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 (Grušček 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 (McCleatchey and Feohan, 2009). The scaffold protein Kibra interacts with Merlin and Expanded both genetically and physically and the Merlin-Expanded-Kibra apical complex promotes Hippo activity (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 PIP5 (PIP2). 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 FCs. Importantly, the apical localization of Merlin in P4KIIalpha mutant FCs, 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 (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 (Boedighheimer 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/UbA-FLP/GAL4 system (Duffy et al., 1998). OE 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/kekko-kac-
- P4KIIalphaGS27 FRT19A/FRT19A; e22c-Gal4, UAS-Flp/+
- P4KIIalphaGS27 FRT19A/FRT19A; e22c-Gal4, UAS-Flp/+
- P4KIIalphaGS27 FRT19A/FRT19A; e22c-Gal4, UAS-Flp/ex-

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-β-gal (1:1000, Aves Labs), guinea pig anti-Expanded [1:200 (Maistra et al., 2006)], guinea pig anti-merlin [1:500 (McCartney and Felon, 1996)], rabbit anti-Kibra [1:100 (Genevet et al., 2010)], rabbit anti-phospho-ERM (Cell Signaling, 1:100) and guinea pig anti-arrd9 (1:2000 [D’Alterio et al., 2005]). Alexa Fluor 568- and 568-conjugated secondary antibodies were from Molecular Probes and pig anti-Cad99C [1:2000 (D’Alterio et al., 2005)], rabbit anti-phospho-ERM (Cell Signaling, 1:100) and guinea pig anti-Kibra [1:100 (Genevet et al., 2010)]. Alexa Fluor 568- and 568-conjugated secondary antibodies were from Molecular Probes and pig anti-Cad99C [1:2000 (D’Alterio et al., 2005)].

RESULTS AND DISCUSSION

PI4KIIalpha 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. We identified a set of candidate genes 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 PIP4. Phosphoinositides, including PIP4, are important phospholipids in the cell membrane that participate in numerous signaling events (Skwark and Boulam, 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 P4K genes have been annotated in the fly genome: four wheel drive (fwd; P4KIIIb) (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 was frequently mislocalized to the center of the oocyte or became dispersed in the oocyte (Figs. 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.

PI4KIIalpha 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 et al., 2009; Xi et al., 2003; Meignin et al., 2007; Polesello et al., 2009).
Cut and Hindsight (Hnt; Pebbled – FlyBase). In wild-type FCs, Cut We also examined the expression of two Notch signaling targets, structure and had smaller nuclei than neighboring cells (Fig. 2D).

PI4KIIIalpha mutant PFCs often lost their monolayered epithelial PI4KIIIalpha (PH3), which is only seen up to stage 6 in wild-type FCs (Fig. 2C; sustained staining of the mitotic marker phosphorylated Histone H3 maintained a mitotic cell cycle after stage 6, as indicated by the n stage 7 and 8 in response to JAK/STAT activation. We detected GFP (Bach et al., 2007) is normally turned on in the PFCs during stage 7 and 8 as a result of EFGR 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; 81.3%, n=76) and upregulate Hnt expression (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.

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 EGFR activation by Grk (Pai et al., 2000). In PFCs for PI4KIIIalpha, 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 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.
Merlin localization is affected in P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha 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. 2. Effects of P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha mutant PFCs, as indicated by the normal levels of β-gal staining (A,A’) and GFP signal (B,B’). (C-F) Egg chambers containing P14KIIIalpha 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). P14KIIIalpha 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). P14KIIIalpha mutant PFCs failed to downregulate Cut (E) and to upregulate Hnt (F) after stage 6. These results indicate that Notch signaling is compromised in P14KIIIalpha mutant PFCs. (D,E) Note that P14KIIIalpha 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 P14KIIIalpha 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 P14KIIIalpha mutant FCs. Scale bars: 10 μm.
adults. Eye discs from late L2 larvae exhibited pyknotic nuclei staining in \textit{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-PLC\(\delta\)-GFP reporter (Gervais et al., 2008), we observed a complete absence of PIP2 from \textit{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 \textit{PI4KIIIalpha} mutant FCs by phalloidin staining, they exhibited abnormal actin-enriched spike structures on their apical domain (Fig. 3H), 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 \textit{PI4KIIIalpha} mutant cells is distinct from that caused by mutations in the Hippo pathway (Fig. 3H), suggesting that the loss of \textit{PI4KIIIalpha} might also have a Hippo-independent effect on apical membrane structure.

How could \textit{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...
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 microvillar region as indicated by a phosopho-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 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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