**PI4KIIIα** is required for cortical integrity and cell polarity during *Drosophila* oogenesis

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Phosphoinositides regulate myriad cellular processes, acting as potent signaling molecules in conserved signaling pathways and as organelle gatekeepers that recruit effector proteins to membranes. Phosphoinositide-generating enzymes have been studied extensively in yeast and cultured cells, yet their roles in animal development are not well understood. Here, we analyze Drosophila melanogaster phosphatidylinositol (PI) 4-kinase IIIα (PI4KIIIα) during oogenesis. We demonstrate that PI4KIIIα is required for production of plasma membrane PI4P and PI(4,5)P2 and is crucial for actin organization, membrane trafficking and cell polarity. Female germ cells mutant for PI4KIIIα exhibit defects in cortical integrity associated with failure to recruit the cytoskeletal-membrane crosslinker Moesin and the exocyst subunit Sec5. These effects reflect a unique requirement for PI4KIIIα, as egg chambers from flies mutant for either of the other Drosophila PI4Ks, fwd or PI4KII, show Golgi but not plasma membrane phenotypes. Thus, PI4KIIIα is a critical regulator of a functionally distinct pool of PI4P that is essential for PI(4,5)P2-dependent processes in Drosophila development.

Abstract: 159 words (max 160)
Introduction

Although phosphoinositides constitute only a small percentage of membrane lipids, they exert powerful effects on many cellular processes. The seven phosphoinositide species are named according to the combination of phosphate groups present on the 3-, 4-, and 5-positions of the inositol ring. Phosphatidylinositol (PI) 4-kinases (PI4Ks) catalyze conversion of PI to PI 4-phosphate (PI4P), the first step in generating the majority of PI phosphates (PIPs) in the cell. PI4P itself has emerged as a key regulator of membrane trafficking at the Golgi by binding and recruiting effectors such as clathrin adaptors, coat proteins, and lipid transfer proteins (D’Angelo et al., 2012). Interestingly, despite the importance of PI4P in intracellular membrane compartments, it has been suggested that the majority of PI4P resides at the plasma membrane (PM) (Hammond et al., 2009).

PI4P is the metabolic precursor to PI 4,5-bisphosphate [PI(4,5)P2], which mainly resides at the PM, where it regulates diverse processes such as cytokinesis, cell migration, cell polarization, cell adhesion, and cell morphogenesis (Brill et al., 2011; Echard, 2012; Saarikangas et al., 2010; Shewan et al., 2011; Zhang et al., 2012). Some of these processes are governed by second messengers that are formed by phospholipase C (PLC)-dependent hydrolysis of PI(4,5)P2. Others are accomplished by recruitment of effector proteins that specifically bind PI(4,5)P2. For example, PI(4,5)P2 recruits AP-2 and dynamin during endocytosis and the exocyst complex during exocytosis (Gaidarov and Keen, 1999; Martin, 2012; Vallis et al., 1999). In flies and mammalian cells, PI(4,5)P2 localizes the exocyst to sites of polarized exocytosis, likely by binding directly to the polybasic domains of the Sec3 and Exo70 subunits, as was shown in yeast (Fabian et al., 2010; He et al., 2007; Thapa et al., 2012; Xiong et al., 2012). Furthermore, PI(4,5)P2 recruits and activates actin regulators, including the cytoskeletal-PM crosslinker Moesin, which requires PI(4,5)P2-binding and subsequent phosphorylation to relieve autoinhibited occlusion of its F-actin binding site (Fievet et al., 2004). The critical role of PI(4,5)P2 at the PM lends interest to recent evidence showing that its precursor, PI4P, has independent dynamics from PI(4,5)P2 and suggesting that PI4P itself can play a role in defining PM identity (Hammond et al., 2012). Although functional studies are beginning to emerge in vitro, few experiments have addressed the role of PM PI4P and the extent to which it is tied to PI(4,5)P2 during animal development.

Much of what we know about PI4P has been elucidated through studying PI4Ks. These
enzymes fall into two classes: type III PI4Ks, which share biochemical properties with the PI3K family of enzymes, and type II PI4Ks, which are unrelated (Balla and Balla, 2006). PI4Ks III\(\alpha\) and II\(\beta\) exert their functions at the PM, whereas PI4Ks III\(\beta\) and II\(\alpha\) affect Golgi and endosomes. Budding yeast has three PI4Ks, STT4 (PI4KIII\(\alpha\)), PIK1 (PI4KIII\(\beta\)), and LSB6 (PI4KII). STT4 and PIK1 have non-overlapping essential roles (Audhya and Emr, 2002; Audhya et al., 2000), whereas LS6B is dispensable (Han et al., 2002). STT4 localizes at the PM, where it regulates actin organization, vacuole morphology and PKC1-MAPK signaling. In contrast, PIK1 has essential functions in the nucleus and at the Golgi, where it directs secretion (Strahl et al., 2005). These disparate functions of yeast PI4Ks are roughly paralleled in mammalian cells, where PI4KIII\(\alpha\) controls a hormone-sensitive pool of PI4P at the PM and PI4KIII\(\beta\) and PI4KII\(\alpha\) control Golgi PI4P and post-Golgi trafficking (Balla et al., 2005; Jovic et al., 2012; Weixel et al., 2005).

PI4Ks are crucial for cell homeostasis, yet only a handful of studies address their functions in multicellular organisms (Brill et al., 2000; Burgess et al., 2012; Khuong et al., 2010; Ma et al., 2009; Polevoy et al., 2009; Raghu et al., 2009; Simons et al., 2009; Yan et al., 2011; Yavari et al., 2010). A recent report examining mouse PI4KIII\(\alpha\) revealed transient localization of PI4KIII\(\alpha\) to the PM (Nakatsu et al., 2012). However, because PI4KIII\(\alpha\) is essential, it was possible to examine genetic nulls only in primary cultures of induced knockout embryonic fibroblasts (MEFs). Hence, the role of PI4KIII\(\alpha\) during animal development has remained a mystery.

The fruit fly Drosophila melanogaster provides a tractable system to examine cellular roles of essential genes; using genetic tools that are more cumbersome to generate in mammals, it is possible to analyze mutant tissues in otherwise normal flies. Drosophila has three PI4Ks. We previously showed that the PIK1 and LSB6 homologues Four wheel drive (Fwd) and PI4KII play roles in post-Golgi trafficking, but are not essential (Brill et al., 2000; Burgess et al., 2012). Fwd localizes to the Golgi where it is required for spermatocyte cytokinesis (Polevoy et al., 2009), whereas PI4KII localizes to Golgi and endosomes and is required for secretory granule biogenesis in the larval salivary gland (Burgess et al., 2012). Here, we examine the requirement for PI4KIII\(\alpha\), which we show is essential and needed for structural integrity of the PM during oogenesis. PI4KIII\(\alpha\) is required for activation and recruitment of Moesin and Sec5, effector proteins that organize the cell cortex. These roles are specific to PI4KIII\(\alpha\), as mutations in \(fwd\)
and PI4KII affect Golgi but not PM morphology. Moreover, PI4KIIIα is required for normal levels of PM PI4P and PI(4,5)P2. Because loss of PI4KIIIα phenocopies mutations in the PI(4,5)P2 regulators sktl and pten, and titration of PI(4,5)P2 recapitulates PI4KIIIα mutant phenotypes, this suggests a crucial role for PI4KIIIα in synthesizing PI4P that acts as a precursor to PI(4,5)P2. Our results highlight PI4KIIIα as a key regulator of cortical integrity and trafficking events at the PM, and emphasize that different pools of the same phosphoinositide can serve drastically different physiological and cellular functions.
Results

