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Drosophila PI4KIIIalpha is required in follicle cells for oocyte polarization and Hippo signaling

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SUMMARY

In a genetic screen we isolated mutations in *CG10260*, which encodes a phosphatidylinositol 4-kinase (PI4KIIIalpha), and found that PI4KIIIalpha is required for Hippo signaling in *Drosophila* ovarian follicle cells. *PI4KIIIalpha* mutations in the posterior follicle cells lead to oocyte polarization defects similar to those caused by mutations in the Hippo signaling pathway. *PI4KIIIalpha* mutations also cause misexpression of well-established Hippo signaling targets. The Merlin-Expanded-Kibra complex is required at the apical membrane for Hippo activity. In *PI4KIIIalpha* mutant follicle cells, Merlin fails to localize to the apical domain. Our analysis of *PI4KIIIalpha* mutants provides a new link in Hippo signal transduction from the cell membrane to its core kinase cascade.

KEY WORDS: *Drosophila*, Hippo signaling, Merlin, PI4 kinase, Oocyte polarity

INTRODUCTION

The Hippo signaling pathway has been identified as a tumor suppressor pathway that is conserved from *Drosophila* to mammals (Edgar, 2006; Pan, 2007). At its core is a series of phosphorylation events that lead to the inhibition of the transcriptional regulator Yorkie (Yki). The proteins involved in these phosphorylation events include the Sterile 20-like kinase Hippo (Hpo), the scaffold protein Salvador (Sav), the DBF family kinase Warts (Wts) and its associated protein Mats. Phosphorylation of Yki prevents it from being transported into the nucleus and activating the transcription of genes that promote cell proliferation and inhibit apoptosis. Loss of *hpo*, *wts*, *sav* or *mats* leads to Yki hyperactivation and causes tissue overgrowth (Justice et al., 1995; Xu et al., 1995; Tapon et al., 2002; Harvey et al., 2003; Pantalacci et al., 2003; Udan et al., 2003; Wu et al., 2003; Huang et al., 2005; Lai et al., 2005; Dong et al., 2007; Wei et al., 2007).

Several upstream inputs of the Hippo pathway have been identified (Grusche et al., 2010). Merlin and Expanded, two FERM (4.1, Ezrin, Radixin and Moesin) domain-containing proteins, are required for Hippo pathway activity (McCartney et al., 2000; Hamaratoglu et al., 2006). FERM domain-containing proteins are important signaling mediators at the membrane-cytoskeleton interface (McClatchey and Fehon, 2009). The scaffold protein Kibra interacts with Merlin and Expanded both genetically and physically and the Merlin-Expanded-Kibra apical complex promotes Hippo activity (Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Fat, an atypical cadherin localized on the apical cell membrane, modulates the Hippo signaling pathway (Bennett and Harvey, 2006; Cho et al., 2006; Silva et al., 2006; Willecke et al., 2006; Rogulja et al., 2008). In addition, components of cortical cell polarity complexes, such as Crumbs, send input to the Hippo pathway through Expanded (Grzeschik et al., 2010; Robinson et al., 2010). It remains to be determined whether other components of the apical cell membrane and cytoskeleton participate in the regulation of the Hippo pathway. Specifically, it is unclear how the Merlin-Expanded-Kibra complex is apically localized and regulated.

The Hippo pathway is involved in other developmental processes in addition to proliferation control (Mikeladze-Dvali et al., 2005; Emoto et al., 2006). During *Drosophila* oogenesis, Hippo signaling activity is required for oocyte polarization (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). The *Drosophila* oocyte is a highly polarized cell with distinct anterior-posterior (AP) and dorsal-ventral (DV) axes. The polarity is manifested in the structure of the cytoskeleton and the asymmetric distribution of cortical proteins and maternal RNAs. Residing in an egg chamber, the oocyte is surrounded by a layer of epithelial cells called follicle cells (FCs). Interactions between the oocyte and the FCs are crucial for the establishment and maintenance of oocyte polarity (reviewed by van Eeden and St Johnston, 1999; Roth and Lynch, 2009). During mid-oogenesis, multiple signaling pathways, including Notch, EGFR, JAK/STAT and Hippo, are required in the posterior follicle cells (PFCs) for sending an unidentified signal to initiate an oocyte repolarization process. In response, the oocyte nucleus migrates from the posterior to the dorsal-anterior corner of the oocyte, establishing the DV asymmetry of the egg and embryo (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Deng et al., 2001; Lopez-Schier and St Johnston, 2001; Xi et al., 2003; Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). Mutations in Hippo components in the PFCs lead to defects in this oocyte repolarization event, at least in part by interfering with Notch signaling (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008).

In a genetic screen to identify *Drosophila* genes required in FCs for oocyte polarization, we isolated alleles of *CG10260*, which encodes a phosphatidylinositol 4-kinase (PI4KIIIalpha) that catalyzes the production of phophatidylinositol-4-phosphate (PIP_4), an important cell membrane phospholipid and a precursor for other phosphoinositide species such as PI(4,5)P_2 (PIP_2). Loss of PI4KIIIalpha in the PFCs leads to oocyte polarization defects similar to those caused by mutations in the Hippo pathway. Moreover, *PI4KIIIalpha* mutations affect the expression of the

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Hippo signaling targets *expanded* (*ex*) and *diap1* (*thread* – FlyBase) in the FCs. Importantly, the apical localization of Merlin is lost in *PI4KIIIalpha* mutant FCs, indicating a potential direct link between membrane composition and Hippo signaling.

MATERIALS AND METHODS

Fly stocks and genetics

Six *PI4KIIIalpha* mutant alleles were isolated from a previously described genetic screen (Denef et al., 2008). Duplication, deficiency and P-element lines were from the Bloomington Stock Center. *sav[3]* FRT82B flies (Tapon et al., 2002) were a kind gift from Dr Ken Irvine (Rutgers University, NJ, USA). Reporter lines used to assay various signaling pathways and other transgenic fly lines included *kekkon-lacZ* (Pai et al., 2000), 10×STAT-GFP (Bach et al., 2007), *ex-lacZ* (Boedigheimer and Laughon, 1993), *diap1-lacZ* (Hay et al., 1995), *Kin-lacZ* (Clark et al., 1994) and Ubi-PH-PLCδ-GFP (Gervais et al., 2008). FC clones were generated using the FRT/UAS-FLP/GAL4 system (Duffy et al., 1998). Eye disc clones were generated using FRT/eyFlp. Genotypes of dissected females were:

PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/+;
PI4KIIIalpha^{FQ88} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/+;
PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/+; *Kin-lacZ*/+;
PI4KIIIalpha^{GS27} FRT19A/FRT19A; e22c-Gal4, UAS-Flp/+; 10×STAT-GFP/+;
PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/*kekkon-lacZ*;
PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/*ex-lacZ*;
PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-Flp/+; *diap1-lacZ*/+;
PI4KIIIalpha^{GS27} FRT19A/FRT19A; e22c-Gal4, UAS-Flp/Ubi-PH-PLCδ-GFP;
hsFLP; *sav*^{3} FRT82B/Ubi-GFP FRT82B; and
PI4KIIIalpha^{GS27} FRT19A/Ubi-GFP FRT19A; ey-Flp/+.

