Type II phosphatidylinositol 4-kinase regulates trafficking of secretory granule proteins in Drosophila

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SUMMARY
Type II phosphatidylinositol 4-kinase (PI4KII) produces the lipid phosphatidylinositol 4-phosphate (PI4P), a key regulator of membrane trafficking. Here, we generated genetic models of the sole Drosophila melanogaster PI4KII gene. A specific requirement for PI4KII emerged in larval salivary glands. In PI4KII mutants, mucin-containing granules failed to reach normal size, with glue protein aberrantly accumulating in enlarged Rab7-positive late endosomes. Presence of PI4KII at the Golgi and on dynamic tubular endosomes indicated two distinct foci for its function. First, consistent with the established role of PI4P in the Golgi, PI4KII is required for sorting of glue granule cargo and the granule-associated SNARE Snap24. Second, PI4KII also has an unforeseen function in late endosomes, where it is required for normal retromer dynamics and for formation of tubular endosomes that are likely to be involved in retrieving Snap24 and Lysosomal enzyme receptor protein (Lerp) from late endosomes to the trans-Golgi network. Our genetic analysis of PI4KII in flies thus reveals a novel role for PI4KII in regulating the fidelity of granule protein trafficking in secretory tissues.

KEY WORDS: PtdIns(4)P, Mucin granule, Salivary gland, Regulated secretion, PI 4-kinase, SNAP-24, LERP

INTRODUCTION
Phosphatidylinositol 4-kinases (PI4Ks) synthesize phosphatidylinositol 4-phosphate (PI4P), a crucial regulator of membrane trafficking (reviewed by Balla and Balla, 2006; D’Angelo et al., 2008; Graham and Burd, 2011). Mammals have two type II and two type III PI4Ks (PI4KIIα, PI4KIIβ, PI4KIIα, PI4KIIIβ), whereas budding yeast, flies and worms each have a single PI4KII and two type III enzymes. Type II PI4Ks and PI4KIIIβ regulate intracellular trafficking in yeast and mammalian cells. However, the role of these enzymes in animal development remains largely unknown.

Budding yeast PI4KIIIβ (Pik1p) localizes to the Golgi, where it synthesizes an essential pool of PI4P required for post-Golgi secretion (Flanagan et al., 1993; Garcia-Bustos et al., 1994; Hama et al., 1999; Walch-Solimena and Novick, 1999; Audhya et al., 2000). In mammalian cells, PI4KIIIβ and PI4KIIα localize to the Golgi (Wong et al., 1997; Wang et al., 2003; Weixel et al., 2005) and are implicated in secretory trafficking. Manipulating PI4KIIIβ by overexpression or dominant-negative constructs affects post-Golgi trafficking (Godi et al., 1999; Hausser et al., 2005), whereas depleting PI4KIIα blocks trans-Golgi network (TGN) recruitment of the clathrin adaptor protein-1 (AP-1) complex and decreases constitutive secretion from the TGN to the plasma membrane (Wang et al., 2003). We previously showed that Drosophila melanogaster PI4KIIIβ [Four wheel drive (Fwd)] localizes to the Golgi, where it synthesizes a pool of PI4P required for spermatocyte cytokinesis and male fertility (Brill et al., 2000; Polevoy et al., 2009). However, fvd is non-essential, raising the question of whether PI4KII also participates in the synthesis of Golgi PI4P.

In addition to the Golgi, type II PI4Ks also localize to endosomes. Budding yeast PI4KII (Lsb6p) is non-essential and has a non-catalytic role in endosome motility (Han et al., 2002; Shelton et al., 2003; Chang et al., 2005). Mammalian PI4KIIα is palmitoylated and localizes to dynamic endosomal tubules, where it promotes the transport and degradation of EGFR (Minogue et al., 2006; Barylko et al., 2009). PI4KIIα requires adaptor protein complex-3 (AP-3) for its endosomal localization, and depleting PI4KIIα from HeLa cells causes redistribution of AP-3 to the cytoplasm and accumulation of AP-3 cargo proteins and SNAREs on enlarged late endosomes (LEs) (Salazar et al., 2005; Croce et al., 2008). Less is known about mammalian PI4KIIIβ, which localizes to endosomes and translocates to the plasma membrane in response to activated Rac (Balla et al., 2002; Wei et al., 2002).