PI4KIIIα is essential and allelic to zeste-white 2

To investigate the role of PI4KIIIα during Drosophila development, we generated a deletion in the corresponding gene. The deletion, henceforth referred to as PI4KIIIαΔ123, removes the entire predicted kinase domain, the majority of the upstream phosphatidylinositol 3-kinase (PIK) accessory domain and part of the first exon of shaggy (sgg, also known as zeste-white3 [zw3]) (Fig. 1A; see Materials and Methods). PI4KIIIαΔ123 was recessive lethal, and hemizygous males died shortly after embryogenesis as first instar larvae. Lethality was due to disruption of PI4KIIIα since viability was fully rescued with a PI4KIIIα transgene (Fig. 1A).

PI4KIIIα is located at polytene interval 3A8 (Marygold et al., 2013). Since saturation mutagenesis had previously been performed in this region of the X chromosome, which lies between zeste and white (Judd et al., 1972), we tested PI4KIIIαΔ123 for allelism to any of the previously identified lethal complementation groups. PI4KIIIαΔ123 failed to complement zeste-white2c21 (zw2c21), but complemented sgg and zw6, two neighboring complementation groups (Fig 1B). Like PI4KIIIαΔ123, males hemizygous for zw2c21 or other zw2 alleles die as first instar larvae (Shannon et al., 1972). These data indicate that PI4KIIIα is allelic to zw2. In addition, because PI4KIIIαΔ123 complemented sgg, the small region of sgg removed in PI4KIIIαΔ123 has no effect on sgg function.

PI4KIIIα is required for normal egg chamber morphology

To determine whether a role for PI4KIIIα in embryogenesis was masked by maternal contribution, female germline clones (GLCs) homozygous for PI4KIIIαΔ123 were generated using the FLP-FRT system and the dominant female-sterile mutation ovoD (Chou and Perrimon, 1992). No eggs were recovered upon induction of PI4KIIIαΔ123 GLCs, consistent with a report that no embryos were produced when maternal zw2 was eliminated (Perrimon et al., 1989). Hence, PI4KIIIα is required during oogenesis.

Examination of PI4KIIIαΔ123 GLCs by DAPI staining revealed that in 50% of stage 9 or later egg chambers (n=36), nurse cell nuclei were found in the ooplasm, rather than being restricted to the anterior of the egg chamber (Fig. 1C,D). In addition, organization of the overlying layer of follicle cells appeared irregular, unlike the regular spacing observed in wild
type (WT) (Fig. 1C–D’). Mutant egg chambers at later stages showed evidence of border cell migration (see below) and nurse cell dumping (Fig. 1E), but lacked dorsal appendages (not shown). In addition, late-stage GLCs exhibited pycnotic nuclei (Fig. 1E) and appeared to degenerate.

**PI4KIIIα is required for actin organization and Moesin activation**

To understand the cellular basis for morphological defects in PI4KIIIα123 GLCs, egg chambers were stained with rhodamine-phalloidin to visualize F-actin. In WT egg chambers, F-actin was found along the cortex of the germ cells and in ring canals (Fig. 2A,D). In early PI4KIIIα123 GLCs, cortical F-actin was greatly reduced and ring canals clustered toward the center of the cyst (Figs 2C, 3D,F). These defects appeared to correlate, as cortical F-actin was observed in cysts where ring canals were not tightly coalesced into a single cluster (Fig. 2C,) or were found in more than one cluster (Fig. 2B). Of GLCs with more than one cluster of ring canals, 92% (n=13) showed cortical F-actin, compared with 45% (n=33) in GLCs with one cluster. In late-stage PI4KIIIα123 GLCs, F-actin was present between some nurse cell nuclei but not others, and aggregations of F-actin often protruded into or across the oocyte (Fig. 2E). F-actin along the oocyte cortex was buckled and disorganized, in contrast to the smooth and rigid appearance of the WT cortex (Fig. 2D). These F-actin phenotypes were never observed in control GLCs from ovoD/+ females and were rescued by the PI4KIIIα transgene, indicating that they are due to loss of PI4KIIIα (Fig. 2F; 100% penetrance in rescuing viability, fertility, and F-actin phenotypes).

Delamination of F-actin from the PM suggested a possible defect in crosslinking the cortical cytoskeleton to the overlying membrane (Jankovics et al., 2002; Polesello et al., 2002; Verdier et al., 2006). Since phosphorylation of the cytoskeletal-membrane crosslinker Moesin (Moe) is required for maintaining cortical actin in the oocyte, and PI(4,5)P2 binding is required for Moe phosphorylation (Fievet et al., 2004; Roch et al., 2010), we examined localization of activated, phosphorylated Moe (pMoe) in PI4KIIIα123 GLCs. In WT stage 10B egg chambers, pMoe colocalized with F-actin along both the oocyte PM and the juxtaposed apical membranes of follicle cells (Fig. 2G). In similarly staged PI4KIIIα123 GLCs, pMoe was greatly reduced at the oocyte cortex and F-actin was more diffuse (Fig. 2G,H), whereas apical localization appeared normal in adjacent follicle cells (Fig. 2G,H).

To examine whether loss of PI4KIIIα affects overall levels of pMoe in addition to pMoe
localization, *Drosophila* S2 cells were treated with double-stranded RNAs (dsRNAs) to knock down *PI4KIIIα* expression by RNA interference (RNAi) and levels of pMoe were assessed by immunoblotting. Treatment with any of three non-overlapping *PI4KIIIα* dsRNAs reduced the amount of pMoe to ~25-40% compared to mock treatment or RNAi directed against green fluorescent protein (GFP), but was not as dramatic as RNAi directed against the Moe kinase Slik (Hipfner et al., 2004) (Fig. 2I,J). Total levels of Moe remained unchanged, demonstrating that *PI4KIIIα* does not affect Moe production or stability. The effect on pMoe levels was specific to *PI4KIIIα*, as RNAi directed against the other *Drosophila* PI4Ks, *fwd* and *PI4KII*, had no effect (Figs 2K,L, S1; see Materials and Methods). In immunofluorescence experiments, pMoe levels were slightly reduced at the cortex of stage 10B *fwd* mutant oocytes (Fig. S2B). However, no actin defects were observed in *fwd* egg chambers (see below). pMoe localization in *PI4KII* mutant oocytes resembled WT (Fig. S2C). Depletion of the PI4P-5 kinase (PIP5K) Skittles (Sktl), which converts PI4P to PI(4,5)P2, also blocked pMoe accumulation in S2 cells (Roubinet et al., 2011) (Fig. S2D,E).