Immunofluorescence staining and microscopy

Ovaries were dissected, fixed and stained following standard procedures. Primary antibodies used were: mouse anti-Gurken (1D12, 1:10, DSHB), mouse anti-Cut (2B10, 1:20, DSHB), mouse anti-Hindsight (1G9, 1:20, DSHB), rabbit anti-phospho-Histone H3 (Ser28) (1:500, Millipore), rabbit anti-β-galactosidase (β-gal) (1:1000, Millipore), rabbit anti-Staufen [1:2000 (St Johnston et al., 1991)], chicken anti-GFP (1:1000, Aves Labs), guinea pig anti-Expanded [1:200 (Maitra et al., 2006)], guinea pig anti-Merlin [1:500 (McCartney and Fehon, 1996)], rabbit anti-Kibra [1:100 (Genevet et al., 2010)], rabbit anti-phospho-ERM (Cell Signaling, 1:100) and guinea pig anti-Cad99C [1:2000 (D'Alterio et al., 2005)]. Alexa Fluor 568- and 647-conjugated secondary antibodies were from Molecular Probes and used at 1:1000. Alexa Fluor 546-phalloidin (1:1000) and Hoechst (1 μg/ml; Molecular Probes) were used to stain actin and DNA, respectively. Images were taken on Zeiss LSM510 and LSM700 confocal microscopes.

Mutation mapping and sequencing

The lethality of the *PI4KIIIalpha* complementation group was rescued by Dp(1;Y)w+303. We used 15 deficiency lines to further map the lethality to the 3A4-3A8 chromosomal region. Deficiency lines used were: *Df(1)dm75e19*, *Df(1)N-8*, *Df(1)N-264-105*, *Df(1)N-81k1*, *Df(1)w-N71a*, *Df(1)ED6630*, *Df(1)w258-42*, *Df(1)w258-11*, *Df(1)JC19*, *Df(1)X12*, *Df(1)64c18*, *Df(1)TEM7*, *Df(1)ED6584*, *Df(1)ED411* and *Df(1)ED11354* (for details, see http://flybase.org). According to public databases, the 3A4-3A8 region contains 16 genes. After performing complementation tests with known genes in the region, we sequenced PCR products from the coding regions of the remaining candidate genes. DNA for sequencing was derived from two independent genomic preparations from homozygous mutant first/second instar larvae (identified by the lack of fluorescence from mutant stocks balanced over an *FM7,Kr>GFP* chromosome). Sequences were compared with the control sequences of the starting chromosome (*yw FRT19A*) to identify molecular lesions.

RESULTS AND DISCUSSION

PI4KIIIalpha mutations affect oocyte polarization during mid-oogenesis

DV asymmetry of the *Drosophila* oocyte is established during mid-oogenesis through a repolarization process initiated in the PFCs. In response to an unknown signal from the PFCs the oocyte nucleus migrates from the posterior end to the dorsal-anterior corner of the oocyte. As a consequence, the Gurken (Grk) protein no longer accumulates at the posterior cortex of the oocyte, but is now found in the dorsal-anterior membrane overlying the oocyte nucleus where it activates EGFR to initiate DV patterning (Gonzalez-Reyes et al., 1995; Roth et al., 1995). In a genetic screen directed at FC components affecting this repolarization process (Denef et al., 2008), we isolated a complementation group with six lethal mutant alleles, initially named after a representative allele, *GS27*. When the PFCs were mutant for the *GS27* gene product, the oocyte nucleus frequently remained at the posterior end of the oocyte (Fig. 1B,C; 47.7%, *n*=111). This phenotype was confirmed by the abnormal posterior localization of Grk in late egg chambers (Fig. 1D,E).

We mapped the lethality of the *GS27* complementation group through duplication and deficiency mapping to the X-chromosomal region 3A4-3A8, which contains 16 genes. Sequencing of candidate genes showed that four alleles of the *GS27* complementation group contained mutations that lead to premature stop codons in the coding region of *CG10260* (Fig. 1A), a predicted phosphatidylinositol 4-kinase (http://flybase.org). Phosphatidylinositol 4-kinases (PI4Ks) catalyze the generation of PIP_4. Phosphoinositides, including PIP_4, are important phospholipids in the cell membrane that participate in numerous signaling events (Skwarek and Boulianne, 2009). Four classes of PI4Ks have been identified in mammalian cells that localize to different cellular compartments and are likely to perform non-redundant functions (Balla and Balla, 2006). Three PI4K genes have been annotated in the fly genome: *four wheel drive* (*fwd*; *PI4KIIIbeta*) (Polevoy et al., 2009), *CG2929* (*PI4KIIalpha*) (Raghu et al., 2009) and *CG10260* (*PI4KIIIalpha*).

To investigate the oocyte polarization defects caused by *PI4KIIIalpha* mutations, we checked the localization of well-established oocyte polarity markers. The microtubule cytoskeleton is polarized in the oocyte. We examined the microtubule plus-end marker Kinesin (Kin, or Khc) fused to β-gal (Kin-β-gal), which normally forms a crescent at the posterior of the oocyte after stage 8 (Clark et al., 1994) (Fig. 1F). When the PFCs were mutant for *PI4KIIIalpha*, Kin-β-gal either localized to the center of the oocyte or was diffuse in the oocyte (Fig. 1G; 66.7%, *n*=24). Staufen localizes to the posterior pole of wild-type oocytes after stage 8 and is required for the localization of maternal RNAs (St Johnston et al., 1991). In PFC clones mutant for *PI4KIIIalpha*, Staufen also frequently mislocalized to the center of the oocyte or became dispersed in the oocyte (Fig. 1H; 73.4%, *n*=74). Therefore, in combination with the mislocalization of the oocyte nucleus, our results demonstrate that PI4KIIIalpha is required in the PFCs for all aspects of the establishment of correct oocyte polarity.