PI4KIIα also associates with a range of secretory organelles, including immature secretory granules, chromaffin granules, glucose transporter 4-containing vesicles and synaptic vesicles (Del Vecchio and Pilch, 1991; Kristiansen et al., 1998; Barylko et al., 2001; Panaretou and Toole, 2002; Guo et al., 2003; Xu et al., 2006). Nonetheless, despite data implicating PI4KIIα in EGFR signaling, neurotransmission and regulated secretion, homozygous mutant mice lacking the catalytic domain of PI4KIIα show no obvious developmental defects, but rather exhibit late onset neurodegeneration (Simons et al., 2009). The cellular functions of PI4KII enzymes during animal development remain unknown.

Drosophila salivary glands provide an excellent system with which to investigate membrane trafficking pathways (Tojo et al., 1987; Xu et al., 2002; Abrams and Andrew, 2005; Wendler et al., 2010; Burgess et al., 2011). At the mid-third instar larval stage (mid-L3), salivary glands begin producing highly glycosylated glue proteins (cargo) that traffic through the endoplasmic reticulum and Golgi before being incorporated into regulated secretory granules (glue granules) at the TGN (Jamieson and Palade, 1967a; Jamieson...
and Palade, 1967b; Beckendorf and Kafatos, 1976; Korge, 1977; Thonopoulos and Ka stricter, 1979; Lehmann, 1996; Burgess et al., 2011). Indeed, we previously showed that clathrin and AP-1, which colocalize with the adaptor EpsinR (Liquid facets-related – FlyBase) at the TGN, are essential for granule formation (Burgess et al., 2011). Glue granules accumulate in salivary cell cytoplasm, where they undergo growth by accretion and homotypic fusion until a high-titer pulse of edcsynone triggers their release at the end of L3 (Zhimulev and Ko sienikov, 1975; Farkas and Suakova, 1999). The secreted mucin-like glue then serves to adhere the pupal case to a solid substrate during metamorphosis.

Here, we investigate PI4KII function in Drosophila. Flies bearing null mutations in PI4KII (PI4KII – FlyBase) are viable, but have strikingly small glue granules and accumulate glue protein in enlarged LEs. Moreover, loss of PI4KII leads to missorting of Snap24, a SNARE implicated in granule fusion (Niemen y and Schwarz, 2000). Catalytic activity of PI4KII is required for glue granules and LEs of normal size, as well as for the formation of endosomal tubules. Based on our data, we propose that PI4KII is required for the proper sorting and retrieval of secretory granule proteins.

MATERIALS AND METHODS

Fly genetics

Fly husbandry, crosses and generation of transgenic flies and mosaic clones followed standard procedures (Golic and Lindquist, 1989; Mullins et al., 1989; Ashburner, 1990). Deletions in PI4KII (CG2929) were generated by imprecise excision of P[EP]CG17246 (GenExel, Daejeon, South Korea) as described (Timakov et al., 2002). Individual fly lines exhibiting altered eye color (indicating P-element mobilization) were screened by PCR, identifying a deletion that removes CG14671 and the first three predicted exons of the somatic PI4KII transcript. The P-element present in this deletion was further mobilized to generate seven larger deletions, including Df(3R)730, which removes the coding regions of both genes. Df(3R)730 was recombinated with P[neoFRT]82B and P[w^`, CG14671] to generate P[w^`, CG14671]. P[neoFRT]82B, Df(3R)730. This chromosome is referred to as PI4KIIA.