*PI4KIIIα* is required for plasma membrane integrity and exocyst localization

The presence of nurse cell nuclei in the ooplasm suggested that membrane barriers might be compromised in *PI4KIIIα*<sup>Δ123</sup> GLCs. To examine PM integrity in early and late-stage egg chambers, membranes were visualized using fluorophore-conjugated tomato lectin, which binds glycoproteins on intracellular and cell surface membranes (Dollar et al., 2002; Murthy and Schwarz, 2004; Verdier et al., 2006). In early WT egg chambers, lectin-positive membranes largely colocalized with cortical F-actin (Fig. 3A,B). In contrast, early-stage *PI4KIIIα*<sup>Δ123</sup> GLCs displayed thinner, discontinuous lectin staining along membranes (Fig. 3C,D), as well as lectin-positive aggregates within the cytoplasm, often concentrated near clustered ring canals (Fig. 3C,D). Thinner lectin staining reflects loss of PM, which was evident in transmission electron micrographs. In contrast to prominent membranes separating WT nurse cell nuclei (Fig. 3G-I), *PI4KIIIα*<sup>Δ123</sup> GLCs showed vesiculated membranes around clustered ring canals (Fig. 3J), thin membranes between some nurse cell nuclei (Fig. 3K,L), and none between others (Fig. 3K). In addition, membranes terminated within the cytoplasm in *PI4KIIIα*<sup>Δ123</sup> GLCs (Fig. 3K,L). Loss of cortical F-actin paralleled loss of membranes; 7/8 GLCs with cortical F-actin between germ cell nuclei also had overlying membrane (Fig. 3D). In early egg chambers with more severe
phenotypes, the somatic follicular epithelium that normally encapsulates the cyst also
degenerated or was missing (Fig. 3E,F). This somatic phenotype could be an indirect effect of
the germline on the follicle cells, or could result from defects due to the presence of unmarked
mutant follicle cell clones. Thus, only GLCs with an intact follicular epithelium and the presence
of cortical F-actin were scored as mildly affected (52%, n=58). In late-stage GLCs, membranes
were seen between some nuclei but not others (Fig. 4A,B), and at times formed a large whorl that
colocalized with F-actin in the oocyte (Fig. 4B). These effects were specific to PI4KIIIα GLCs,
as fwd and PI4KII mutant egg chambers showed distinct phenotypes (Fig. 4C,D; see below).
Given the presence of membranes and evidence of border cell migration in some late-stage GLCs
(Fig. 4B), we hypothesize that mildly affected early mutant GLCs are able to mature to late
stages, whereas severely affected early GLCs (e.g., Fig. 3F) fail to survive.
Since lack of cortical F-actin, clustering of ring canals and disintegration of PM are also
seen in GLCs of mutants that affect membrane addition, including the exocyst subunits Sec5 and
Sec6 (Beronja et al., 2005; Murthy et al., 2005; Murthy and Schwarz, 2004), we examined Sec5
distribution. In WT stage 6-8 egg chambers, Sec5 was found at the PM and enriched on oocyte
membranes (Murthy and Schwarz, 2004) (Fig. 5A,B). In PI4KIIIαΔ123 GLCs, this localization
was lost, even in mildly affected egg chambers that retained cortical F-actin (Fig. 5D,E). Sec5
was reduced on both the oocyte and nurse cell membranes (Fig. 5B,E). In contrast to WT (Fig.
5C), the level of Sec5 at the oocyte-follicle cell interface in PI4KIIIα GLCs (Fig. 5F) was no
stronger than on lateral follicle cell membranes, indicating that PI4KIIIα GLCs fail to recruit or
retain the exocyst.

PI4KIIIα, fwd and PI4KII have differential effects on cellular membranes
To test whether these PM defects result from a specific requirement for PI4KIIIα or a general
requirement for PI4P, we examined egg chambers in fwd and PI4KII null mutants. Overall, the
PM was intact and F-actin appeared normal (Fig. 4C,D), indicating that actin organization and
PM integrity during oogenesis are independent of Fwd and PI4KII. Additionally, Sec5
localization was not affected in fwd or PI4KII mutants (Fig. S3). However, in late-stage fwd and
PI4KII egg chambers, nurse cells showed large lectin-positive structures in the cytoplasm (Fig.
4C,D) that were not visible in late-stage WT egg chambers or PI4KIIIαΔ123 GLCs (Fig. 4A,B).
These structures were more prominent in PI4KII than fwd, with large puncta visible in early
PI4KII egg chambers that had otherwise normal lectin staining (Fig. 6D).

To further define these intracellular membrane defects, PI4KIIIαΔ123 GLCs and fwd and PI4KII mutant egg chambers were immunostained for the cis-Golgi marker Lava lamp (Lva). In stage 7 or earlier WT egg chambers, Lva decorated discrete puncta in the cytoplasm of germ cells, with ~40% of Lva structures partially overlapping with lectin puncta and vice versa (Fig. 6A,F). Lva puncta varied in size, with larger Lva structures being associated with larger lectin-positive structures (Fig. 6A, compare insets 1-3). In contrast, fwd and PI4KII mutant egg chambers showed obvious, yet distinct, defects in Golgi morphology. In fwd, Lva puncta were significantly smaller than WT (Fig. 6C,E). Lectin puncta that partially overlapped with larger Lva puncta were either irregularly shaped (Fig. 6C, inset 1) or elongated (Fig. 6C, inset 2). 22.4% (n=241) of fwd puncta exhibited abnormal shapes compared to 8.5% in WT (n=188) and 7.8% in PI4KII (n=90). Many of the small Lva puncta appeared to be in close proximity to, but did not overlap with, small lectin puncta (Fig. 6C, inset 3). As tomato lectin is predicted to bind glycosylated proteins at the trans-Golgi network (TGN), this may indicate fragmentation of the TGN. Lva puncta in PI4KII were slightly larger and more varied in size (Fig. 6D,E). The largest structures were often still associated with lectin, but many appeared to be clusters of several Lva puncta that could not be resolved at the level of confocal microscopy (Fig. 6D, inset 1). Within some clusters, discrete Lva puncta were distinguishable and scored as separate units (Fig. 6D, inset 3; scored as three Lva bodies). Overall, the average numbers of Lva puncta in fwd (112.3 ± 17.5) and PI4KII (114.0 ± 1.4) egg chambers were similar to WT (124.7 ± 58.8; n=2-3 egg chambers each). These results suggest fwd and PI4KII affect Golgi morphology and the manner in which cis-Golgi associate with the TGN.

In contrast, in PI4KIIIαΔ123 GLCs, Lva puncta were of similar size, shape, and number (118.7 ± 28.7 per GLC, n=3) to those in WT (Fig. 6E), and appeared as individual units rather than clusters. Lva puncta located away from the centre cluster partially overlapped with adjacent lectin puncta, as in WT (Fig. 6B, insets 1-3). However, in contrast to the perinuclear localization of Golgi in WT, most Lva puncta were localized in the centre of the cyst near the clustered lectin-positive membranes (Fig. 6B). This may be a secondary consequence of PM breakdown, as other organelles were concentrated in this region as well (Fig. 3J and not shown). Membrane clustering made it difficult to assess specific association between lectin and Lva puncta. While the normal size, shape, and number of Lva puncta suggest that cis-Golgi morphology is not
grossly affected in the absence of PI4KIIIα, inability to assess most of the lectin puncta  
precludes a conclusion about the effect of $PI4KIII^{\Delta 123}$ on overall Golgi organization.