PI4KIIIalpha mutations and mutations in Hippo pathway components produce similar phenotypes during oogenesis

Oocyte polarization relies on the integrity of four signaling pathways in the PFCs: Notch, JAK/STAT, EGFR and Hippo (Gonzalez-Reyes et al., 1995; Roth et al., 1995; Lopez-Schier and St Johnston, 2001; Xi et al., 2003; Meignin et al., 2007; Polesello

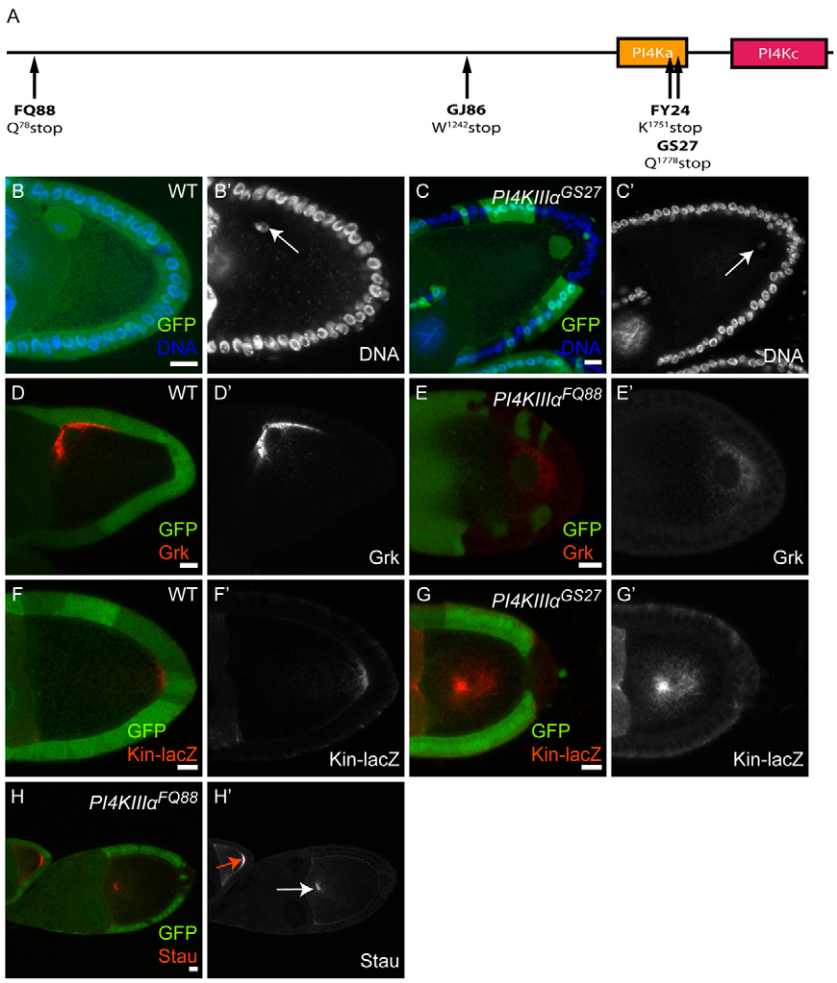

Fig. 1. Mutations in *PI4KIIIalpha* lead to oocyte polarity defects. (**A**) *CG10260*, the *Drosophila PI4KIIIalpha* homolog. Alleles *PI4KIIIalpha*^{FQ88}, *PI4KIIIalpha*^{GJ86}, *PI4KIIIalpha*^{FY24} and *PI4KIIIalpha*^{GS27} contain early stop codons at Gln78, Trp1242, Lys1751 and Gln1778, respectively. Protein domains are annotated with CDD search (Marchler-Bauer et al., 2009). PI4Ka, phosphoinositide 4-kinase (PI4K) accessory domain; PI4Kc, PI4K, type III, alpha isoform, catalytic domain. (**B-E'**) Wild-type egg chambers (B,D) and egg chambers containing *PI4KIIIalpha*^{GS27} (C) or *PI4KIIIalpha*^{FQ88} (E) mutant posterior follicle cells (PFCs), marked by the absence of GFP (green) and stained for DNA (blue, B,C) or Grk (red, D,E). The DNA (B′,C′) and Grk (D′,E′) channels are also shown separately. The oocyte nucleus is located in the dorsal-anterior corner in a wild-type egg chamber (B′, arrow). In mutant PFCs, the oocyte nucleus remains at the posterior of the oocyte (C′, arrow). Similarly, Grk localization is disrupted in the mutant egg chamber (E). (**F-G'**) A wild-type egg chamber (F) and an egg chamber containing *PI4KIIIalpha*^{GS27} mutant PFCs (G) expressing *Kin-lacZ* and stained for β-gal (red). The β-gal channel is shown separately in F′,G′. In the wild-type egg chamber, Kin-β-gal localizes to the posterior of the oocyte (F). In the egg chamber containing a large *PI4KIIIalpha*^{GS27} PFC clone, Kin-β-gal is mislocalized to the center of the oocyte (G). (**H**,**H'**) Egg chambers stained for Staufen (red), which forms a tight crescent at the posterior of a wild-type egg chamber (H′, red arrow), but is mislocalized to the center of the oocyte in an egg chamber containing a *PI4KIIIalpha*^{FQ88} PFC clone (H′, white arrow). Scale bars: 10 μm.

and Tapon, 2007; Yu et al., 2008). To examine whether the polarization defect we observed in *PI4KIIIalpha* mutants was caused by disruption of one of these signaling pathways, we examined well-established downstream targets of each pathway in *PI4KIIIalpha* mutants.

The EGFR signaling reporter *kekkon-lacZ* (*kek-lacZ*) is highly expressed in the PFCs at stage 7 and 8 as a result of EFGR activation by Grk (Pai et al., 2000). In PFCs mutant for *PI4KIIIalpha*, the *kek-lacZ* expression level was comparable to that of wild-type PFCs, indicating that EFGR signaling was unaffected (Fig. 2A; n>50). The JAK/STAT signaling reporter 10×STAT92E-GFP (Bach et al., 2007) is normally turned on in the PFCs during stage 7 and 8 in response to JAK/STAT activation. We detected apparently normal levels of GFP in the nuclei of *PI4KIIIalpha* mutant PFCs, suggesting that JAK/STAT signaling was also intact (Fig. 2B; n>30).

Notch signaling is required for FCs to exit the mitotic cell cycle at stage 6 and switch to an endocycle (Deng et al., 2001; Lopez-Schier and St Johnston, 2001). *PI4KIIIalpha* mutant PFCs maintained a mitotic cell cycle after stage 6, as indicated by the sustained staining of the mitotic marker phosphorylated Histone H3 (PH3), which is only seen up to stage 6 in wild-type FCs (Fig. 2C; n>30). Consistent with a failure to exit the mitotic cycle, the *PI4KIIIalpha* mutant PFCs often lost their monolayered epithelial structure and had smaller nuclei than neighboring cells (Fig. 2D). We also examined the expression of two Notch signaling targets, Cut and Hindsight (Hnt; Pebbled – FlyBase). In wild-type FCs, Cut expression is downregulated whereas Hnt expression is upregulated upon Notch activation at stage 6 (Sun and Deng, 2005; Sun and Deng, 2007). *PI4KIIIalpha* mutant PFCs frequently failed to downregulate Cut (Fig. 2E; 81.6%, n=76) and upregulate Hnt (Fig. 2F; 66.7%, n=57) expression. Interestingly, *PI4KIIIalpha* mutant cells on the lateral side of the egg chambers showed no defect in Notch signaling (Fig. 2D-E). These results suggest that *PI4KIIIalpha* mutations compromise Notch signaling in the PFCs only.

