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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 glue 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, PI4KIIIα, 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, *fwd* 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 HEK293 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; Craige et al., 2008). Less is known about mammalian PI4KIIβ, 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 Tooze, 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

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and Palade, 1967b; Beckendorf and Kafatos, 1976; Korge, 1977; Thomopoulos and Kastritis, 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 glue 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 ecdysone triggers their release at the end of L3 (Zhimulev and Kolesnikov, 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* (*Pi4KIIα* – 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 (Niemeyer 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}CG12746*^{GE28807} (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 recombined 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 *PI4KIIΔ*.

Mosaic clones were generated in flies of genotype *y*^{1}*, w*^{1118}*, P{70FLP}3F/w*^{1118}*;; P{neoFRT}82B, P{Ubi-GFP.D}83/PI4KIIΔ*. 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; TM3/TM6B*. *P{w*$^+$*, αtub-GFP-LAMP}* (Pulipparacharuvil et al., 2005) and *P{w*$^+$*, αtub-garnet-GFP}* (Burgess et al., 2011) were described previously. Additional stocks were *P{w*$^+$*, β-tubulin-EGFP}* (Inoue et al., 2004) (gift of Y. Akiyama-Oda and H. Oda, Osaka, Japan), *P{w*$^+$*, Sgs3-DsRed}* and *P{w*$^+$*, Sgs3-GFP}* (Biyasheva et al., 2001; Costantino et al., 2008) (gift of A. Andres, Las Vegas, NV, USA), *P{w*$^+$*, YFP-Rab7}* (Marois et al., 2006) (gift of S. Eaton, Dresden, Germany) and *P{w*$^+$*, YFP-Golgi}* (YFP fused to the N-terminal 81 amino acids of human β-1,4-galactosyltransferase) (LaJeunesse et al., 2004) (Bloomington Stock Center, Bloomington, IL, USA).

Molecular biology

For genomic rescue constructs, *PI4KII* and *CG14671* were amplified from BACR24024 (BACPAC Resources Center, Oakland, CA, USA) and subcloned into pCaSpeR4 (Pirrotta, 1988). *P{w*$^+$*, CG14671}* begins 1.3 kb upstream of the predicted start site of *CG14671* and ends before *PI4KII*. *P{w*$^+$*, PI4KII}* begins in exon 2 of *CG14671* (after the first methionine) and ends ~1 kb downstream of the last exon of *PI4KII*.

For low-level expression of fluorescent fusion proteins, *PI4KII* (LD24833) and *Vps29* (*CG4764*) (GH25884) cDNAs (Canadian Drosophila Microarray Centre, Mississauga, ON, Canada) were subcloned into a modified pCaSpeR4 vector containing the α*Tub84B* promoter (*αtub*) (Marois et al., 2006) and mCherry or mEGFP (Zacharias et al., 2002; Shaner et al., 2004) (gift of R. Tsien, San Diego, CA, USA). GFP-Lerp (Hirst et al., 2009) (gift of J. Hirst, Cambridge, UK) was cloned directly into the *αtub* vector. PI4KII catalytic (CAT; D465A) and ATP-binding (ATP; K311M) mutations (Barylko et al., 2002) were generated using QuikChange 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 (MSGATDQTDPPLLQLEDEVD) 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 (N357, 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-1γ (Benhra 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 (Niemeyer 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-disk confocal microscope equipped with an SD 63× LCI Plan-NEOFLUAR 1.3 DIC Imm Kor (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 LSM510 inverted laser-scanning confocal microscope equipped with LSM objectives (20×, FLUAR NA 0.75; 40×, Plan-APOCHROMA NA 1.3; 63×, Plan-APOCHROMAT NA 1.4; or 100×, Plan-APOCHROMAT NA 1.4) and LSM510 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.