**PI4KIIIα is required for PM PI4P and PI(4,5)P$_2$**

Since $PI4KIII^{\Delta 123}$ exhibited drastic effects on the PM and actin cytoskeleton, we reasoned that  
$PI4KIIIα$ might affect PM levels of PI4P or PI(4,5)P$_2$. To detect PI(4,5)P$_2$, we examined  
PLCδPH-GFP, a fluorescent reporter that has also been used to titrate PI(4,5)P$_2$ (Raucher et al.,  
2000). Low-level ubiquitous expression of PLCδPH-GFP marked the PM and colocalized with  
cortical F-actin in WT developing egg chambers (Fig. 7A,B). However, 50% of the egg  
chambers exhibited nurse cell nuclei in the ooplasm, indicating that titration of PI(4,5)P$_2$ can  
recapitulate a $PI4KIII^{\Delta 123}$ phenotype (Fig. 7B).

To assess PM phosphoinositide levels without eliciting phenotypes by titration, we used  
anti-PI4P or anti-PI(4,5)P$_2$ antibodies to immunostain WT egg chambers and $PI4KIII^{\Delta 123}$  
GLCs. In WT, PI4P was detected along the PM and in ring canals (Fig. 7C). In contrast,  
$PI4KIII^{\Delta 123}$ GLCs showed reduced PI4P staining at the PM (Fig. 7D; 76.7% of egg chambers,  
n=30). Since PM integrity is compromised in $PI4KIII^{\Delta 123}$ GLCs, reduced PI4P staining might  
be due to loss of membranes. To account for this, we examined the PI4P signal in relation to  
cortical F-actin. If the decrease in PI4P staining intensity was due to loss of membrane, and not a  
decrease in the level of PI4P, we would expect GLCs to exhibit a similar PI4P:F-actin ratio  
compared to WT. However, of the GLCs that showed reduced PI4P, the average PI4P:F-actin  
ratio was 55.5% of WT (Fig. 7E; n=23, p<0.01), indicating that $PI4KIIIα$ controls PI4P levels at  
the PM. Like PI4P, PI(4,5)P$_2$ was detected along the PM and in ring canals in WT (Fig. 7F).  
PI(4,5)P$_2$ was also reduced in $PI4KIIIα$ GLCs, although to a lesser extent (Fig. 7G; 46.9% of egg  
chambers, n=49); the PI(4,5)P$_2$:F-actin ratio was 70.5% of WT (Fig. 7E; n=23, p<0.05). Thus,  
PI4KIIIα is needed for normal PI4P and PI(4,5)P$_2$ levels at the PM.

**PI4KIIIα is required for egg chamber polarity**

Since egg chambers with reduced levels of PI(4,5)P$_2$ due to mutation of the PIP5K Sktl have  
oocyte polarity defects (Gervais et al., 2008), we examined whether $PI4KIII^{\Delta 123}$ GLCs also  
exhibit polarity defects. Oskar is localized to the posterior pole in stage 9 or later WT egg
chambers (Fig. 8A,A’). In \textit{PI4KIII}^{\Delta 123} \text{GLCs}, Oskar was either reduced (Fig. 8B,B’) or missing (Fig. 8C,C’) at the posterior pole, correlating with the degree of F-actin disruption. Two other polarity indicators are successful migration of the oocyte nucleus and localization of Gurken to the dorsal-anterior of the oocyte at stage 8 (Poulton and Deng, 2007) (Fig. 8D). In \textit{PI4KIII}^{\Delta 123} \text{GLCs}, Gurken was either not visibly concentrated within the egg chamber (Fig. 8E) or not associated with the oocyte nucleus (Fig. 8F). In addition, the oocyte nucleus failed to localize at the dorsal-anterior position (3/8 were properly localized, compared to 10/10 in WT) (Fig. 8D,F,G, asterisks; quantified in Fig. 8H). Hence, \textit{PI4KIII}^{\Delta 123} phenocopies \textit{sktl} polarity defects.
Discussion
Many cellular processes at the PM depend on phosphoinositides, although it has remained unclear whether these processes are coordinately regulated. Here, we show that during oogenesis, PI4KIIIα is essential for coordinating membrane trafficking and actin organization at the cortex as well as for integrity of the PM itself. Moreover, our data suggest that PI4KIIIα is the PI4K that affects PM phosphoinositides. We provide several lines of evidence indicating that a major role for this enzyme is production of PI4P for conversion into PI(4,5)P₂ at the PM.

First, PI4KIIIαΔ₁₂₃ GLCs exhibit nurse cell nuclei in the ooplasm and accumulation of intracellular F-actin, distinctive phenotypes also observed in GLCs mutant for the PI(3,4,5)P₃ phosphatase PTEN (von Stein et al., 2005), loss of which would also result in decreased levels of PI(4,5)P₂. The similar phenotypes of PI4KIIIα and dPTEN mutants strongly suggest they impinge upon a common pool of PI(4,5)P₂. Second, ubiquitous expression of the PLCδPH-GFP, which titrates PI(4,5)P₂, recapitulated this phenotype in 50% of otherwise WT egg chambers. Third, immunostaining revealed reduced levels of PI4P and PI(4,5)P₂ in the PM of PI4KIIIα GLCs. Fourth, PI4KIIIα GLCs fail to activate and recruit proteins previously shown to require PI(4,5)P₂. pMoe was dramatically reduced at the oocyte cortex in GLCs, and Moe phosphorylation was attenuated in PI4KIIIα knockdown cells. Additionally, PI4KIIIα GLCs failed to recruit or retain the exocyst subunit Sec5 at the PM. Consistent with these observations, PI4KIIIα GLCs phenocopy the disruption of cortical F-actin seen in mutants for Moe and the Moe activator dRok, and exhibit PM defects found in GLCs mutant for the exocyst subunits Sec5 and Sec6, as well as Rab6, a regulator of secretion, and Rab11, which binds the exocyst component Sec15 to promote vesicle recycling (Bogard et al., 2007; Coutelis and Ephrussi, 2007; Jankovics et al., 2002; Januschke et al., 2007; Langevin et al., 2005; Murthy et al., 2005; Polesello et al., 2002; Verdier et al., 2006; Wu et al., 2005). Thus, PI4KIIIα, by synthesizing the precursor to PI(4,5)P₂, exerts profound effects on PM signaling and stability.

Consistent with this, PI4KIIIαΔ₁₂₃ GLCs resemble GLCs for the PIP5K Sktl. For example, pMoe localization is also defective in sktl hypomorphic GLCs (Gervais et al., 2008). However, PI4KIIIα oogenesis defects are not identical to those of sktl. Although marked GLCs of sktl null alleles have been reported (Gervais et al., 2008), PI4KIIIαΔ₁₂₃ GLCs made in this manner fail to thrive amongst WT follicles (our unpublished observations). This suggests Sktl
may be at least partially redundant with another *Drosophila* PIP5K during oogenesis. Support for this idea stems from observations that PI4KIII\(\alpha\) and PI5K59B carry out similar functions in Rho activation during mesoderm migration (Murray et al., 2012), and that expression of dominant negative versions of Rho GTPase family members during oogenesis causes aggregation of ring canals and loss of cortical F-actin, similar to *PI4KIII\(\alpha\)\(^{\Delta 123}\)* GLCs (Murphy and Montell, 1996). Hence, we suggest that in *Drosophila* oogenesis PI4KIII\(\alpha\) acts upstream of Sktl, and perhaps also PIP5K59B, to produce a pool of PI4P that feeds PM PI(4,5)P\(_2\).