Mosaic clones were generated in flies of genotype y^1, w^1118, P[70FLP]12F[w^1128]; P[neoFRT]82B, P[ubi-GFP.D]83PI4KIIA. To generate salivary clones, 0- to 60-minute embryos were aged for 2.5 hours at room temperature, heat shocked for 90 minutes at 37°C in a water bath, then incubated at 25°C to allow further development. New transgenes were mapped and balanced using w^1118, Sco/CyO; TMTM66. P[w^`, atub-GFP-LAMP] (Pulippararavichul and Bose, 2005) (gift of J. Hirst, Cambridge, UK) was cloned directly into the P{w+, CG14671} vector. PI4KII catalytic (CAT; D465A) and ATP-binding (ATP; K311M) mutations (Barylko et al., 2002) were generated using QuickChange XL (Stratagene, La Jolla, CA, USA).

For kinase assays, FLAG-tagged PI4KII, PI4KII CAT and PI4KII ATP were subcloned into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). To detect PI4KII transcripts, RNA was prepared from five adults, and primers specific to either the somatic or testis cDNA were used for reverse transcriptase-coupled PCR (RT-PCR) as described (Burgess et al., 2011).

Primers used for molecular cloning and PCR are listed in supplementary material Table S1.

PI4KII antibody and biochemistry

Rabbit anti-Drosophila PI4KII antibodies (from Dr Rama Ranganathan, Dallas, TX, USA) generated against residues 1-20 (MSGAQDQPDDPLQLEDEVD) of the somatic PI4KII sequence (absent from the testis isoform) were affinity purified on SulfoLink pre-packed columns with crosslinked peptide, as instructed (Pierce, Thermo Scientific, Rockford, IL, USA). For immunoblotting, samples containing 40 μg protein extract were dissolved in 4× sample buffer, separated in 10% SDS-polyacrylamide gels and transferred to nitrocellulose (GE Healthcare, Amersham, UK) using a Trans-Blot semi-dry transfer apparatus (Bio-Rad, Mississauga, ON, Canada). Blots were probed sequentially with rabbit anti-PI4KII (this work) and mouse monoclonal anti-β-tubulin antibodies (NDS75, Amersham) at 1:200 and 1:4000, respectively. HRP-conjugated goat anti-rabbit and donkey anti-mouse secondary antibodies (Jackson Labs) were diluted 1:10,000 and visualized using chemiluminescence (ECL Plus Kit, Amersham).

Kinase activity was measured on membrane extracts from untransfected or transiently transfected COS-7 cells as described (Barylko et al., 2001).

Microscopy

L3 salivary glands were prepared for immunostaining as described (Burgess et al., 2011). Antibodies were 1:1000 rabbit anti-Lva (Sisson et al., 2000) (gift of J. Sisson and O. Papoulas, Austin, TX, USA), 1:500 mouse anti-AP-γ (Benhrira et al., 2011; Burgess et al., 2011) (gift of R. Le Borgne, Rennes, France), 1:350 rabbit anti-PI4KII (this work), 1:100 rabbit anti-EpsinR (Burgess et al., 2011) (gift of P. Leventis and G. Boulianne, Toronto, ON, Canada), 1:100 rabbit anti-Snap24 (Niemen y and Schwarz, 2000) (gift of T. Schwarz, Boston, MA, USA) and 1:500 mouse anti-GFP monoclonal 3E6 (Molecular Probes, Eugene, OR, USA). Secondary antibodies conjugated to Alexa 488, 568 or 633 were from Molecular Probes.

For live imaging, salivary glands in 25 μl PBS (pH 7.4) on a microscope slide were sealed using a coverslip edged with high-vacuum M grease (Apiezon, Manchester, UK). For Lysotracker staining, glands were incubated in PBS containing 1:1000 Lysotracker (Molecular Probes) for 1 minute and mounted in PBS for imaging. Salivary glands were imaged for no longer than 10 minutes after being mounted.