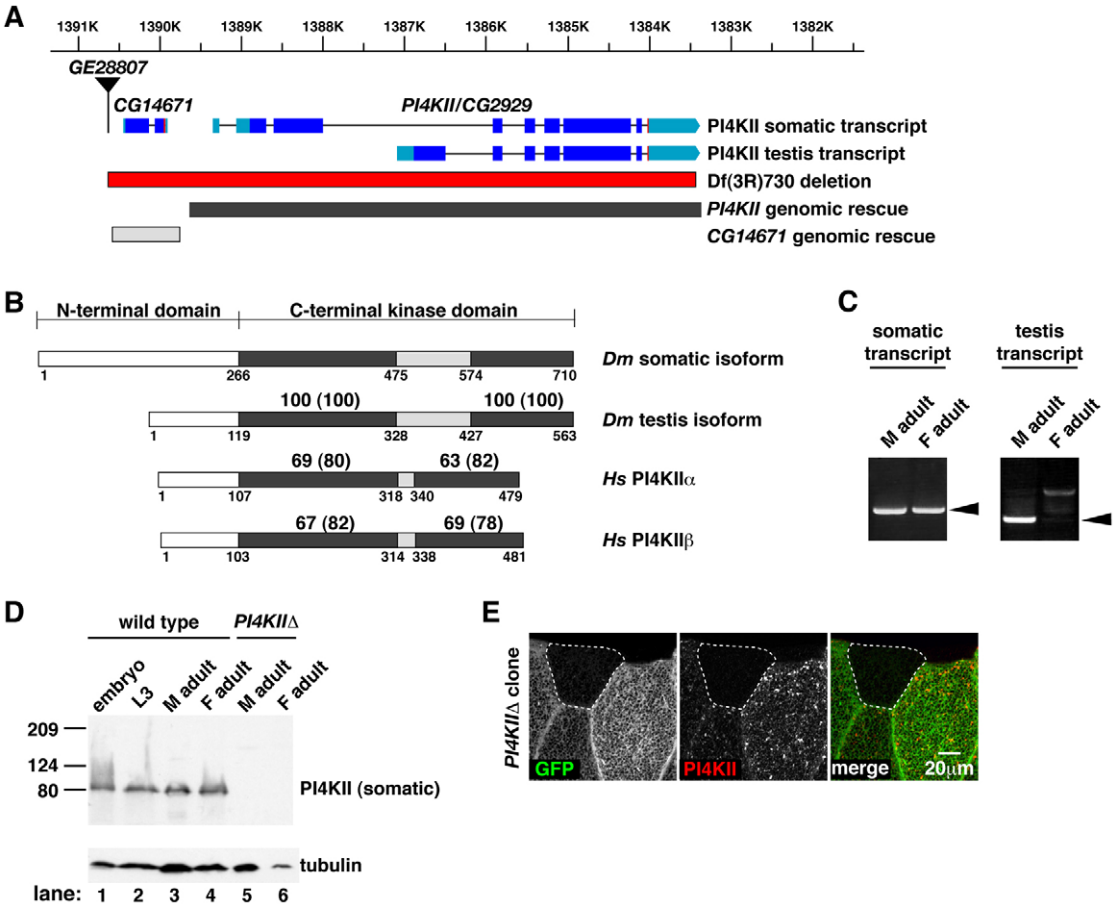

Fig. 1. Deletions in *PI4KII* are protein null. (**A**) Physical map of *Drosophila PI4KII* (*CG2929*, *Pi4KIIα*) showing somatic and testis-specific transcripts and predicted exons (dark blue, coding regions; light blue, untranslated regions; red, stop codons). P-element GE28807 (triangle) was excised 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 *PI4KIIΔ* mutant (lanes 5, 6). (**E**) Scanning confocal micrograph of late L3 salivary gland stained for endogenous PI4KII shows a punctate distribution that is absent from a *PI4KIIΔ* cell (marked by absence of GFP and outlined by white dashed line).

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 *Df(3R)730*, which removes the entire coding region of *PI4KII* and the upstream gene *CG14671* (Fig. 1A). Flies homozygous for *Df(3R)730* died as larvae. Lethality was rescued by a genomic transgene containing *CG14671* (Fig. 1A). This transgene was recombined with *Df(3R)730*, generating a chromosome that is effectively mutant only for *PI4KII* (henceforth *PI4KIIΔ*; see Materials and methods). Phenotypes of *PI4KIIΔ* homozygotes (see below) were fully rescued by a *PI4KII* genomic transgene.

Antibodies directed against the somatic PI4KII isoform detected a single polypeptide in immunoblots of wild-type extracts from different developmental stages that was not detected in extracts from *PI4KIIΔ* flies (Fig. 1D). Polypeptide migration was slower than the predicted 79 kDa, probably owing to a strongly acidic tract in the N-terminus and post-translational modifications such as palmitoylation (Barylko et al., 2002; Barylko et al., 2009). Immunofluorescence of larval salivary glands in which mosaic clones (single mutant cells marked by the absence of GFP) had been induced by FLP-FRT-mediated recombination revealed localization of PI4KII to intracellular puncta (Fig. 1E) that were absent from *PI4KIIΔ* cells (Fig. 1E, 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)