Loss of *PI4KIII\(\alpha\)* has a greater effect on PI4P than on PI(4,5)P\(_2\); a smaller percentage of GLCs showed reduced PI(4,5)P\(_2\), and those that did were less strongly affected. Interestingly, in *PI4KIII\(\alpha\)* knockout MEFs, PI4P and PI(4,5)P\(_2\) reporters are dramatically reduced at the PM, whereas global levels of PI(4,5)P\(_2\) are only modestly affected when assessed by metabolic labeling (Nakatsu et al., 2012). This is likely due to upregulation of two PIP5Ks, PIPKI\(\beta\) and PIPK1\(\gamma\). Hence, it is possible that compensatory upregulation of *Drosophila* PIP5Ks accounts for the weaker effect of *PI4KIII\(\alpha\)* on PI(4,5)P\(_2\). Alternatively, in the absence of PI4KIII\(\alpha\), one of the other PI4Ks could supply a small amount of PI4P that serves as a precursor to PI(4,5)P\(_2\).

Our results leave open the possibility of PI(4,5)P\(_2\)-independent functions for PM PI4P. It is noteworthy that some functions previously attributed to PM PI(4,5)P\(_2\) were found to be reliant on a negative charge that could be provided by either PI4P or PI(4,5)P\(_2\) (Hammond et al., 2012). Thus, in *PI4KIII\(\alpha\)\(^{\Delta 123}\)* GLCs, it is possible that lack of PI4P is directly responsible for the some of the observed phenotypes. However, the fact that *sktl* GLCs show similar phenotypes indicates that either PI4P alone is not sufficient or that PI(4,5)P\(_2\) is specifically required. Since PI4KIII\(\alpha\) regulates both PI4P and PI(4,5)P\(_2\), the mechanism by which PI4P contributes to PM function in *Drosophila*, whether as a direct regulator, a precursor, or both, remains an open question.

Several aspects of cell polarity were disrupted in *PI4KIII\(\alpha\)\(^{\Delta 123}\)* GLCs. Failure of the oocyte nucleus to migrate or anchor at the dorsoanterior and mislocalization of Gurken are phenotypes shared with *sktl* mutants (Gervais et al., 2008). Oskar protein was either reduced or missing from the posterior pole, suggesting *PI4KIII\(\alpha\)* like *sktl* and *dPTEN*, may also affect *oskar* mRNA localization (Gervais et al., 2008; von Stein et al., 2005). A similar effect on *oskar* mRNA and protein was frequently observed in moesin mutant egg chambers (Jankovics et al., 2002; Polesello et al., 2002). We noted that the extent of the Oskar defect correlated with the
degree of F-actin disruption, suggesting the effect of PI4KIIIα on Oskar may be mediated in part via Moesin-dependent F-actin organization. Indeed, based on previously reported links between Oskar and F-actin (Krauss et al., 2009; Tanaka et al., 2011), PI4KIIIα may stimulate a positive feedback loop that coordinates phosphoinositides, actin organization, membrane trafficking and cell polarization.

PI4KIIIα was previously shown to be required in posterior follicle cells (PFCs) to control Hippo signaling, which in turn regulates oocyte nucleus migration and localization of the posterior polarity determinant Staufen (Yan et al., 2011). These defects are similar to those seen in PI4KIIIα GLCs. However, our observations suggest that PI4KIIIα likely regulates distinct molecular events in PFCs and the oocyte to control oocyte nucleus migration. In egg chambers with PI4KIIIα mutant PFCs, the oocyte nucleus is consistently positioned tightly at the posterior, indicating that mutant PFCs fail to send the unknown signal that initiates oocyte repolarization (Yan et al., 2011). In contrast, the oocyte nucleus in PI4KIIIα GLCs is found in the middle of the oocyte, suggesting that a PI4KIIIα mutant germline is capable of receiving the unknown signal initiating nucleus migration, but fails to complete the process. Alternatively, tethering of the nucleus to the dorsal-anterior or posterior of the oocyte could be defective in PI4KIIIα GLCs.

When half of the PFCs are WT and half are mutant for Hippo signaling, normal posterior Staufen and Oskar localization is observed adjacent only to the WT PFCs (Meignin et al., 2007; Polesello and Tapon, 2007; Yu et al., 2008), suggesting that PI4KIIIα may be required in both the oocyte and the PFCs for continued communication and maintenance of posterior polarity determinants.

Our study identifies PI4KIIIα as the essential PM PI4K in flies, and shows that it performs a non-overlapping cellular function. Indeed, our results underscore a recurrent theme in phosphoinositide biology: Enzymes that nominally act to produce the same lipid can have vastly different physiological and cellular effects. For example, the class II PI3Ks PI3K-C2α and PI3K-C2β both produce PI3P and are co-expressed; however, only the latter is necessary for LPA-dependent migration of cultured human ovarian and cervical cells (Maffucci et al., 2005).

Differential regulation of the enzymes likely results in production of different pools of the same lipid within the cell, emphasizing the importance of identifying factors that control specific pools. Indeed, genetic interactions imply that Hedgehog relieves Patched inhibition of

Drosophila PI4KIIIα, suggesting PI4KIIIα activity is regulated (Yavari et al., 2010). PI4KIIIα
also promotes FGF signaling in zebrafish (Ma et al., 2009), Hippo signaling in flies (Yan et al., 2011; Yavari et al., 2010) and MAPK signaling in yeast (Garrenton et al., 2010). Hence, a critical and conserved property of this enzyme is to control lipids at the PM, thereby relaying signals to molecular effectors at the cell cortex. Given the multifaceted roles of phosphoinositide signaling in metazoans, it appears that functional diversity of PI4K isoforms has evolved as a mechanism for spatial coordination of phosphoinositide-dependent processes in the cell, with PI4KIIIα acting as a key regulator at the PM.
17

Materials and Methods

Fly stocks and genetic crosses

Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). Visible markers and balancer chromosomes are described in Lindsley and Zimm (1992). Germline transformation of \emph{w^{1118}} embryos was carried out as in Spradling and Rubin (1982). P-element GE3785 was obtained from GenExel (Taejon, Korea). \emph{w;; \Delta 2-3 Sb/TM2, Ubx} was from Ted Erclik and Howard Lipshitz (University of Toronto, Toronto, ON, Canada). \emph{w;P\{w\+, UASp::PLC\&PH-GFP\}} was from Lynn Cooley (Yale University, New Haven, CT) and \emph{w;P\{w\+, tubP-GAL4\}/TM3, Sb} was from Eyal Schejter (Weizmann Institute, Rehovot, Israel). \emph{fwd} and \emph{PI4KII} mutants were described previously (Brill et al., 2000; Burgess et al., 2012). Stocks from the Bloomington \emph{Drosophila} Stock Center (Bloomington, IN) were:

\begin{itemize}
  \item \emph{l(1)3Ah^{21}/FM7a/Dp(1;2;Y)w\+}
  \item \emph{l(1)3Bb^{4}/FM7a/Dp(1;2;Y)w\+}
  \item \emph{sgg^{7}/FM7a/Dp(1;2;Y)w\+}
  \item \emph{P\{FRT(w^{hs})\}14A-B}
  \item \emph{w ovoD P\{FRT(w^{hs})\}14A-B/C(1)DX/Y; P\{hsFLP\}38}
  \item \emph{y w P\{Ubi-GFP\}ID-1 P\{FRT(w^{hs})\}14A-B}
  \item \emph{FM7i, y w B, P\{Act-GFP\}JMR3/C(1)DX, y f}
  \item \emph{l(1)3Ah^{21} is zw2^{21}}, an allele of \emph{zeste-white2 (zw2)} and \emph{l(1)3Bb^{4} is an allele of zw6}.
\end{itemize}