The phenotypes described above are similar to those caused by mutations in Hippo pathway components (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). In particular, the observation that only PFCs appear affected is characteristic of mutations in the Hippo pathway, which are reported to affect Notch signaling only in this group of FCs (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008). When we checked the expression of a Hippo pathway target, *ex*, using the enhancer trap line *ex-lacZ* (Boedigheimer and Laughon, 1993), we detected a much higher level of β-gal in *PI4KIIIalpha* mutant FCs than in wild-type cells (Fig. 2G; 81.4%, n=86). This upregulation was observed in all FCs, regardless of their position. Another Hippo pathway target, Diap1, monitored with the enhancer trap line *diap1-lacZ* (Hay et al., 1995), was mildly upregulated in the *PI4KIIIalpha* mutant FCs (Fig. 2H; 43.4%, n=53). These results indicate that the polarization defect in the *PI4KIIIalpha* mutants is likely to be caused by defective Hippo signaling.

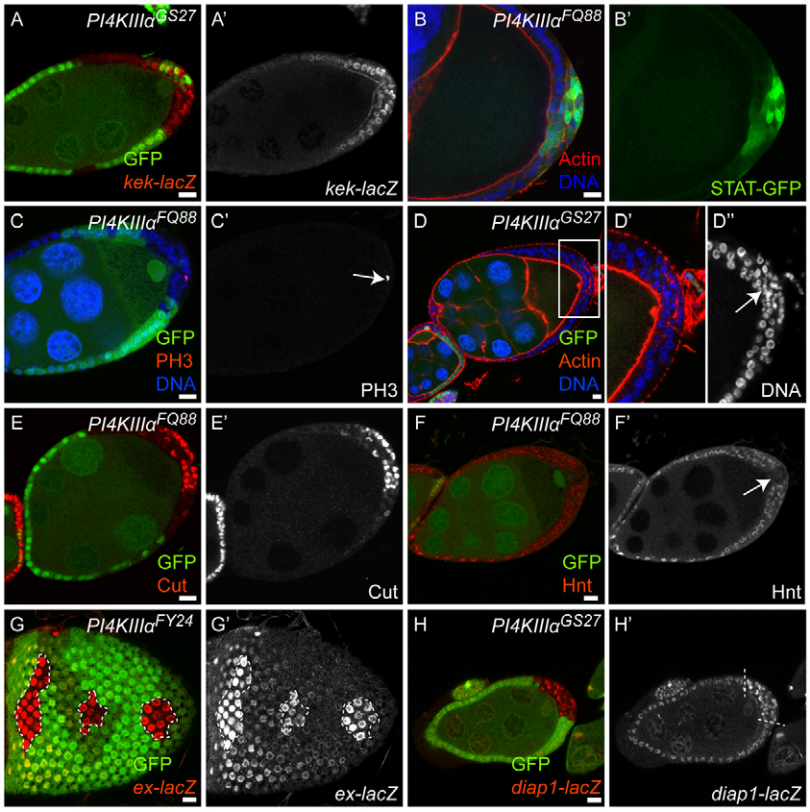

Fig. 2. Effects of *PI4KIIIalpha* mutations on different signaling pathways. Mutant cells are marked by the absence of GFP (green), except in B where we generated unmarked clones in order to visualize the 10×STAT-GFP reporter and *PI4KIIIalpha* mutant cells were identified by the loss of their monolayered epithelial structure outlined by actin staining. (**A**,**A′**) A *Drosophila* egg chamber containing *PI4KIIIalpha* mutant PFCs, expressing the EGFR signaling reporter *kekkon-lacZ* and stained for β-gal (red in A, gray in A′). (**B**,**B′**) An egg chamber containing *PI4KIIIalpha* mutant PFCs, expressing the JAK/STAT signaling reporter 10×STAT-GFP (green). Both EGFR and JAK/STAT signaling pathways were correctly activated and transduced in the *PI4KIIIalpha* mutant PFCs, as indicated by the normal levels of β-gal staining (A,A′) and GFP signal (B,B′). (**C-F′**) Egg chambers containing *PI4KIIIalpha* mutant PFCs stained for phosphorylated Histone H3 (PH3, red, C), Actin (red, D), DNA (blue, C,D), Cut (red, E) and Hnt (red, F); the PH3, Cut and Hnt channels are also shown separately (C′,E′,F′); the boxed region in D is shown at higher magnification in D′ and D″ (DNA channel only). *PI4KIIIalpha* mutant PFCs remained in the mitotic cycle after stage 6, as indicated by the presence of PH3-positive cells (C′, arrow) and multilayered cells with smaller nuclei (D″, arrow). *PI4KIIIalpha* mutant PFCs failed to downregulate Cut (E) and to upregulate Hnt (F) after stage 6. These results indicate that Notch signaling is compromised in *PI4KIIIalpha* mutant PFCs. (D,E) Note that *PI4KIIIalpha* mutant cells at the lateral side of the egg chambers show normal epithelial structure (D) and correctly downregulated Cut expression (E), as in the wild-type cells. (**G-H′**) Egg chambers containing *PI4KIIIalpha* mutant FCs, expressing the Hippo signaling reporters *ex-lacZ* (G) and *diap1-lacZ* (H), stained for β-gal (red in G,H; gray in G′,H′). Upregulation of both reporters indicates that Hippo signaling is disrupted in *PI4KIIIalpha* mutant FCs. Scale bars: 10 μm.

Merlin localization is affected in *PI4KIIIalpha* mutant follicle cells and eye disc cells

Multiple lines of evidence suggest that the apical localization of the Expanded-Merlin-Kibra complex is crucial for Hippo signaling activity (Baumgartner et al., 2010; Genevet et al., 2010; Grzeschik et al., 2010; Robinson et al., 2010; Yu et al., 2010) as it is proposed to function as a platform to bring the core Hippo components into close proximity and facilitate the phosphorylation reactions (Baumgartner et al., 2010; Genevet et al., 2010; Grzeschik et al., 2010; Robinson et al., 2010; Yu et al., 2010). In addition, it has been reported that Expanded directly interacts with Yki and functions to sequester Yki in the cytoplasm (Badouel et al., 2009).