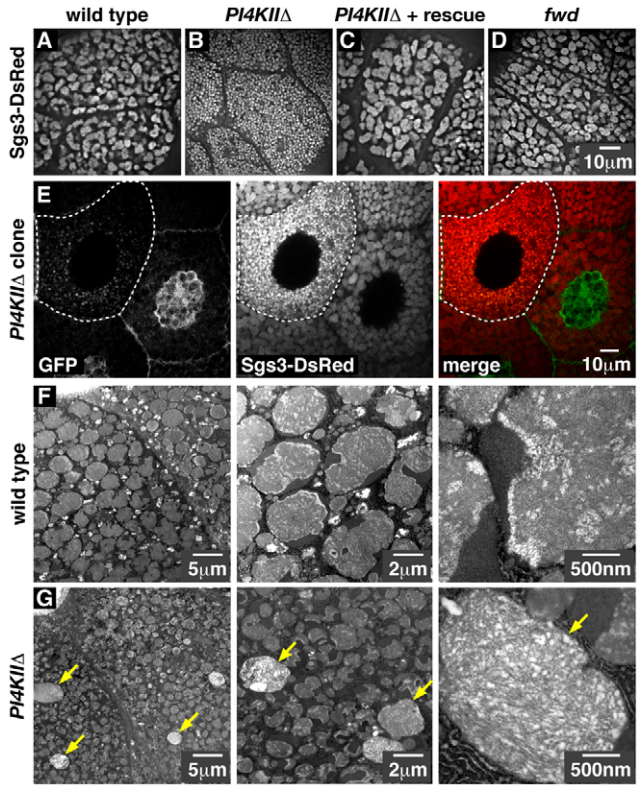

Fig. 2. PI4KII is required for proper glue granule biogenesis. (**A-D**) Spinning-disk confocal micrographs of late L3 salivary cells expressing the glue granule marker Sgs3-DsRed. Compared with wild type (A) or *fwd* mutant (D), *PI4KIIΔ* exhibits strikingly small glue granules (B) that can be rescued by a wild-type *PI4KII* genomic transgene (C). (**E**) Scanning confocal micrograph showing that the small granule phenotype of the *PI4KIIΔ* mutant is cell-autonomous (mutant cell marked by absence of GFP and outlined by white dashed line). (**F**,**G**) Transmission electron micrographs of wild-type (F) and *PI4KIIΔ* mutant (G) late L3 salivary glands. In wild type, granules are large and well organized, with electron-dense material near the membrane and filamentous material near the center of each granule. *PI4KIIΔ* salivary glands exhibit small granules, as well as vacuolated structures that contain filamentous material (arrows).

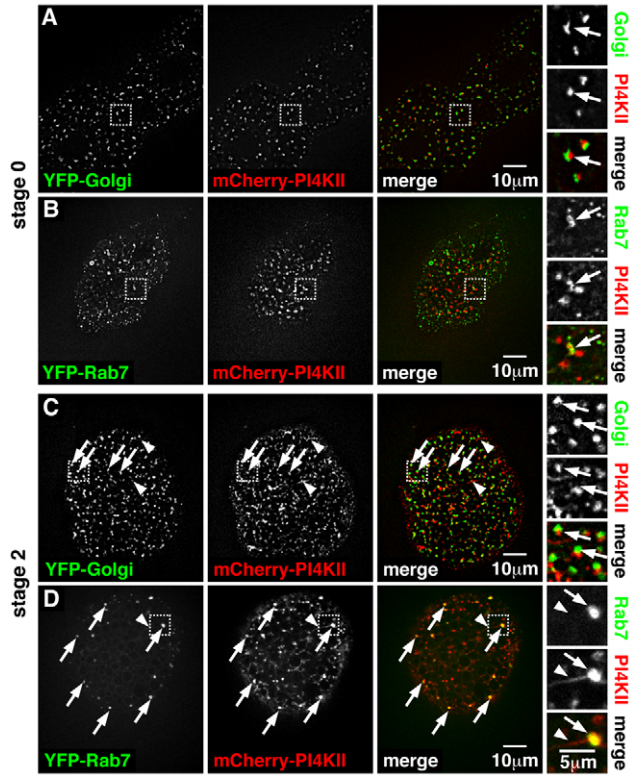

Fig. 3. PI4KII localizes to the Golgi and LEs in live salivary gland cells. Spinning-disk confocal micrographs of live L3 salivary cells expressing mCherry-PI4KII (red) and YFP-Golgi or YFP-Rab7 (green). Boxed regions are magnified in insets. (**A**,**B**) In mid-L3 (stage 0) salivary cells, mCherry-PI4KII localizes adjacent to YFP-Golgi (A, arrows). mCherry-PI4KII also colocalizes with YFP-Rab7 at late endosomes (LEs) in stage 0 salivary cells (B, arrows). (**C**,**D**) In late L3 (stage 2) salivary cells, mCherry-PI4KII localizes adjacent to YFP-Golgi (C, arrows) and to tubular structures lacking YFP-Golgi (C, arrowhead). mCherry-PI4KII 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.

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 *PI4KIIΔ* granules were smaller (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 granule phenotype is specific to *PI4KIIΔ* 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 *PI4KIIΔ* exhibited small granules (Fig. 2E, dashed line).