To generate GLCs by the method of Chou and Perrimon (1992), the \emph{PI4KIII\alpha} deletion was recombined with \emph{P\{FRT(w^{hs})\}14A-B} and balanced over \emph{FM7i}. GLCs were produced by crossing virgin females to \emph{w ovoD P\{FRT(w^{hs})\}14A-B/Y; P\{hsFLP\}38} males. Females were allowed to lay eggs for 24 hours and emerging larvae were heat-shocked for 2 hours in a 37°C water bath at 48, 72, and 96 hours. Non-Bar female progeny were aged 7-8 days on yeast paste before dissection.

Generation of \emph{PI4KIII\alpha} deletion

To generate a deletion in \emph{PI4KIII\alpha}, GE3785 (Fig. 1A) was excised imprecisely using \emph{Δ2-3} transposase. Deletions were identified by PCR of genomic DNA extracted from 750 candidate flies, pooled into groups of 10. An initial deletion removed the intergenic region between \emph{PI4KIII\alpha} and the first exon of \emph{sgg}, while leaving GE3785 intact (GE3785-9). Subsequent
mobilization of GE3785-9 removed an additional 1.8 kb from the 3’ end of

\( \text{PI4KIII} \alpha \). (\( \text{PI4KIII} \alpha^{123} \)).

Neither homozygous \( \text{PI4KIII} \alpha^{123} \) females nor hemizygous \( \text{PI4KIII} \alpha^{123} \) males were
recovered, indicating \( \text{PI4KIII} \alpha \) is essential. To determine the lethal period of \( \text{PI4KIII} \alpha^{123} \)
mutants, \( \text{PI4KIII} \alpha^{123}/\text{FM7i} \) females were crossed to \( \text{FM7i Act-GFP/Y} \) males. Non-GFP (male)
embryos were collected onto agar juice plates and allowed to develop. All embryos hatched, but
only \( \text{FM7i/Y} \) larvae reached the adult stage. The others died as L1 larvae, and were presumed to
be \( \text{PI4KIII} \alpha^{123}/\text{Y} \). To assess the ability of the \( \text{PI4KIII} \alpha \) transgene to rescue viability, \( \text{FM7i Act-}
\text{GFP/Y} \) males were crossed with \( \text{PI4KIII} \alpha^{123};; P\{w^+; \text{PI4KIII} \alpha\} \) females. Viable and fertile
non-Bar males of genotype \( \text{PI4KIII} \alpha^{123}/\text{Y};; P\{w^+; \text{PI4KIII} \alpha\}/+ \) were recovered in equal
numbers to female siblings.

Molecular biology

The \( \text{PI4KIII} \alpha \) rescue transgene was generated as a genomic-cDNA fusion. The 5’ half,
consisting of genomic DNA encoding \( \text{PI4KIII} \alpha \), was joined to the 3’ half, containing cDNA
(EST clone SD12145; Canadian Drosophila Microarray Centre, Mississauga, Ontario, Canada).
Genomic DNA containing the 5’ rescuing region was amplified from \( \text{w}^{1118} \) flies and cloned into
pBluescript with \( XbaI \) and \( KpnI \). cDNA subcloned from SD12145 as a \( KpnI-MluI \) fragment was
ligated with a \( MluI-XmaI \) PCR product containing the 3’UTR from SD12145 into pBluescript
with \( KpnI \) and \( XmaI \). Genomic and cDNA fragments were fused at a unique internal \( KpnI \) site
and cloned into pBluescript with \( XbaI \) and \( XmaI \). The genomic-cDNA construct was subcloned
into pCaSpeR4 using \( XbaI \) and \( SmaI/StuI \).

\( \text{PI4KIII} \alpha \), \( \text{fwd} \), \( \text{PI4KII} \), and \( \text{sktl} \) double-stranded RNA (dsRNA) templates were prepared
by PCR amplification of genomic DNA from \( \text{w}^{1118} \) flies. \( \text{slik} \) dsRNA was amplified from EST
LD34405. As a negative control, \( \text{GFP} \) dsRNA was amplified from the pEGFP-N2 plasmid.
Oligonucleotides included (top strand) a 5´ T3 promoter sequence or (bottom strand) a 5´ T7
promoter sequence fused to gene-specific sequences for \( \text{PI4KIII} \alpha \) (exon 5, exon 6 start, exon 6
end), \( \text{fwd} \), \( \text{PI4KII} \), \( \text{sktl} \) (exon 1 start, exon 1 middle), \( \text{slik} \), and \( \text{GFP} \). dsRNA was prepared using
MegaScript T7 and T3 in vitro transcription kits (Ambion, Applied Biosystems, Carlsbad, CA).
Equal amounts of the T3 and T7 transcription products were mixed, heated to 95°C for 10
minutes and cooled slowly to RT to anneal. Double knockdown of \textit{fwd} and \textit{PI4KII} was verified by qRT-PCR (Fig. S1; see Supplementary Materials and Methods). Oligonucleotide sequences of primers used for molecular cloning and dsRNA experiments are listed in Table S1.

**Cell culture and dsRNA treatment**

S2 cell culture, dsRNA treatments, cell lysis, and immunoblotting were performed essentially as described (Hipfner et al., 2004). Blots were probed with rabbit anti-phospho-Ezrin/Radixin/Moesin (#3141) (Cell Signaling Technology Inc., Danvers, MA) and then re-probed with rabbit anti-\textit{Drosophila} Moe [a gift from Daniel Kiehart (Duke University, Durham, NC)]. Signals in immunoblots were quantified using the “Gel” function of ImageJ 1.42q. Moe phosphorylation levels were expressed as the ratio of phosphorylated to total Moe signals, and were normalized to the ratio observed in \textit{GFP} dsRNA treated cells. Statistical analysis for comparison of pMoe levels in \textit{PI4KII\textalpha} versus \textit{fwd} and \textit{PI4KII} knockdown experiments was performed with results from triplicate dsRNA treatments.

**Immunocytochemistry**

Immunolocalization was performed using standard procedures (Mathe, 2004). Ovaries were fixed in Buffer B fixative (3:2:1 solution of dH2O:16% paraformaldehyde:Buffer B) for 15 minutes. Buffer B is 100 mM KH2PO4/K2HPO4 pH 6.8, 450 mM KCl, 150 mM NaCl, 20 mM MgCl2. Rhodamine-phalloidin was used at 4 U/mL, dried and resuspended in ethanol before addition (Invitrogen Corp., Carlsbad, CA). ToPro DNA dye was used at 1:1000 (Invitrogen) and samples were mounted in PPD (0.1x PBS, 90% glycerol, 1 mg/mL \textit{p}-phenylenediamine). Immunolocalization of PIPs was performed as described (Hammond et al., 2009).