Images of all live and some fixed samples were acquired on a Quorum spinning-disc confocal microscope equipped with an SD 63× LCI Plan-NEOFLAR 1.3 DIC Imm Korr (water) objective (Carl Zeiss) and Volocity software (PerkinElmer) (SickKids Imaging Facility). Serial optical sections were acquired at 0.3 μm intervals unless otherwise indicated, and deconvolved using the Iterative Restoration function of Volocity 4. Fluorescence micrographs of fixed samples were acquired on a Zeiss LSM 510 inverted laser-scanning confocal microscope equipped with LSM 510 options (20×, FLUAR NA 0.75; 40×, Plan-APoCHROMAT NA 1.3; 63×, Plan-APoCHROMAT NA 1.4; or 100×, Plan-APoCHROMAT NA 1.4) and LSM 510 software (SickKids Imaging Facility). Images were exported using Volocity.

Live male germ cells were prepared in testis isolation buffer (Casal et al., 1990) and squashed with a coverslip (Wei et al., 2008) before imaging. Salivary gland samples were prepared for transmission electron microscopy as described (Bazinet and Rollins, 2003; Burgess et al., 2011). Images were obtained using AmtV542 acquisition software (Advanced Microscopy Techniques, Woburn, MA, USA).

Images were adjusted for brightness and contrast using Adobe Photoshop CS2.
RESULTS

**PI4KII null mutant flies are viable**

*Drosophila* PI4KII has two predicted transcripts (Stapleton et al., 2002a; Stapleton et al., 2002b): a longer somatic transcript and a shorter testis-specific transcript, which encode PI4KII isoforms with unique N-termini and identical kinase domains (Fig. 1A,B). These were confirmed by RT-PCR, with the shorter testis transcript detected exclusively in males (Fig. 1C).

To investigate PI4KII function, we excised a nearby P-element, GE28807 (Fig. 1A), to generate a homozygous lethal deletion *Df(3R)730* (red) that removes PI4KII and CG14671. Lethality can be rescued with genomic DNA encoding CG14671 (light gray) but not PI4KII (dark gray). (B) *Drosophila* (Dm) PI4KII C-terminal kinase domain (dark gray) is homologous to human (Hs) PI4KIIα and PI4KIIβ, whereas N-terminal regions (white) are not conserved. Percentage sequence identity (and similarity) is shown for homologous portions relative to the somatic isoform. (C) RT-PCR detects a somatic transcript in males (M) and females (F) (left panel, arrowhead) and a testis-specific transcript in males (right panel, arrowhead). (D) Immunoblot probed with anti-PI4KII (top) and anti-tubulin (bottom). Anti-PI4KII recognizes a single ~90 kDa band in wild type (lanes 1-4), but not in the PI4KII Δ cells (marked by absence of GFP and outlined by white dashed line).

**PI4KII is required during glue granule biogenesis**

*Drosophila* PI4KII is 5.5-fold enriched in larval salivary glands compared with whole flies (FlyAtlas) (Chintapalli et al., 2007), suggesting a potential role in glue granule biogenesis. Using a fluorescent fusion to the glue cargo protein Sgs3 (Sgs3-DsRed)
under control of the Sgs3 promoter, we previously defined three stages of granule biogenesis: stage 0, early to mid-L3 salivary glands lacking granules; stage 1, mid-L3 salivary glands containing small granules in distal cells; and stage 2, late-L3 salivary glands with fully mature granules in most cells (Burgess et al., 2011).

Wild-type stage 2 glue granules had an average diameter of 3.8±1.0 μm (n=222), whereas PI4KII Δ mutants had smaller granules (1.7±0.3 μm, n=292) (Fig. 2A,B). Granule size was restored (4.0±0.9 μm, n=249) by a PI4KII genomic transgene (Fig. 2C). The phenotype is specific to PI4KII Δ mutants, as fwd mutants exhibited granules of normal diameter (4.3±1.3 μm, n=307) (Fig. 2D). Moreover, the phenotype is cell-autonomous, as single cells homozygous for PI4KII Δ exhibited small granules (Fig. 2E, dashed line).