To investigate how mutations in *PI4KIIIalpha* lead to defective Hippo signaling, we examined the apical localization of the Merlin-Expanded-Kibra complex. The complex is confined to the apical domain in wild-type FCs. In the *PI4KIIIalpha* mutant cells, we observed a loss of apical Merlin staining (Fig. 3A; n>30), whereas Expanded and Kibra were upregulated at the apical membrane (Fig. 3C,E; n>30). In addition to being Hippo pathway regulators, Expanded and Kibra are also targets of the Hippo signaling pathway. Mutations in Hippo pathway components lead to upregulation of Expanded and Kibra (Fig. 3D,F; n>30) (Hamaratoglu et al., 2006; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). In addition, it has been reported that the apical sorting of Merlin, Expanded and Kibra occur independently of each other (McCartney et al., 2000; Baumgartner et al., 2010; Genevet et al., 2010; Yu et al., 2010). Therefore, the absence of Merlin from the apical membrane in *PI4KIIIalpha* mutant cells is the likely cause of the signaling defect, and the upregulation of Expanded and Kibra would be an expected secondary consequence of the disrupted Hippo signaling.

When we examined *PI4KIIIalpha* mutant clones in the imaginal eye discs of early second instar larvae, we also observed an absence of Merlin from the apical and junctional region (Fig. 3B; n>10). However, we did not observe an overgrowth phenotype typical of Hippo pathway mutations (data not shown). In fact, adults with mutant eye clones had smaller eyes than wild-type

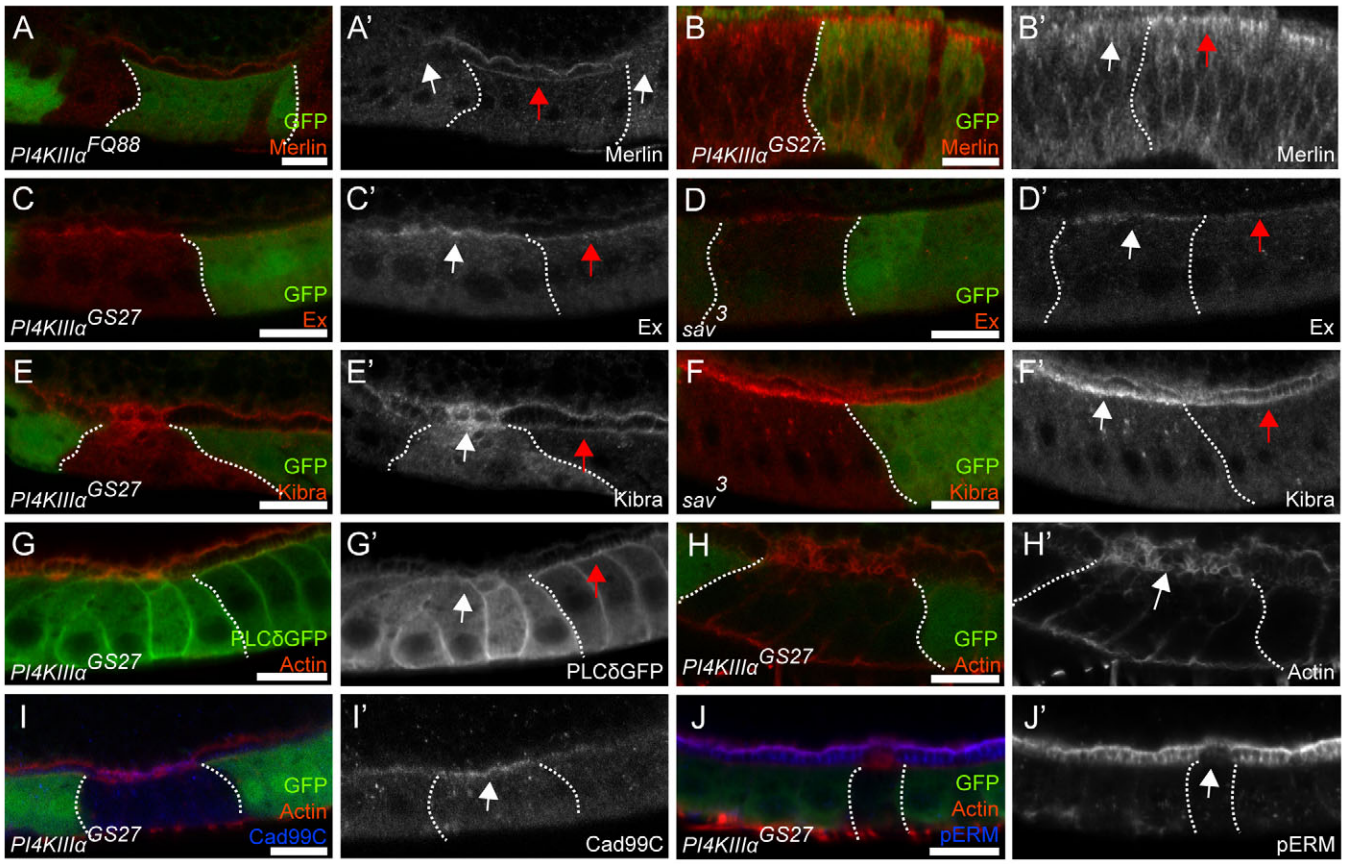

Fig. 3. Merlin mislocalization from the apical membrane in *PI4KIIIalpha* mutant follicle cells and eye disc cells. Clone boundaries are marked by dotted white lines. Mutant cells are marked by the absence of GFP (green), except in G where we generated unmarked clones in order to visualize the Ubi-PH-PLCδ-GFP reporter. *PI4KIIIalpha* mutant cells were identified by the abnormal actin structure on the apical domain. FCs and eye disc cells are orientated with the apical side up. (**A-B'**) The follicular epithelium (A) and the imaginal eye disc epithelium (B) containing *PI4KIIIalpha* mutant cells stained for Merlin (red in A,B; gray in A',B'). (**C-F'**) Egg chambers containing *PI4KIIIalpha* (C,E) or *sav* (D,F) mutant FCs stained for Expanded (red in C,D; gray in C',D') and Kibra (red in E,F; gray in E',F'). Merlin, Expanded and Kibra localization in wild-type cells is indicated with red arrows. Merlin disappears from the apical and junctional region of the mutant cells (A',B', white arrow), whereas Expanded (C', white arrow) and Kibra (E', white arrow) are upregulated but remain localized. Upregulation of Expanded (D', white arrow) and Kibra (F', white arrow) is similarly observed in *sav* mutant FCs. (**G-J'**) Egg chambers containing *PI4KIIIalpha* mutant cells, expressing the PI(4,5)P_2 reporter Ubi-PH-PLCδ-GFP (G), stained for GFP (green in G; gray in G'), actin (red in G-J; gray in H'), Cad99C (blue in I; gray in I') and phospho-ERM proteins (blue in J; gray in J'). The PI(4,5)P_2 reporter is absent from the apical membrane of the mutant cells (G', white arrow), in contrast to the wild-type cells (G', red arrow). *PI4KIIIalpha* mutant cells exhibit abnormal actin spikes on the apical side (H', white arrow) as marked by the microvillus marker Cad99C (I', white arrow). The apical microvilli region of *PI4KIIIalpha* mutant cells shows depletion of phospho-ERM proteins (J', white arrow). Scale bars: 10 μm.

adults. Eye discs from late L2 larvae exhibited pyknotic nuclei staining in *PI4KIIIalpha* mutant clones, indicating death of the mutant cells (data not shown).