The following antibodies were used: rabbit anti-Oskar (a gift from Paul Lasko, 1:700), rabbit anti-phospho-Moe (Cell Signaling, 1:700), mouse anti-Sec5 22A2 (DSHB, 1:200) (Murthy et al., 2003), mouse anti-Gurken 1D12 (DSHB, 1:300) (Queenan et al., 1999), mouse anti-FasIII 7G10 (DSHB, 1:50) (Patel et al., 1987), rabbit anti-Lva (a gift from John Sisson, 1:2000), mouse anti-PIP IgM antibodies (Echelon Biosciences Inc., Salt Lake City, UT) were used at 1:100 (anti-PI4P) or 1:400 (anti-PI(4,5)P2). Fluorescein- or Texas Red-labeled \textit{Lycopersicon esculentum} (tomato) lectin (Vector Laboratories Inc., Burlingame, CA) was used at 150 μg/mL. Anti-rabbit and anti-mouse IgG or IgM secondary antibodies conjugated to Alexa Fluor 488 or 568
(Molecular Probes, Invitrogen, Carlsbad, CA) were used at 1:1000.

**Imaging and Analysis**

Images were acquired on either a Zeiss Axiovert 100 inverted laser scanning confocal microscope using LSM510 software or on a Zeiss Axioplan 2 upright fluorescence microscope with a Zeiss Axiocam CCD camera using Axiovision 4.8 software (Oberkochen, Baden-Württemberg, Germany). Images of PIP staining were acquired on a Nikon Eclipse Ti inverted scanning confocal microscope using NIS Elements AR software (Melville, NY). When necessary, images used for comparison were adjusted for levels, brightness and contrast in an identical manner using Adobe Photoshop CS5. Staging of WT egg chambers was performed according to Spradling (1993). Mutant GLCs were staged based on the most prominent developmental hallmark(s). Oocyte nucleus position was scored in WT and $PI4KIII\alpha$ GLCs judged to be stage 8 or later by vitellogenesis, size and shape of the oocyte relative to the egg chamber, and follicle cell morphology.

Ovaries were prepared for transmission electron microscopy as described (Bazinet and Rollins, 2003). Images were obtained using AmtV542 acquisition software (Advanced Microscopy Techniques, Woburn, MA, USA).

Quantification of Lva and lectin puncta size was performed using Volocity 4. Puncta within one plane of a representative egg chamber were scored using the line measurement tool across the greatest cross-sectional distance for each spot. Statistical analysis was performed using one-way and two-way ANOVA followed by Tukey’s pairwise comparison post-test. Quantification of Sec5, PI4P, PI(4,5)P2, and F-actin intensity levels was performed using ImageJ 1.43u using representative egg chambers. Statistical analysis of PIP:F-actin intensity ratios between WT and $PI4KIII\alpha$ was performed with the paired student’s t-test using average intensities normalized to WT.
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References


Figure Legends

Fig. 1. PI4KIIIα is essential and required for oogenesis. (A) Schematic of PI4KIIIα (CG10260) locus (top), PI4KIIIαΔ123 deletion (middle), and PI4KIIIα rescue construct (bottom). Blue bars, PI4KIIIα exons; white bars, sgg exons; orange bars, cDNA. PI4KIIIαΔ123 was generated by imprecise excision of the P-element GE3785. (B) Physical location of PI4KIIIα within the region defined by the zw2 complementation group (Shannon et al., 1972). Modified with permission from Genetics. zw3 is sgg. (C-E) Epifluorescence micrographs of egg chambers stained with DAPI to mark nuclei. Compared to WT (C), PI4KIIIα GLCs exhibit a buckled oocyte cortex, as revealed by the position of follicle cell nuclei (boxed areas are enlarged 2.5X in C’, D’), and nurse cell nuclei are found in the ooplasm (D, arrowhead). (E) Late-stage GLCs exhibit pycnotic nuclei. Scale bar: 20 μm. Panels (C’, D’) were adjusted similarly for brightness and contrast.

Fig. 2. PI4KIIIα mutant germlines display defects in F-actin organization and Moesin activation. (A–F) Confocal sections of early- (A–C) and late-stage (D–F) egg chambers stained for F-actin (rhodamine-phalloidin; red) and DNA (ToPro; blue). Early PI4KIIIα GLCs have reduced cortical F-actin (B–C, arrows) and clustered ring canals (arrowheads), while late-stage GLCs display F-actin protrusions (E, arrow). Actin defects are rescued by a PI4KIIIα rescue construct (F). (G, H) Confocal micrographs of egg chambers stained with anti-pMoe (green), rhodamine-phalloidin (red) and ToPro (blue). Right panels are 2X-magnified views of the boxed areas, showing the oocyte cortex (arrows) and the apical side of the follicular epithelium (arrowheads). Compared to WT (G), pMoe is severely reduced in PI4KIIIα GLCs (H, arrows), but not in the apical regions of follicle cells (H, arrowheads). (I–L) Immunoblotting of lysates from dsRNA-treated S2 cells (I, K) and quantification of immunoblots (J, L). Depletion of PI4KIIIα with any of three dsRNAs reduces pMoe levels without affecting total Moe protein levels (I, J). Co-depletion of fwd and PI4KII has little effect on pMoe levels (K, L). slik and gfp dsRNAs serve as positive and negative controls, respectively. Scale bars: 50 μm.

Fig. 3. Membrane integrity is defective in PI4KIIIα germline clones. (A–F) Confocal sections of early-stage egg chambers stained with fluorescein-tomato lectin (green), rhodamine-phalloidin (red) and ToPro (blue). Boxed areas in A, C, E are enlarged in B, D, F. Insets in D are magnified 3X and adjusted for brightness and contrast. In WT (A, B), lectin staining marks the
PM and colocalizes with cortical F-actin (B, arrows). In contrast, PI4KIIIα GLCs (C-F) show reduced lectin staining along the PM and decreased cortical F-actin (D, arrows, insets), while the remaining lectin-positive membrane localizes around the cluster of ring canals (D, arrowheads).

In severely affected egg chambers (E,F), aggregates of membrane are found throughout the egg chamber and follicle cells appear to degenerate (F). Scale bars: 50 μm. (G-L) Transmission electron micrographs of WT (G-I) and PI4KIIIα GLCs (J-L). Stage 5 egg chambers showing a single ring canal in WT (G) and a cluster of ring canals in a PI4KIIIα GLC (J) (arrows; compare insets). Stage 7 egg chambers show robust PM separating each nurse cell nuclei in WT (H-I, arrowheads), but thin (K,L, black arrowheads) or no (K, bottom two nuclei) PM between nuclei of PI4KIIIα GLCs. Thin membranes in PI4KIIIα terminate within the cytoplasm (K,L, white arrowheads). Inset in (I) is from a different region of the same egg chamber. fc, follicle cells; m, mitochondria; n, nurse cell nucleus; rc, ring canal(s).