At the ultrastructural level, *PI4KIIΔ* mutants revealed small glue granules of grossly normal morphology (Fig. 2F,G). Intriguingly, *PI4KIIΔ* 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 *PI4KIIΔ* 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 localized adjacent to, but did not overlap with, a medial Golgi marker (YFP-Golgi) in stage 0 and stage 2 salivary glands (Fig. 3A,C and supplementary material Movie 1). Indeed, at the earliest stages of glue production (stage 1), mCherry-PI4KII showed partial colocalization with Sgs3-GFP in the vicinity of the TGN (supplementary material Fig. S3A). mCherry-PI4KII also localized to YFP-Rab7-positive LEs (Fig. 3B,D and supplementary material

Movie 2), but was not detected on the limiting membrane of mature granules containing Sgs3-GFP (supplementary material Fig. S3B). Strikingly, in stage 2 salivary glands, mCherry-PI4KII was found on numerous tubular structures emanating from LEs (Fig. 3D, inset), whereas YFP-Rab7 was excluded from these endosomal tubules.

To further dissect the association of PI4KII with endosomes and endosomal tubules, we examined stage 2 salivary cells. Endogenous and mCherry-tagged PI4KII localized to LEs and endosomal tubules marked with GFP-LAMP (Fig. 4A and supplementary material Fig. S2B). These LEs were connected by an mCherry-PI4KII-containing tubular endosomal network that appeared most dense near the cell periphery (Fig. 4B,C). Importantly, this network was not induced by overproduction of PI4P, as catalytically inactive mCherry-PI4KII^{ATP} (see below) also localized to a tubular endosomal network in wild-type salivary gland cells (supplementary material Fig. S3C). PI4KII and GFP-LAMP tubules were not observed in salivary cells prior to glue production (compare Fig. 4B and Fig. 3D with Fig. 3B; also compare supplementary material Fig. S2A,C with S2B,D), nor in granule non-producing duct cells at the proximal end of the salivary gland, imaginal disc cells or spermatocytes (data not shown).

Tubules containing mCherry-PI4KII colocalized extensively with β-tubulin-GFP (Fig. 4D). In real-time imaging experiments, PI4KII-containing tubules emanated from LEs and extended and retracted along microtubules (Fig. 4E and supplementary material Movie 3). The tubules formed transient connections that were associated with saltatory displacement of endosomes (Fig. 4F and supplementary material Movie 4). Small mCherry-PI4KII-containing vesicles could also be seen moving rapidly along microtubules (Fig. 4G and supplementary material Movie 5). Together, these observations suggest that PI4KII functions at the Golgi/TGN and on LEs/endosomal tubules during granule formation.

PI4KIIΔ mutants missort granule proteins to enlarged LEs

To determine the site of action of PI4KII during glue 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). Similarly, 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