Multiple classes of PI4Ks exist in eukaryotic cells that participate in producing various phosphoinositide species in distinct cellular compartments (Balla and Balla, 2006). Three PI4K genes have been annotated in the fly genome. When we examined the intracellular distribution and level of PIP_2 using a Ubi-PH-PLCδ-GFP reporter (Gervais et al., 2008), we observed a complete absence of PIP_2 from *PI4KIIIalpha* mutant FCs in rare cases (2 out of 40 clones). In most cases, the PIP_2 reporter was specifically lost from the apical plasma membrane in the mutant cells (Fig. 3G; 82.5%, n=40). The yeast homolog of PI4KIIIalpha, Stt4p, localizes to patches on the plasma membrane where it is required for normal actin cytoskeleton organization (Audhya et al., 2000; Audhya and Emr, 2002). When we examined the actin cytoskeleton of *PI4KIIIalpha* mutant FCs by phalloidin staining, they exhibited abnormal actin-enriched spike structures on their apical domain (Fig. 3H; n>100) that were positively marked by the microvillus marker Cad99C (D'Alterio et al., 2005) (Fig. 3I; n>30), suggesting that the spikes were malformed microvilli. As mutations in the Hippo pathway have been reported to lead to apical domain expansion (Justice et al., 1995; Wu et al., 2003; Genevet et al., 2009), one possibility is that the malformed microvilli are caused by defective Hippo signaling. However, the morphology of the actin-enriched spikes in *PI4KIIIalpha* mutant cells is distinct from that caused by mutations in the Hippo pathway (Fig. 3H), suggesting that the loss of *PI4KIIIalpha* might also have a Hippo-independent effect on apical membrane structure.

How could *PI4KIIIalpha* mutations cause Merlin mislocalization? Expanded and Merlin are ERM (Ezrin, Radixin and Moesin)-related proteins, which are key linkers of the plasma membrane and cytoskeleton. Classical ERM proteins bind to PIP_2 in the membrane to switch from a closed to an open conformation

for their activation (Nakamura et al., 1999; Fievet et al., 2004; Fehon et al., 2010). Significantly, in *PI4KIIIalpha* mutant cells, phosphorylated ERM proteins were absent from the apical microvilli region as indicated by a phospho-ERM-specific antibody (Fig. 3J; n>20). The malformed microvillus structure might therefore indicate a general failure of ERM protein activation in the *PI4KIIIalpha* mutant cells (Takeuchi et al., 1994). For Merlin, the closed conformation is the active form, opposite to other ERM proteins (Okada et al., 2007; McClatchey and Fehon, 2009). Nevertheless, Merlin undergoes a similar conformational switch to the other ERM proteins (Gonzalez-Agosti et al., 1999) and contains an ERM PIP_2-binding site (Barret et al., 2000). Given our observations, it is possible that PIP_2 binding activates and/or stabilizes Merlin in the apical membrane, and a depletion of this lipid species due to the absence of PI4KIIIalpha might directly lead to the loss of Merlin.

In summary, we have shown that PI4KIIIalpha is required in the FCs for Merlin localization and Hippo signaling. *PI4KIIIalpha* mutations in the PFCs lead to a Notch signaling defect and the subsequent failure of oocyte repolarization, which are precisely the phenotypes reported for Hippo mutations in the FCs. This effect is likely to be caused by a change in lipid composition in the membrane. How the abnormal actin structures are generated in the mutant cells, and whether they have a direct role in Merlin localization, remain to be investigated.

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Competing interests statement

The authors declare no competing financial interests.

References

Audhya, A. and Emr, S. D. (2002). Stt4 PI 4-kinase localizes to the plasma membrane and functions in the Pkc1-mediated MAP kinase cascade. *Dev. Cell* **2**, 593-605.

Audhya, A. M., Foti, M. and Emr, S. D. (2000). Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics. *Mol. Biol. Cell* **11**, 2673-2689.

Bach, E. A., Ekas, L. A., Ayala-Camargo, A., Flaherty, M. S., Lee, H., Perrimon, N. and Baeg, G. H. (2007). GFP reporters detect the activation of the Drosophila JAK/STAT pathway in vivo. *Gene Expr. Patterns* **7**, 323-331.

Badouel, C., Gardano, L., Amin, N., Garg, A., Rosenfeld, R., Le Bihan, T. and McNeill, H. (2009). The FERM-domain protein expanded regulates Hippo pathway activity via direct interactions with the transcriptional activator Yorkie. *Dev. Cell* **16**, 411-420.

Balla, A. and Balla, T. (2006). Phosphatidylinositol 4-kinases: old enzymes with emerging functions. *Trends Cell Biol.* **16**, 351-361.

Barret, C., Roy, C., Montcourrier, P. and Niggli, V. (2000). Mutagenesis of the phosphatidylinositol 4,5-bisphosphate (PIP(2)) binding site in the NH(2)-terminal domain of ezrin correlates with its altered cellular distribution. *J. Cell Biol.* **151**, 1067-1080.

Baumgartner, R., Poernbacher, I., Buser, N., Hafen, E. and Stocker, H. (2010). The WW domain protein Kibra acts upstream of Hippo in Drosophila. *Dev. Cell* **18**, 309-316.

Bennett, F. C. and Harvey, K. F. (2006). Fat cadherin modulates organ size in Drosophila via the Salvador/Warts/Hippo signaling pathway. *Curr. Biol.* **16**, 2101-2110.

Boedigheimer, M. and Laughon, A. (1993). Expanded: a gene involved in the control of cell proliferation in imaginal discs. *Development* **118**, 1291-1301.

Cho, E., Feng, Y., Rauskolb, C., Maitra, S., Fehon, R. and Irvine, K. D. (2006). Delineation of a fat tumor suppressor pathway. *Nat. Genet.* **38**, 1142-1150.