Fig. 4. PI4KIIIα exhibits membrane defects not seen in other PI4K mutants. (A,B) Confocal micrographs of a late-stage WT egg chamber (A) and a PI4KIIIα GLC (B) stained with fluorescein-lectin (green), rhodamine-phalloidin (red), and ToPro (blue). Compared to WT (A, arrowheads), nurse cell membranes are disrupted or missing in PI4KIIIα GLCs (B, arrowheads) and a whorl of membrane colocalizes with F-actin in the oocyte (arrows). Border cells, dashed circles. (C,D) Confocal sections of fwd (C) and PI4KII (D) mutant egg chambers stained with Texas Red-lectin (green), Alexa488-phalloidin (red), and ToPro (blue). In contrast to PI4KIIIα GLCs (B), fwd and PI4KII nurse cell membranes are intact. However, prominent membrane aggregates are found in the nurse cell cytoplasm. Scale bar: 50 μm.

Fig. 5. Loss of PI4KIIIα disrupts Sec5 localization. (A,B,D,E) Confocal micrographs of egg chambers stained with anti-Sec5 (green) and rhodamine-phalloidin (red). Boxed areas in A,D are enlarged in B,E. In WT, Sec5 is enriched at the oocyte PM and along nurse cell membranes adjacent to follicle cells (B, arrows). In PI4KIIIα GLCs, enrichment of Sec5 at these membranes is lost, i.e., the Sec5 signal at the nurse cell-follicle cell interface is similar to that in lateral follicle cell membranes (E, compare arrows, arrowheads). Scale bars: 50 μm. (C,F)

Quantification of Sec5 intensity across the lateral follicle cell membranes (grey; average intensity taken from positions indicated by yellow bars in A,B) and across the oocyte-posterior
follicle cell membranes (red; average intensity taken from positions indicated by red bars in A,B) for WT (C) and \textit{PI4KIII\textalpha} (F). Error bars represent standard deviation.

**Fig. 6. Fwd and PI4KII regulate Golgi morphology and organization.** (A-D) Confocal images of egg chambers stained with anti-Lva (red) and fluorescein-tomato lectin (green). Insets at far right are 8X enlargements of boxed areas. Dotted lines trace the shape of lectin puncta, where discernable. (A) Discrete Lva puncta are visible in WT egg chambers and partially colocalize to lectin-positive puncta. The sizes of Lva puncta were proportional to the sizes of lectin puncta (insets 1-3); small Lva puncta had either barely discernible or no adjacent lectin puncta (inset 3). (B) Lectin-positive membranes in \textit{PI4KIII\textalpha} GLCs localize towards the centre of the egg chamber. Elsewhere, the sizes of Lva and associated lectin puncta resemble those in WT, although the lectin puncta appear more diffuse (boxes 1-3). (C) \textit{fwd} egg chambers exhibit smaller Lva puncta that show greater overlap with either irregularly-shaped (box 1) or elongated (box 2) lectin puncta. The smallest Lva puncta were either not associated with lectin or were in close proximity to, but not overlapping with, small lectin puncta (box 3). (D) Lva puncta in \textit{PI4KII} mutants overlap with engorged lectin puncta (boxes 1, 2). Often, several Lva puncta associate with a single lectin spot (boxes 1-3). (E) Mean diameter of individual Lva puncta found in egg chambers of the genotypes listed. WT, 0.65 μm ± 0.23, n=251 puncta (3 egg chambers); \textit{PI4KIII\textalpha}, 0.68 ± 0.27, n=554 (6 egg chambers); \textit{fwd}, 0.43 μm ± 0.22, n=399 (4 egg chambers); \textit{PI4KII}, 0.73 μm ± 0.40, n=132 (2 egg chambers). (F) Percentage colocalization of lectin puncta with Lva (black bars) and Lva puncta with lectin (grey bars). WT, n=388 lectin and 374 Lva puncta (3 egg chambers); \textit{PI4KIII\textalpha}, n=291 lectin and 356 Lva puncta (3 egg chambers); \textit{fwd}, n=436 lectin and 337 Lva puncta (3 egg chambers); \textit{PI4KII}, n=139 lectin and 228 Lva puncta (2 egg chambers). Bars represent standard error. *P<0.05, **P<0.01, ***P<0.001. Scale bar: 10 μm.

**Fig. 7. PI4KIII\textalpha is required for plasma membrane PI4P and PI(4,5)P2.** (A-B) Confocal sections of WT egg chambers expressing the PI(4,5)P2 marker PLCδPH-GFP under control of α\textsubscript{1}-tubulin-GAL4 (green) and stained with rhodamine-phalloidin (red) and ToPro (blue). PLCδPH-GFP labels the PM and colocalizes with F-actin (A,B, arrowheads). Approximately 50% of these egg chambers exhibit nurse cell nuclei within the ooplasm, similar to \textit{PI4KIII\textalpha}.
GLCs (B, arrow). (C,D,F,G) WT egg chambers (C,F) or PI4KIIIα GLCs (D,G) stained with anti-PI4P (C,D) or anti-PI(4,5)P₂ (F,G) antibodies (green) and rhodamine-phalloidin (red). PI4P and PI(4,5)P₂ are detected on the PM (arrows) and in ring canals. Overall intensity of PI4P and PI(4,5)P₂ were reduced along the PM in PI4KIIIα GLCs compared to WT (compare arrows in C with D, F with G). (E,H) (Top) Representative plot of average intensity of PI4P (E) or PI(4,5)P₂ (H) staining in WT egg chamber (black line) compared to PI4KIIIα GLC (red line). Average intensity was plotted using values taken from positions indicated by yellow bars in C,D (for E) and in F,G (for H). (Bottom) Average PI4P:F-actin (E, n=23; 76.7% of egg chambers examined) and PI(4,5)P₂:F-actin (H, n=23; 46.9% of egg chambers examined) intensity ratios normalized to WT. *P<0.05, **P<0.001. Scale bars: 50 μm.

Fig. 8. Polarity defects in PI4KIIIα GLCs. (A-C) Egg chambers stained with anti-Oskar antibodies (green), rhodamine-phalloidin (red), and ToPro (blue). (A’,B’,C’) 2X magnified views of boxed areas. Oskar protein is concentrated at the oocyte posterior in stage 9 or later egg chambers in WT (A’, arrow), but is either reduced or absent in PI4KIIIα (B’,C’, arrows). Variability of Oskar localization in PI4KIIIα correlated with the degree of F-actin disruption throughout the rest of the egg chamber (compare rhodamine-phalloidin in B,C). Oskar staining of the follicle cells is non-specific. (D-F) Egg chambers stained with anti-Gurken (green), rhodamine-phalloidin (red) and ToPro (blue). (D) In WT, Gurken is found directly above the oocyte nucleus, which is anchored in the dorsal anterior corner of the oocyte in stage 10 egg chambers (outline, asterisk). In PI4KIIIα oocytes with an anchored nucleus, Gurken is either dispersed (E) or mislocalized (F) relative to the oocyte nucleus. (G) PI4KIIIα egg chamber with mislocalized oocyte nucleus. (H) Frequency of normal (blue bar) and abnormal (red bar) nuclear position in WT versus PI4KIIIα oocytes. Scale bars: 50 μm in (A-G).
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A

WT
lectin DNA

F-actin
merge

B

Pl4KIIα

C

fwd

D

Pl4KII
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