**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 phosphatidylinositol-4-phosphate (PIP₄), an important cell membrane phospholipid and a precursor for other phosphoinositide species such as P(1,4,5)P₃ (PIP₃). 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
Hippo signaling targets expanded (ex) and diap1 (thread–FlyBase) in the PFCs. Importantly, the apical localization of Merlin is lost in P4kIIalpha mutant PFCs, indicating a potential direct link between membrane composition and Hippo signaling.

MATERIALS AND METHODS
Fly stocks and genetics
Six P4kIIalpha mutant alleles were isolated from a previously described genetic screen (Deneff 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 kkekton-lacZ (Pai et al., 2000), 10×STAT-GFP (Bach et al., 2007), exclacZ (Boedigheimer and Laughon, 1993), diap1-lacZ (Hay et al., 1995), Kin-lacZ (Clark et al., 1994) and Ubi-PH-PLC6-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:

P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/+;
P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/kkekton-lacZ;
P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/ex-lacZ;
P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/diap1-lacZ+;
P4kIIalphaGS27 FRT19A/FRT19A; e22c-Gal4, UAS-FLP/+; 10×STAT-GFP/+;
P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/+;
P4kIIalphaGS27 FRT19A/Ubi-GFP FRT19A; e22c-Gal4, UAS-FLP/+;

Immunofluorescence staining and microscopy
Ovaries were dissected, fixed and stained following standard procedures. Primary antibodies used were: mouse anti-Gurken (1D12, 1:1000, 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-β-galactosidase (1:1000, Aves Labs), guinea pig anti-Expanded (1:2000 (Maitra et al., 2006)), guinea pig anti-Merlin [1:500 (McCarty and Felon, 1996)], rabbit anti-Kibra [1:100 (Genevet et al., 2010)], rabbit anti-phospho-ERM (Cell Signaling, 1:100) and guinea pig anti-Cut99C [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-phallolidin (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.

RESULTS AND DISCUSSION
P4kIIalpha 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 (Deneff 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 PIPα, Phosphoinositides, including PIPα, are important phospholipids in the cell membrane that participate in numerous signaling events (Skwark 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 (Ball and Balla, 2006). Three PI4Ks have been annotated in the fly genome: four wheel drive (fwd; P4kIIibeta) (Polevoy et al., 2009), CG2929 (P4kIIalpha) (Raghu et al., 2009) and CG10260 (P4kIIalpha).

To investigate the oocyte polarization defects caused by P4kIIalpha 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 P4kIIalpha, 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 P4kIIalpha, 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 P4kIIalpha is required in the PFCs for all aspects of the establishment of correct oocyte polarity.

P4kIIalpha 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...
PI4KIIIalpha (PH3), which is only seen up to stage 6 in wild-type FCs (Fig. 2C; n = 76) and upregulate Hnt (Fig. 2F; 66.7%, n = 57) expression. Interestingly, PI4KIIalpha mutant cells on the lateral side of the egg chambers showed no defect in Notch signaling (Fig. 2D-E). These results suggest that PI4KIIalpha 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 PI4KIIalpha 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 PI4KIIalpha mutant FCs (Fig. 2H; 43.4%, n = 53). These results indicate that the polarization defect in the PI4KIIalpha mutants is likely to be caused by defective Hippo signaling.

and Tapon, 2007; Yu et al., 2008). To examine whether the polarization defect we observed in PI4KIIalpha mutants was caused by disruption of one of these signaling pathways, we examined well-established downstream targets of each pathway in PI4KIIalpha mutants.

The EGFR signaling reporter kekkon-lacZ (kek-lacZ) is highly expressed in the PFCs at stage 7 and 8 as a result of EGFR activation by Grk (Pai et al., 2000). In PFCs mutant for PI4KIIalpha, the kek-lacZ expression level was comparable to that of wild-type PFCs, indicating that EGFR signaling was unaffected (Fig. 2A; n > 50). The JAK/STAT signaling reporter 10xSTAT92E-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 PI4KIIalpha 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). PI4KIIalpha 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 PI4KIIalpha 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). PI4KIIalpha mutant PFCs frequently failed to downregulate Cut (Fig. 2E; 81.6%, n = 76) and upregulate Hnt (Fig. 2F; 66.7%, n = 57) expression. Interestingly, PI4KIIalpha mutant cells on the lateral side of the egg chambers showed no defect in Notch signaling (Fig. 2D-E). These results suggest that PI4KIIalpha mutations compromise Notch signaling in the PFCs only.
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-Kibr 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-Kibr 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). 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.
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 PIP2 using a Ubi-PH-PLCδ-GFP reporter (Gervais et al., 2008), we observed a complete absence of PIP2 from PI4KIIIalpha mutant FCs in rare cases (2 out of 40 clones). In most cases, the PIP2 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 PIP2 in the membrane to switch from a closed to an open conformation. Merlin 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.

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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 microvilli 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 PIP2-binding site (Barret et al., 2000). Given our observations, it is possible that PIP2 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.

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