Clark, I., Giniger, E., Ruohola-Baker, H., Jan, L. Y. and Jan Y. N. (1994). Transient posterior localization of a kinesin fusion protein reflects anteroposterior polarity of the Drosophila oocyte. *Curr. Biol.* **4**, 289-300.

D'Alterio, C., Tran, D. D., Yeung, M. W., Hwang, M. S., Li, M. A., Arana, C. J., Mulligan, V. K., Kubesh, M., Sharma, P., Chase, M. et al. (2005). Drosophila melanogaster Cad99C, the orthologue of human Usher cadherin PCDH15, regulates the length of microvilli. *J. Cell Biol.* **171**, 549-558.

Denef, N., Chen, Y., Weeks, S. D., Barcelo, G. and Schüpbach, T. (2008). Crag regulates epithelial architecture and polarized deposition of basement membrane proteins in Drosophila. *Dev. Cell* **14**, 354-364.

Deng, W. M., Althauser, C. and Ruohola-Baker, H. (2001). Notch-Delta signaling induces a transition from mitotic cell cycle to endocycle in Drosophila follicle cells. *Development* **128**, 4737-4746.

Dong, J., Feldmann, G., Huang, J., Wu, S., Zhang, N., Comerford, S. A., Gayyed, M. F., Anders, R. A., Maitra, A. and Pan, D. (2007). Elucidation of a universal size-control mechanism in Drosophila and mammals. *Cell* **130**, 1120-1133.

Duffy, J. B., Harrison, D. A. and Perrimon, N. (1998). Identifying loci required for follicular patterning using directed mosaics. *Development* **125**, 2263-2271.

Edgar, B. A. (2006). From cell structure to transcription: Hippo forges a new path. *Cell* **124**, 267-273.

Emoto, K., Parrish, J. Z., Jan, L. Y and Jan, Y. N. (2006). The tumour suppressor Hippo acts with the NDR kinases in dendritic tiling and maintenance. *Nature* **443**, 210-213.

Fehon, R. G., McClatchey, A. I. and Bretscher, A. (2010). Organizing the cell cortex: the role of ERM proteins. *Nat. Rev. Mol. Cell Biol.* **11**, 276-287.

Fievet, B. T., Gautreau, A., Roy, C., Del Maestro, L., Mangeat, P., Louvard, D. and Arpin, M. (2004). Phosphoinositide binding and phosphorylation act sequentially in the activation mechanism of ezrin. *J. Cell Biol.* **164**, 653-659.

Genevet, A., Polesello, C., Blight, K., Robertson, F., Collinson, L. M., Pichaud, F. and Tapon, N. (2009). The Hippo pathway regulates apical-domain size independently of its growth-control function. *J. Cell Sci.* **122**, 2360-2370.

Genevet, A., Wehr, M. C., Brain, R., Thompson, B. J. and Tapon, N. (2010). Kibra is a regulator of the Salvador/Warts/Hippo signaling network. *Dev. Cell* **18**, 300-308.

Gervais, L., Claret, S., Januschke, J., Roth, S. and Guichet, A. (2008). PIP5K-dependent production of PIP2 sustains microtubule organization to establish polarized transport in the Drosophila oocyte. *Development* **135**, 3829-3838.

Gonzalez-Agosti, C., Wiederhold, T., Herndon, M. E., Gusella, J. and Ramesh, V. (1999). Interdomain interaction of merlin isoforms and its influence on intermolecular binding to NHE-RF. *J. Biol. Chem.* **274**, 34438-34442.

Gonzalez-Reyes, A., Elliott, H. and St Johnston, D. (1995). Polarization of both major body axes in Drosophila by gurken-torpedo signalling. *Nature* **375**, 654-658.

Grusche, F. A., Richardson, H. E. and Harvey, K. F. (2010). Upstream regulation of the Hippo size control pathway. *Curr. Biol.* **20**, R574-R582.

Grzeschik, N. A., Parsons, L. M., Allott, M. L., Harvey, K. F. and Richardson, H. E. (2010). Lgl, aPKC, and Crumbs regulate the Salvador/Warts/Hippo pathway through two distinct mechanisms. *Curr. Biol.* **20**, 573-581.

Hamaratoglu, F., Willecke, M., Kango-Singh, M., Nolo, R., Hyun, E., Tao, C., Jafar-Nejad, H. and Halder, G. (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.

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

Hay, B. A., Wassarman, D. A. and Rubin, G. M. (1995). Drosophila homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* **83**, 1253-1262.

Huang, J., Wu, S., Barrera, J., Matthews, K. and Pan, D. (2005). The Hippo signaling pathway coordinately regulates cell proliferation and apoptosis by inactivating Yorkie, the Drosophila homolog of YAP. *Cell* **122**, 421-434.

Justice, R. W., Zilian, O., Woods, D. F., Noll, M. and Bryant, P. J. (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.

Lai, Z. C., Wei, X., Shimizu, T., Ramos, E., Rohrbaugh, M., Nikolaidis, N., Ho, L. L. and Li, Y. (2005). Control of cell proliferation and apoptosis by mob as tumor suppressor, mats. *Cell* **120**, 675-685.

Lopez-Schier, H. and St Johnston, D. (2001). Delta signaling from the germ line controls the proliferation and differentiation of the somatic follicle cells during Drosophila oogenesis. *Genes Dev.* **15**, 1393-1405.

Maitra, S., Kulikauskas, R. M., Gavilan, H. and Fehon, R. G. (2006). The tumor suppressors Merlin and Expanded function cooperatively to modulate receptor endocytosis and signaling. *Curr. Biol.* **16**, 702-709.

Marchler-Bauer, A., Anderson, J. B., Chitsaz, F., Derbyshire, M. K., DeWeese-Scott, C., Fong, J. H., Geer, R. C., Gonzales, N. R., Gwadz, M. and He, S. (2009). CDD: specific functional annotation with the conserved domain database. *Nucleic Acids Res.* **37**, D205-D210.

McCartney, B. M. and Fehon, R. G. (1996). Distinct cellular and subcellular patterns of expression imply distinct functions for the Drosophila homologues of moesin and the neurofibromatosis 2 tumor suppressor, merlin. *J. Cell Biol.* **133**, 843-852.