At the ultrastructural level, PI4KII Δ mutants revealed small glue granules of grossly normal morphology (Fig. 2F,G). Intriguingly, PI4KII Δ cells also exhibited large vacuolated structures not observed in wild type (Fig. 2G, arrows). These vacuoles contained filamentous material that appeared less dense than normal secretory granules. Nevertheless, glue secretion occurred normally in PI4KII Δ glands (supplementary material Fig. S1). Hence, PI4KII is required during development for the formation of granules of normal size, but is dispensable for regulated secretion.

**PI4KII localizes to the Golgi and to a dynamic tubular endosomal network**

To investigate how PI4KII participates in granule formation, we examined PI4KII localization in wild-type salivary glands. Endogenous PI4KII and a functional mCherry-PI4KII fusion (see below) colocalized with markers for the Golgi and LEs (Fig. 3A-D and supplementary material Fig. S2A,B). mCherry-PI4KII also colocalized with YFP-Rab7 at late endosomes (LEs) in stage 0 salivary cells (B, arrows). In stage 0 salivary cells (B, arrows). In stage 3, late-L3 stage (3) salivary cells, mCherry-PI4KII localizes adjacent to YFP-Golgi (C, arrows) and to tubular structures lacking YFP-Golgi (C, arrowhead). mCherry-PI4KII also colocalizes with YFP-Rab7 at LEs (D, arrows) and to endosomal tubules (D, arrowhead) lacking YFP-Rab7 (inset). See supplementary material Movies 1 and 2.
PI4KIIΔ mutants missort granule proteins to enlarged LEs

To determine the site of action of PI4KII during granule biogenesis, we first examined whether PI4KII is required to recruit regulators of post-Golgi secretory trafficking. Since the clathrin adaptors AP-1 and EpsinR bind PI4P in vitro (Hirst et al., 2003; Mills et al., 2003; Wang et al., 2003; Heldwein et al., 2004), we tested whether their recruitment to the TGN is affected by loss of PI4KII. PI4KIIΔ cells exhibited normal recruitment of endogenous AP-1 and EpsinR to the Golgi region in stage 0 salivary glands (Fig. 5A,B). Similarily, at stage 1, AP-1 and clathrin were recruited normally to immature secretory granules in PI4KIIΔ cells (data not shown). Hence, Drosophila PI4KII is dispensable for recruitment of AP-1 and EpsinR to Golgi membranes.

To investigate whether PI4KII functions at LEs, we examined fluorescent endosomal markers in wild-type and PI4KIIΔ salivary cells. LEs marked with YFP-Rab7 were enlarged in PI4KIIΔ mutants and appeared as individual units rather than clusters (Fig. 6A,B, insets). LEs were also visible throughout the cytoplasm rather than being concentrated near the cell cortex. Acidic LEs or lysosomes marked by Lysotracker were also enlarged (Fig. 6C,D), as were LEs containing GFP fused to Lysosomal enzyme receptor protein (Lerp), which is the Drosophila homolog of the mannose 6-phosphate receptor (Dennes et al., 2005; Hirst et al., 2009) (Fig. 6E,F). This enlargement of endocytic compartments is not a general effect of PI4KIIΔ mutants missort granule proteins to enlarged LEs.
loss of PI4KII function, as YFP-Rab7 LEs appeared normal in spermatagonia (supplementary material Fig. S4A,B), salivary duct cells and imaginal cells (data not shown). These results suggest that defects in glue granule formation might be linked to the enlarged LE phenotype in PI4KIIΔ salivary cells.