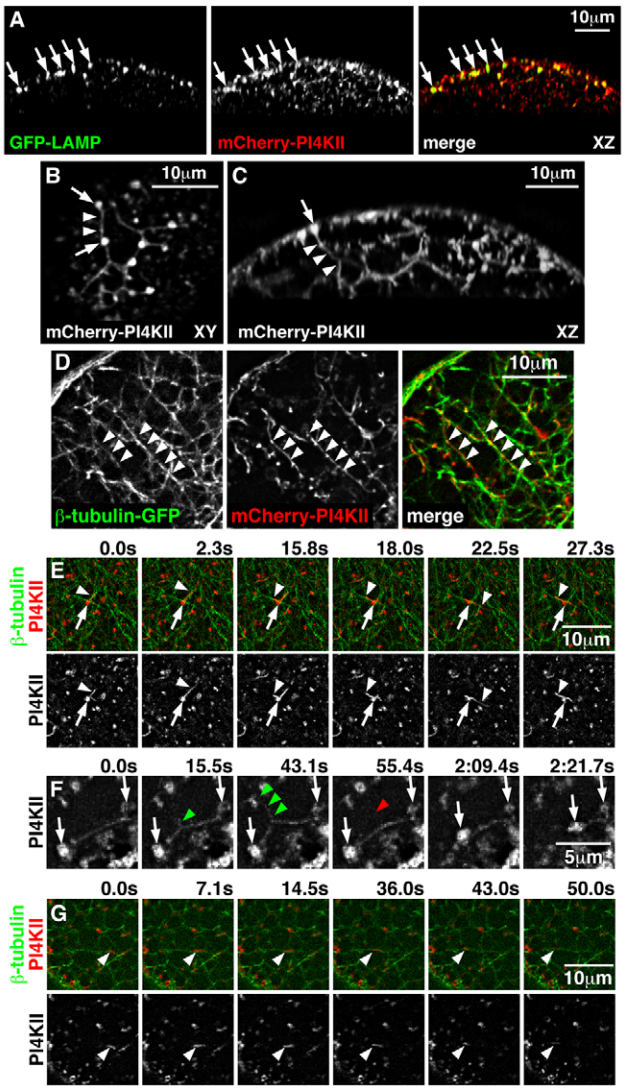

Fig. 4. PI4KII localizes to dynamic tubular endosomes. Spinning-disk confocal micrographs of live L3 (stage 2) salivary cells expressing mCherry-PI4KII alone (B,C,F) or with GFP-LAMP (A) or β-tubulin-GFP (D,E,G). (**A**) Projection constructed from a *z*-stack of 52 confocal sections. The *y*-axis was cropped and the resulting slice turned 90°. mCherry-PI4KII and GFP-LAMP are enriched near the cell cortex and colocalize on LEs (arrows). mCherry-PI4KII is also seen deeper in the cell. (**B**) *xy* projection generated from a *z*-stack of seven optical sections acquired starting from the basal surface. mCherry-PI4KII is present in tubules (arrowheads) linking LEs (arrows). (**C**) *xz* projection generated from a *z*-stack of 60 optical sections. The *y*-axis was cropped and the slice turned 90°. mCherry-PI4KII is enriched at the cell cortex (arrow) and on tubules (arrowheads) that extend perpendicular to the cell surface. (**D**) Projection of nine optical sections. mCherry-PI4KII-containing tubules colocalize with microtubules (β-tubulin-GFP, arrowheads). (**E-G**) Time-lapse fluorescence micrographs (elapsed time in seconds). (E) Single optical section. Tubules containing mCherry-PI4KII (arrowheads) extend and retract from endosomes (arrow) along microtubules. See supplementary material Movie 3. (F) Projections of five optical sections. mCherry-PI4KII-containing tubules rapidly form (frames 2 and 3, green arrowheads) and break (frame 4, red arrowhead). Retracting tubules appear to exert a pulling force, displacing endosomes (frames 4-6, arrows). See supplementary material Movie 4. (G) Projections of three optical sections. mCherry-PI4KII-containing vesicles move rapidly along microtubules (arrowheads). See supplementary material Movie 5.

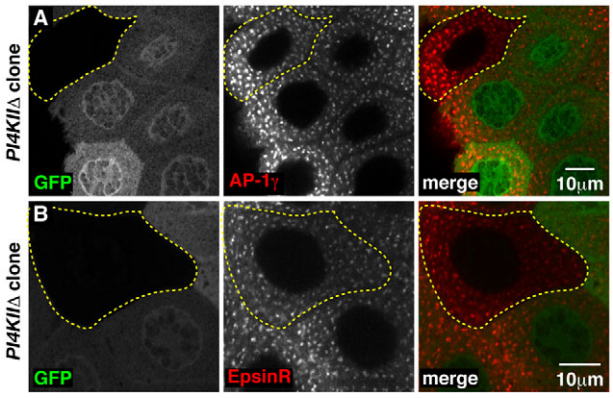

Fig. 5. PI4KII is dispensable for recruiting the clathrin adaptor proteins AP-1 and EpsinR to the Golgi. Scanning confocal micrographs of L3 salivary glands stained for AP-1γ (**A**) or EpsinR (**B**). Localization of these clathrin adaptors appears normal in *PI4KIIΔ* cells (marked by the absence of GFP and outlined by yellow dashed lines).

loss of *PI4KII* function, as YFP-Rab7 LEs appeared normal in spermatogonia (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

