). (C) Eya was detected in *wts*-knockdown cells at stage 7 (yellow arrowheads) but not stage 5 (yellow arrows). (D) Eya was detected in *wts*-knockdown cells at stage 9 (yellow arrowheads). Length of scale bar is 20 μm for all panels.