To determine whether PI4KII affects trafficking of glue granule cargo in the endosomal pathway, we examined colocalization of the glue marker Sgs3-DsRed with YFP-Rab7. Although Sgs3 was not detected in Rab7-positive LEs in wild type (Fig. 6G), Sgs3 was found in the lumen of LEs in PI4KIIΔ mutants (Fig. 6H). To test whether sorting of granule membrane proteins also requires PI4KII, we examined localization of the SNARE Snap24, which is implicated in glue granule biogenesis. Snap24 and Sgs3 were co-expressed and colocalized at stage 1, as cells initiate glue biogenesis (data not shown). In stage 2, Snap24 localized uniformly to granule membranes in wild type, but accumulated dramatically on the limiting membranes of organelles lacking Sgs3 in PI4KIIΔ cells (Fig. 6I). Hence, PI4KII is required for the proper trafficking of integral membrane and luminal granule proteins.

**Formation of PI4KII-containing endosomal tubules is independent of retromer or AP-3 function, but retromer dynamics at LEs depends on PI4KII**

The accumulation of granule proteins in LEs suggested that transport out of this compartment might be disrupted. The retromer complex, which mediates retrograde transport of cargo from endosomes to the TGN, was recently shown to localize to tubular endosomal structures (Rojas et al., 2008). To determine whether retromer localizes to PI4KII-containing tubules, we generated a fluorescent fusion to the retromer subunit Vps29. In stage 2
salivary cells, mCherry-Vps29 colocalized with YFP-Rab7 on LEs (arrows) but mCherry-Vps29 was largely excluded from mCherry-PI4KII-containing tubules (arrowheads). See supplementary material Movie 6. (B,C) Projections of 22 optical slices. Vps29-GFP is uniformly distributed around enlarged endosomes in the PI4KIIΔ mutant (C) relative to wild type (B). (D) Time-lapse fluorescence micrographs (elapsed time in seconds) reveal that Vps29-GFP localizes to dynamic foci associated with the limiting membranes of LEs in wild type (left), but is more uniformly and stably distributed around the periphery of an enlarged LE in the PI4KIIΔ mutant (right). See supplementary material Movie 7. (E,F) Projections of 13 optical slices. Garnet-GFP-labeled endosomes are larger in the PI4KIIΔ mutant (F) than in wild type (E). (G,H) mCherry-PI4KII tubular endosomes are of normal morphology in retromer (vps35Δ) (G) and AP-3Δ (g1Δ) (H) mutant salivary cells.

To determine whether PI4KII is required for localization or dynamics of endosomal sorting complexes, we analyzed Vps29-GFP and a fluorescent fusion to the delta subunit of AP-3 (AP-3Δ, or Garnet in Drosophila) in PI4KIIΔ salivary glands. Vps29-GFP was distributed around enlarged LE membranes, in contrast to its localization to tight LE foci in wild-type cells (Fig. 7B,C). In addition, dynamic localization of retromer to rapidly moving LE foci was lost in PI4KIIΔ mutants (Fig. 7D and supplementary material Movie 7). Similarly, Garnet-GFP localized to enlarged LEs in PI4KIIΔ mutants (Fig. 7E,F). These data suggest that LE identity is maintained but that partitioning of LE membranes into dynamic microdomains involved in post-LE trafficking is defective in the absence of PI4KII.

Since AP-3 is required for the proper distribution of mammalian PI4KIIα, we examined whether retromer or AP-3 is needed to initiate formation of PI4KII-containing tubules. To test this, we examined mCherry-PI4KII in retromer (vps35Δ) and AP-3Δ (g1Δ) mutant salivary cells expressing Sgs3-GFP (Fig. 7G,H). mCherry-PI4KII (B) but not mCherry-PI4KIIαΔ (C) fully rescues the glue granule and endosome defects of the PI4KIIΔ mutant. mCherry-PI4KIIαΔ localizes to enlarged organelles that can contain Sgs3-GFP (insets).