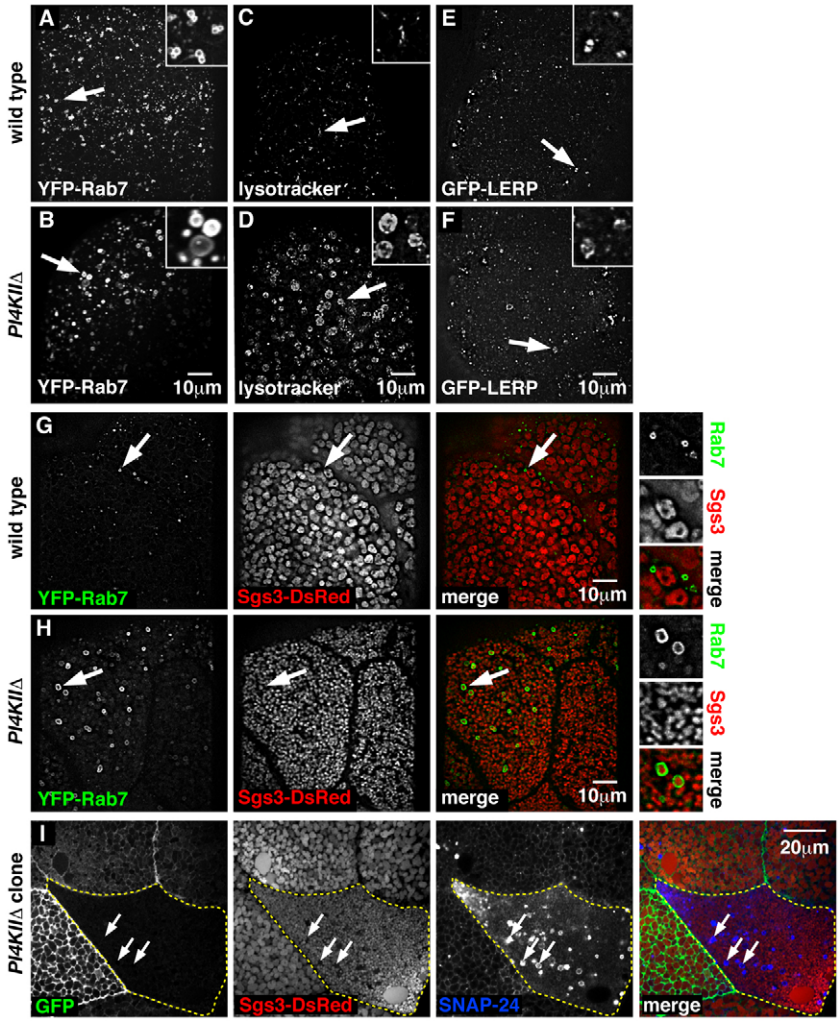

Fig. 6. *PI4KIIΔ* mutants accumulate granule proteins in enlarged LEs. (**A-H**) Spinning-disk confocal micrographs of live late L3 salivary cells. Arrows point to regions magnified in insets (A-F) or shown on right (G,H). (A,B) Projections of 32 (A) or 101 (B) slices. YFP-Rab7-positive endosomes are larger in the *PI4KIIΔ* mutant (B) than in wild type (A). (C,D) Projections of 19 optical slices. Acidic LEs or lysosomes stained with Lysotracker are larger in the *PI4KIIΔ* mutant (D) than in wild type (C). (E,F) Single optical sections. GFP-Lerp localizes to enlarged organelles in the *PI4KIIΔ* mutant (F) as compared with wild type (E). (G,H) Single optical sections of cells expressing YFP-Rab7 and Sgs3-DsRed. YFP-Rab7-positive LEs lacking luminal Sgs3-DsRed are present near the cell cortex in wild type (G). Enlarged YFP-Rab7-positive LEs accumulate luminal Sgs3-DsRed and are scattered throughout the cell in the *PI4KIIΔ* mutant (H). (**I**) Scanning confocal micrographs of a fixed salivary gland showing altered distribution of the SNARE Snap24 (blue) on organelles lacking Sgs3-DsRed (red) in a *PI4KIIΔ* cell (marked by absence of GFP and outlined by yellow dashed lines). Arrows indicate Snap24 accumulation on enlarged organelles lacking Sgs3.

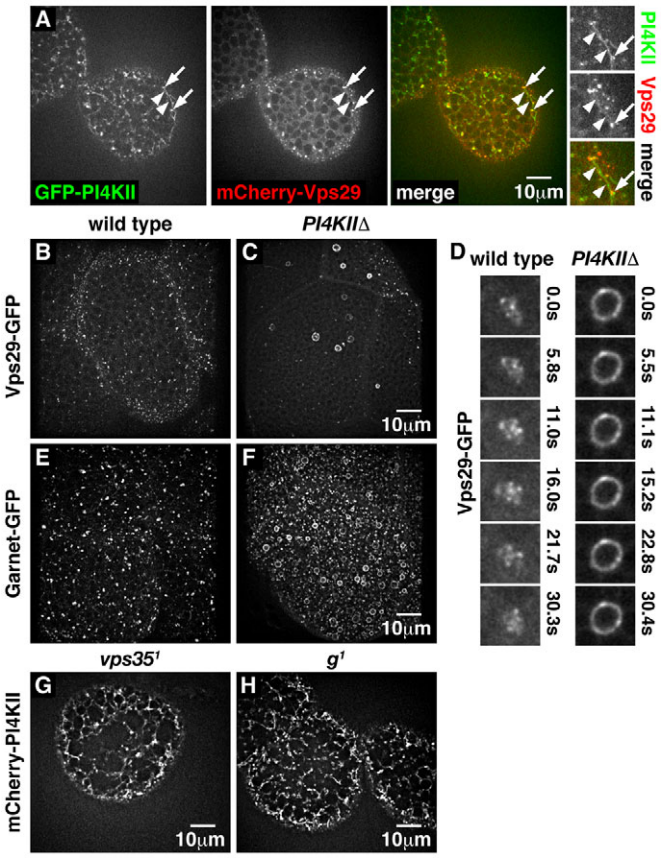

Fig. 7. Retromer localization and dynamics are altered in *PI4KIIΔ* mutants. Spinning-disk confocal micrographs of live L3 (stage 2) salivary cells. (**A**) GFP-PI4KII colocalizes with mCherry-Vps29 on endosomes (arrows), but mCherry-Vps29 is largely excluded from mCherry-PI4KII-containing tubules (arrowheads). 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 (*vps35*^{1}) (G) and AP-3δ (*g*^{1}) (H) mutant salivary cells.