McCartney, B. M., Kulikauskas, R. M., LaJeunesse, D. R. and Fehon, R. G. (2000). The neurofibromatosis-2 homologue, Merlin, and the tumor suppressor expanded function together in Drosophila to regulate cell proliferation and differentiation. *Development* **127**, 1315-1324.
McClatchey, A. I. and Fehon, R. G. (2009). Merlin and the ERM proteins-regulators of receptor distribution and signaling at the cell cortex. *Trends Cell Biol.* **19**, 198-206.
Meignin, C., Alvarez-Garcia, I., Davis, I. and Palacios, I. M. (2007). The salvador-warts-hippo pathway is required for epithelial proliferation and axis specification in Drosophila. *Curr. Biol.* **17**, 1871-1878.
Mikeladze-Dvali, T., Wernet, M. F., Pistillo, D., Mazzoni, E. O., Teleman, A. A., Chen, Y. W., Cohen, S., Desplan, C. et al. (2005). The growth regulators warts/lats and melted interact in a bistable loop to specify opposite fates in Drosophila R8 photoreceptors. *Cell* **122**, 775-787.
Nakamura, F., Huang, L., Pestonjamasp, K., Luna, E. J. and Furthmayr, H. (1999). Regulation of F-actin binding to platelet moesin in vitro by both phosphorylation of threonine 558 and polyphosphatidylinositides. *Mol. Biol. Cell* **10**, 2669-2685.
Okada, T., You, L. and Giancotti, F. G. (2007). Shedding light on Merlin's wizardry. *Trends Cell Biol.* **17**, 222-229.
Pai, L. M., Barcelo, G. and Schüpbach, T. (2000). D-cbl, a negative regulator of the Egfr pathway, is required for dorsoventral patterning in Drosophila oogenesis. *Cell* **103**, 51-61.
Pan, D. (2007). Hippo signaling in organ size control. *Genes Dev.* **21**, 886-897.
Pantalacci, S., Tapon, N. and Léopold, P. (2003). The Salvador partner Hippo promotes apoptosis and cell-cycle exit in Drosophila. *Nat. Cell Biol.* **5**, 921-927.
Polesello, C. and Tapon, N. (2007). Salvador-warts-hippo signaling promotes Drosophila posterior follicle cell maturation downstream of notch. *Curr. Biol.* **17**, 1864-1870.
Polevoy, G., Wei, H. C., Wong, R., Szentpetery, Z., Kim, Y. J., Goldbach, P., Steinbach, S. K., Balla, T. and Brill, J. A. (2009). Dual roles for the Drosophila PI 4-kinase four wheel drive in localizing Rab11 during cytokinesis. *J. Cell Biol.* **187**, 847-858.
Raghu, P., Coessens, E., Manifava, M., Georgiev, P., Pettitt, T., Wood, E., Garcia-Murillas, I., Okkenhaug, H., Trivedi, D., Zhang, Q. et al. (2009). Rhabdomere biogenesis in Drosophila photoreceptors is acutely sensitive to phosphatidic acid levels. *J. Cell Biol.* **185**, 129-145.
Robinson, B. S., Huang, J., Hong, Y. and Moberg, K. H. (2010). Crumbs regulates Salvador/Warts/Hippo signaling in Drosophila via the FERM-domain protein expanded. *Curr. Biol.* **20**, 582-590.
Rogulja, D., Rauskolb, C. and Irvine, K. D. (2008). Morphogen control of wing growth through the Fat signaling pathway. *Dev. Cell* **15**, 309-321.
Roth, S. and Lynch, J. A. (2009). Symmetry breaking during Drosophila oogenesis. *Cold Spring Harb. Perspect. Biol.* **1**, a001891.
Roth, S., Neuman-Silberberg, F. S., Barcelo, G. and Schüpbach, T. (1995). Cornichon and the EGF receptor signaling process are necessary for both anterior-posterior and dorsal-ventral pattern formation in Drosophila. *Cell* **81**, 967-978.
Silva, E., Tsatskis, Y., Gardano, L., Tapon, N. and McNeill, H. (2006). The tumor-suppressor gene fat controls tissue growth upstream of expanded in the hippo signaling pathway. *Curr. Biol.* **16**, 2081-2089.
Skwarek, L. C. and Boulianne, G. L. (2009). Great expectations for PIP: phosphoinositides as regulators of signaling during development and disease. *Dev. Cell* **16**, 12-20.
St Johnston, D., Beuchle, D. and Nüsslein-Volhard, C. (1991). Staufen, a gene required to localize maternal RNAs in the Drosophila egg. *Cell* **66**, 51-63.
Sun, J. and Deng, W. M. (2005). Notch-dependent downregulation of the homeodomain gene cut is required for the mitotic cycle/endocycle switch and cell differentiation in Drosophila follicle cells. *Development* **132**, 4299-4308.
Sun, J. and Deng, W. M. (2007). Hindsight mediates the role of notch in suppressing hedgehog signaling and cell proliferation. *Dev. Cell* **12**, 431-442.
Takeuchi, K., Sato, N., Kasahara, H., Funayama, N., Nagafuchi, A., Yonemura, S., Tsukita, S. and Tsukita, S. (1994). Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J. Cell Biol.* **125**, 1371-1384.
Tapon, N., Harvey, K. F., Bell, D. W., Wahrer, D. C., Schiripo, T. A., Haber, D. A. and Hariharan, I. K. (2002). Salvador Promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. *Cell* **110**, 467-478.
Udan, R. S., Kango-Singh, M., Nolo, R., Tao, C. and Halder, G. (2003). Hippo promotes proliferation arrest and apoptosis in the Salvador/Warts pathway. *Nat. Cell Biol.* **5**, 914-920.
van Eeden, F. and St Johnston, D. (1999). The polarisation of the anterior-posterior and dorsal-ventral axes during Drosophila oogenesis. *Curr. Opin. Genet. Dev.* **9**, 396-404.
Wei, X., Shimizu, T. and Lai, Z. C. (2007). Mob as tumor suppressor is activated by Hippo kinase for growth inhibition in Drosophila. *EMBO J.* **26**, 1772-1781.
Willecke, M., Hamaratoglu, F., Kango-Singh, M., Udan, R., Chen, C. L., Tao, C., Zhang, X. and Halder, G. (2006). The fat cadherin acts through the hippo tumor-suppressor pathway to regulate tissue size. *Curr. Biol.* **16**, 2090-2100.
Wu, S., Huang, J., Dong, J. and Pan, D. (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.
Xi, R., McGregor, J. R. and Harrison, D. A. (2003). A gradient of JAK pathway activity patterns the anterior-posterior axis of the follicular epithelium. *Dev. Cell* **4**, 167-177.
Xu, T., Wang, W., Zhang, S., Stewart, R. A. and Yu, W. (1995). Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. *Development* **121**, 1053-1063.
Yu, J., Poulton, J., Huang, Y. C. and Deng, W. M. (2008). The hippo pathway promotes Notch signaling in regulation of cell differentiation, proliferation, and oocyte polarity. *PLoS One* **3**, e1761.
Yu, J., Zheng, Y., Dong, J., Klusza, S., Dang, W. M. and Pan, D. (2010). Kibra functions as a tumor suppressor protein that regulates Hippo signaling in conjunction with Merlin and Expanded. *Dev. Cell* **18**, 288-299.