PI4KII catalytic activity is required for formation of tubular endosomes
To determine whether PI4KII kinase activity is required for glue granule biogenesis, we examined the ability of wild-type or catalytically inactive mCherry-PI4KII to rescue the salivary gland defects of PI4KIIΔ larvae. We first confirmed that the putative kinase-dead variants PI4KIIαΔ (K311M, mutated in the predicted ATP-binding residue) and PI4KIIαΔ (D465A, mutated in the predicted catalytic residue) (Barylko et al., 2002) expressed in COS-7 cells lacked detectable catalytic activity (Fig. 8A). We then examined these constructs in transgenic flies. In a wild-type
background, endogenous PI4KII and mCherry-PI4KII (wild-type or CAT) fusions were expressed at similar levels (data not shown).

Moreover, catalytically inactive mCherry-PI4KII exhibited normal localization to Golgi and endosomal tubules and did not interfere with granule biogenesis (supplementary material Fig. S3A,C; data not shown).

When introduced into a PI4KIIΔ background, mCherry-PI4KII restored glue granule size to near wild type (3.8±0.9 μm, n=93) and restored normal LE morphology (Fig. 8B). By contrast, mCherry-PI4KIIΔ failed to rescue granule size (1.9±0.3 μm, n=91) or LE morphology (Fig. 8C). mCherry-PI4KII localized to YFP-Rab7 and mCherry-PI4KII show strong overlap on LEs (C, insets), whereas YFP-Rab7 and mCherry-PI4KIIΔ localize to different endosomes (D) or to distinct domains on the same endosomes (D, insets).

**DISCUSSION**

We report the first analysis of PI4KII null mutants in *Drosophila melanogaster. PI4KIIΔ* mutants are viable and do not exhibit any gross morphological defects, although they exhibit defects in membrane trafficking during the formation of regulated secretory granules in the larval salivary gland. Mammalian PI4KII has long been suspected to participate in regulated secretion (Husebye et al., 1990; Del Vecchio and Pilch, 1991; Wiedemann et al., 1996; Kristiansen et al., 1998; Barylko et al., 2001; Panaretou and Tooze, 2002; Guo et al., 2003; Olsen et al., 2003; Ishihara et al., 2006; Xu et al., 2006). Nonetheless, *Drosophila* PI4KII does not localize to the limiting membrane of glue granules and is dispensable for secretion. Instead, PI4KII localizes to the TGN and to endosomes, and PI4KIIΔ mutants exhibit small glue granules and enlarged LEs that aberrantly accumulate glue cargo proteins, Lerp and SNAREs. Our results therefore suggest a role for PI4KII in intracellular trafficking pathways required for normal granule biogenesis.

Our data point to a contribution of PI4KII in regulating the fidelity of sorting events at the Golgi or to retrieval of proteins from LEs to the TGN (Fig. 10). At the Golgi, failure to properly segregate cargo could lead to mixing of glue proteins with lysosomal hydrolases destined for LEs. Similarly, failure to properly segregate SNARE proteins could result in granule-specific SNAREs being missorted to endosomes. TGN localization of AP-1 and EpsinR appears unaffected in PI4KIIΔ cells. This contrasts with the observation that AP-1 becomes cytoplasmic when PI4KIIΔ is depleted from HeLa cells, but is consistent with recent studies demonstrating that PI4KIIΔ is dispensable for AP-1 localization in HEK293 cells (Wang et al., 2003; Craige et al., 2008). PI4KII might exert a subtle influence on AP-1 or EpsinR. For example, changes in the levels or distribution of PI4P might influence the kinetics of AP-1 or EpsinR recruitment to membranes or their association with particular subdomains of the TGN. Indeed, partial loss-of-function AP-1 mutants exhibit small granules similar to those found in PI4KIIΔ mutants (Burgess et al., 2011). Alternatively, PI4KII might affect the Golgi recruitment of other PI4P-binding proteins involved in post-Golgi vesicular trafficking, for example GGA or GOLPH3 (Dippold et al., 2009; Hirst et al., 2009; Kametaka et al., 2010). Nonetheless, the overwhelming majority of Sgs3 appears to traffic normally to small secretory granules, indicating that post-Golgi trafficking of glue cargo proteins is relatively unaffected in PI4KIIΔ mutants. Hence, our data appear more consistent with the idea that PI4KIIΔ mutants are defective in the retrieval of proteins from LEs to the TGN.