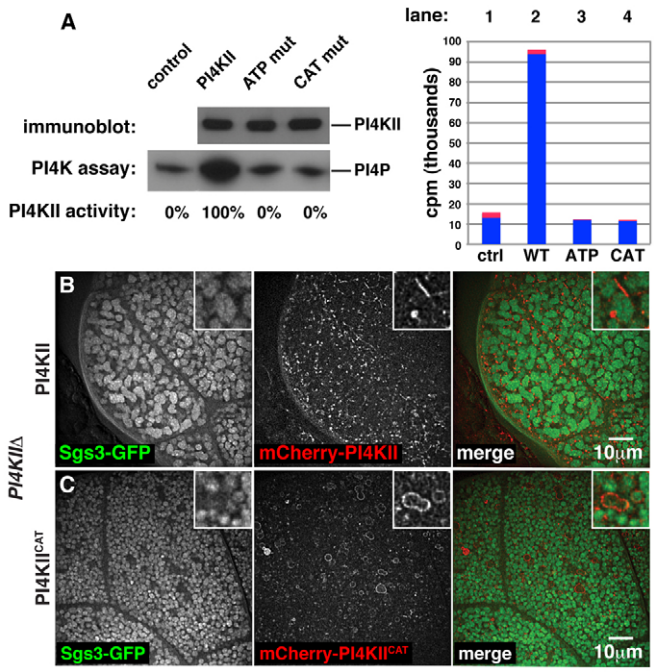

Fig. 8. PI4KII catalytic activity is required for granule biogenesis and normal endosome size. (**A**) Catalytic activity of *Drosophila* PI4KII. Untransfected control (ctrl, lane 1) or transfected (lanes 2-4) COS-7 cells expressing FLAG-tagged wild-type (lane 2), ATP mutant (lane 3) or CAT mutant (lane 4) PI4KII. Immunoblot shows membrane extracts probed with anti-PI4KII antibody. PI4K assay shows an autoradiograph of radiolabeled PI4P analyzed by TLC. PI4K catalytic activity is shown relative to that of control cells (0%) and cells transfected with wild-type PI4KII (100%). PI4KII activity [counts per minute (cpm)] is shown in the bar chart (identical results were obtained in two independent experiments). (**B**,**C**) Spinning-disk confocal micrographs of late L3 salivary cells expressing Sgs3-GFP and mCherry-PI4KII (B) or mCherry-PI4KII^{CAT} (C) in a *PI4KIIΔ* background. mCherry-PI4KII (B) but not mCherry-PI4KII^{CAT} (C) fully rescues the glue granule and endosome defects of the *PI4KIIΔ* mutant. mCherry-PI4KII^{CAT} localizes to enlarged organelles that can contain Sgs3-GFP (insets).

salivary cells, mCherry-Vps29 colocalized with YFP-Rab7 on LEs (data not shown), but was not detected on GFP-PI4KII-containing tubules (Fig. 7A and supplementary material Movie 6).

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 (*vps35*^{1}) and AP-3 (*garnet*; *g*^{1}) mutants. mCherry-PI4KII was still able to form tubules in these mutants (Fig. 7G,H). Additionally, endosome and glue granule size was not obviously altered (data not shown) (Burgess et al., 2011). This strongly suggests that these sorting complexes are not required for PI4KII function in glue granule biogenesis.

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 PI4KII^{ATP} (K311M, mutated in the predicted ATP-binding residue) and PI4KII^{CAT} (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

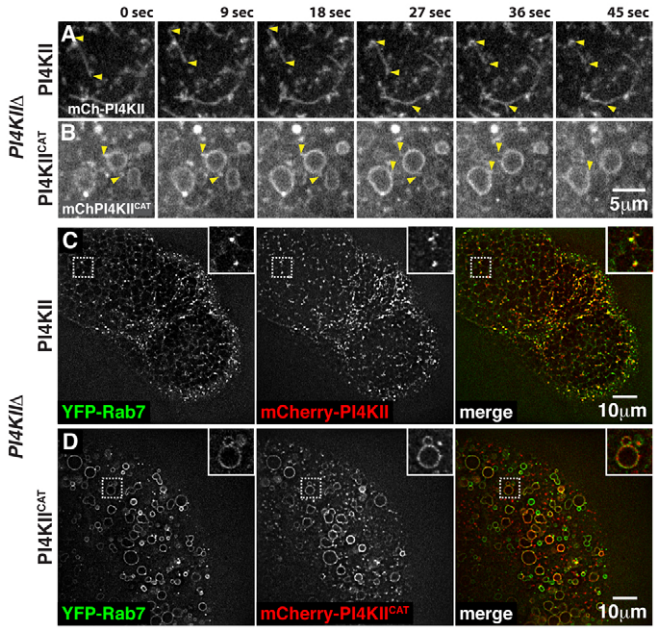

Fig. 9. PI4KII catalytic activity is required for endosomal tubule dynamics and endosome morphology. Spinning-disk confocal micrographs of late L3 salivary cells expressing mCherry-PI4KII (A,C) or mCherry-PI4KII^{CAT} (B,D) in a *PI4KIIΔ* background. (**A**,**B**) Time-lapse fluorescence micrographs (elapsed time in seconds). mCherry-PI4KII localizes to dynamic endosomal tubules (A, arrowheads), whereas mCherry-PI4KII^{CAT} 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-PI4KII^{CAT} localize to different endosomes (D) or to distinct domains on the same endosomes (D, insets).