PI4KII localizes to an extensive network of dynamic, highly interconnected tubular endosomes. Emergence of these tubular endosomes coincides with the onset of glue granule biogenesis, suggesting that the two processes might be linked. Loss of PI4KII catalytic activity results in the accumulation of enlarged LEs and loss of tubule formation. Moreover, the granule SNARE Snap24 and Lerp accumulate on enlarged endosomes, suggesting a defect in retrograde trafficking of proteins involved in granule maturation and lysosomal trafficking. Indeed, retromer dynamics is greatly attenuated at these aberrant endosomes, further supporting a role for PI4P in retrograde transport (Wood et al., 2009). However, PI4KII is not generally required for retromer function, as PI4KIIΔ mutants show no obvious defects in Wingless signaling, unlike retromer mutants (Belenkaya et al., 2008; Franch-Marro et al., 2008; Port et al., 2008).

One possibility is that a defect in retrograde trafficking from LEs to the TGN could explain both the small granule phenotype and the accumulation of Sgs3 in LEs in PI4KIIΔ mutants. In wild type, SNAREs could normally be retrieved from growing granules to LEs by the action of AP-1 and clathrin, which are present on immature granules (Burgess et al., 2011). Retrograde trafficking of SNAREs and Lerp from LEs to the TGN could occur via tubules.

**Fig. 9. PI4KII catalytic activity is required for endosomal tubule dynamics and endosome morphology.** Spinning-disk confocal micrographs of late L3 salivary cells expressing mCherry-PI4KII (A,C) or mCherry-PI4KIIΔ (B,D) in a PI4KIIΔ background. (A,B) Time-lapse fluorescence micrographs (elapsed time in seconds). mCherry-PI4KII localizes to dynamic endosomal tubules (A, arrowheads), whereas mCherry-PI4KIIΔ shows minimal localization to tubules (B, arrowheads). See supplementary material Movies 8 and 9. (C,D) YFP-Rab7 and mCherry-PI4KII show strong overlap on LEs (C, insets), whereas YFP-Rab7 and mCherry-PI4KIIΔ localize to different endosomes (D) or to distinct domains on the same endosomes (D, insets).
that require PI4KII and PI4P. Alternatively, retrograde trafficking of these proteins could occur in carriers that are indirectly affected by loss of PI4KII. Any glue cargo proteins (e.g. Sgs3) inadvertently trafficked to LEs during AP-1/clathrin-dependent SNARE recycling would undergo lysosomal degradation. In PI4KIIΔ mutants, the defect in SNARE recycling could block homotypic granule fusion, leading to the small granule phenotype. Similarly, defects in lysosomal hydrolase trafficking caused by a failure to recycle Lerp could reduce lysosomal function and lead to an accumulation of Sgs3 in LEs in PI4KIIΔ mutants.

Although both Fwd and PI4KII localize to the Golgi (Polevoy et al., 2009) (this work), these PI4Ks carry out distinct functions in Drosophila development. Fwd recruits the recycling endosome regulator Rab11 to Golgi membranes and vesicles during spermatocyte cytokinesis (Brill et al., 2000; Polevoy et al., 2009), but is dispensable for glue granule formation. By contrast, PI4KII is required during glue granule biogenesis, but is dispensable for spermatocyte cytokinesis. At a cellular level, we recently demonstrated distinct roles for mammalian PI4KIIβ and PI4KIIα in trafficking of β-glucocerebrosidase, the lysosomal enzyme that is defective in Gaucher disease (Jovic et al., 2012). Hence, unlike in budding yeast, where the Fwd homolog Ptk1p is essential and the only known requirement for the type II PI4K Lsb6p is in endosome motility, PI4KII appears to have evolved a more prominent role in specialized trafficking events that occur in metazoan development.

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Competing interests statement

The authors declare no competing financial interests.

Supplementary material

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