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-PI4KII^{CAT} failed to rescue granule size (1.9±0.3 μm, *n*=91) or LE morphology (Fig. 8C). mCherry-PI4KII localized to YFP-Rab7-positive LEs and dynamic tubular endosomes, as in wild type (Fig. 9A,C and supplementary material Movie 8). In a *PI4KIIΔ* background, mCherry-PI4KII^{CAT} also localized to LEs, frequently colocalizing with Rab7 (Fig. 9B,D). However, mCherry-PI4KII^{CAT} did not localize to tubular endosomes, but rather formed abortive tubules (Fig. 9B and supplementary material Movie 9). The tubule defect was not due to disruption of microtubules, as β-tubulin-GFP localization appeared normal in *PI4KIIΔ* cells (data not shown). Collectively, the data suggest that PI4KII catalytic activity is needed for the formation of tubular endosomes that might serve to retrieve Lerp and granule SNAREs.

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 granule 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 PI4KIIα is depleted from HeLa cells, but is consistent with recent studies demonstrating that PI4KIIα 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

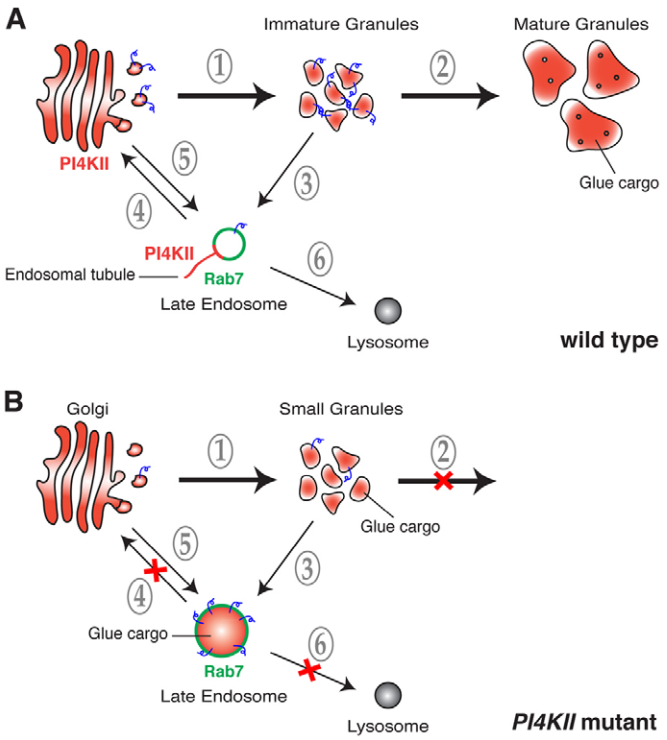

Fig. 10. Model of PI4KII function during glue granule biogenesis. (**A**) At least six trafficking steps (1-6) contribute to normal granule biogenesis in wild-type salivary gland cells. Vesicles containing granule cargo (red shading) and SNAREs (blue) traffic to immature secretory granules (1) that fuse with each other to form mature granules (2). SNAREs are recycled for future rounds of granule biogenesis, potentially via AP-1/clathrin-dependent trafficking to Rab7- and PI4KII-positive LEs (3), followed by retrieval to the trans-Golgi network (TGN) (4). Lerp and its associated lysosomal enzymes traffic from the TGN to LEs (5), from which Lerp is subsequently recycled to the TGN (4). PI4KII-dependent tubular endosomes might be involved in retrograde trafficking from LEs to the TGN. Any granule cargo aberrantly missorted (5) or retrieved (3) to LEs is rapidly degraded in lysosomes (6). (**B**) In the absence of PI4KII, initial stages of granule formation are normal (1). However, granules fail to reach full size (2), presumably owing to reduced availability of SNARES for homotypic fusion of immature granules, a consequence of a defect in the retrieval of SNAREs from LEs (4) or due to missorting of SNAREs from the TGN to LEs (5). Additionally, missorted granule cargo accumulates in LEs, presumably owing to impaired lysosomal degradation (6) caused by a defect in recycling of Lerp (4). Red crosses indicate trafficking steps that appear compromised in *PI4KIIΔ* mutant salivary gland cells.

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 PI4KIIIβ 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 Pik1p 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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