Dual roles for the *Drosophila* PI 4-kinase Four wheel drive in localizing Rab11 during cytokinesis

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Successful completion of cytokinesis relies on addition of new membrane, and requires the recycling endosome regulator Rab11, which localizes to the midzone. Despite the critical role of Rab11 in this process, little is known about the formation and composition of Rab11-containing organelles. Here, we identify the phosphatidylinositol (PI) 4-kinase III β Four wheel drive (Fwd) as a key regulator of Rab11 during cytokinesis in *Drosophila melanogaster* spermatocytes. We show Fwd is required for synthesis of PI 4-phosphate (PI4P) on Golgi membranes and for formation of PI4P-containing secretory organelles that localize to the midzone. Fwd binds and colocalizes with Rab11 on Golgi membranes, and is required for localization of Rab11 in dividing cells. A kinase-dead version of Fwd also binds Rab11 and partially restores cytokinesis to *fwd* mutant flies. Moreover, activated Rab11 partially suppresses loss of *fwd*. Our data suggest Fwd plays catalytic and noncatalytic roles in regulating Rab11 during cytokinesis.

Introduction

For an animal cell to divide, the total surface area must increase by ~25%. In principle, cells could achieve this either by having a reserve of membrane in microvilli or other outpocketings of the plasma membrane, or by trafficking of newly synthesized membrane through the secretory pathway. However, recent studies in dividing tissue culture cells favor an additional mechanism: at metaphase, plasma membrane is endocytosed and is then recycled to the cell surface starting in anaphase (Boucrot and Kirchhausen, 2007).

Endocytosis and recycling are particularly important in late telophase, when recycled membrane is thought to promote cleavage furrow stability and subsequent abscission of the daughter cells. Although the precise pathways taken by endocytosed membranes during cytokinesis are not fully known, endocytic regulators such as Arf6 and Rab11 are required in late stages of cytokinesis in various organisms and cell types (Montagnac et al., 2008; Prekeris and Gould, 2008). Rab11 and its effector FIP3 (Nuclear fallout [Nuf] in *Drosophila melanogaster*) target endosomes to the midzone during terminal stages of cytokinesis and promote cellularization—a specialized form of cytokinesis—in *Drosophila* embryos.

In addition to endocytosis and recycling, secretory trafficking is also implicated in cytokinesis. Trafficking of secreted cargo proteins to the site of cleavage has been observed in yeast, sea urchin embryos, and mammalian tissue culture cells (Prekeris and Gould, 2008). In *Drosophila*, several Golgi proteins, including the golgin Lava lamp (Lva), the Cog5 homologue Four way stop (Fws), and Syntaxin 5 (dSyx5), are required for cytokinesis or cellularization (Albertson et al., 2005). Nonetheless, the contribution of biosynthetic secretory trafficking to cytokinesis remains unknown.

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Abbreviations used in this paper: coIP, co-immunoprecipitation; FAPP, phosphatidylinositol 4-phosphate adaptor protein; Fws, Four way stop; Fwd, Four wheel drive; Gio, Giotto; Lva, Lava lamp; Nuf, Nuclear fallout; PH, pleckstrin homology domain; PI, phosphatidylinositol; PI4K, phosphatidylinositol 4-kinase; PI4Kβ, phosphatidylinositol 4-kinase III β; PI4P, phosphatidylinositol 4-phosphate; PIP$_2$, phosphatidylinositol 4,5-bisphosphate; sGFP, secreted green fluorescent protein.

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Drosophila spermatogenesis is an ideal system for the study of cytokinesis (Fuller, 1993; Giansanti et al., 2001). Although cytokinesis in the male germline is incomplete, the mechanism of cytokinesis is well conserved between spermatocytes and cells that undergo more conventional forms of cleavage (Eggert et al., 2006). In spermatocytes, meiotic divisions occur in rapid succession, necessitating a total increase in cell surface area of ~60% in less than two hours. For this reason, it is perhaps not surprising that meiotic cytokinesis is susceptible to mutations that affect regulators of Golgi trafficking (Syntaxin 5 and Fws), endocytosis and recycling (Arf6 and Rab11), and phosphatidylinositol (PI) metabolism (Giotto [Gio] and Four wheel drive [Fwd]) (Brill et al., 2000; Xu et al., 2002; Farkas et al., 2003; Gatt and Glover, 2006; Giansanti et al., 2006, 2007; Dyer et al., 2007).

PI lipids constitute a small proportion of the membrane lipids in a cell, yet they play critical roles in cell signaling, polarity, membrane trafficking, cytoskeletal organization, and cytokinesis. Previous genetic studies revealed roles for the phosphatidylinositol transfer protein (PITP) Gio (also called Vib) and the PI 4-kinase (PI4K) Fwd in spermatocyte cytokinesis (Brill et al., 2000; Gatt and Glover, 2006; Giansanti et al., 2006). PITPs transfer PI between cellular membranes and therefore likely provide the substrate for PI4K enzymes (Cockcroft and Carvou, 2007). PI4Ks, in turn, phosphorylate PI on the D-4 position of the inositol ring, producing phosphatidylinositol 4-phosphate (PI4P), one of seven different PI phosphates (also called phosphoinositides). PI4P serves as a precursor for phosphatidylinositol 4,5-bisphosphate (PIP_2), which is also required for cytokinesis (Janetopoulos and Devreotes, 2006; Logan and Mandato, 2006). In addition, PI4P directly regulates membrane dynamics by binding and recruiting factors involved in both post-Golgi vesicular trafficking and nonvesicular lipid transport (D'Angelo et al., 2008). For example, 4-phosphate adaptor proteins (FAPPs) contain a conserved pleckstrin homology (PH) domain that binds PI4P (Dowler et al., 2000). Fluorescent fusions to PH domains have been used to examine the subcellular localization of particular pools of phosphoinositides, and to assess effects of enzymes that control their abundance or distribution (Balla and Várnai, 2002).

Our previous experiments demonstrated that the *fwd* gene, which is required for spermatocyte cytokinesis, encodes the sole predicted *Drosophila* PI4KIIIβ (PI4Kβ) (Brill et al., 2000). Although the homologous yeast *PIK1* genes are essential (Flanagan et al., 1993; Garcia-Bustos et al., 1994; Park et al., 2009), *fwd* null flies are viable and female fertile. Male flies are sterile, exhibiting multinucleate cells characteristic of a meiotic cytokinesis defect. Mutations in *fwd*, like those in other membrane-trafficking genes, cause defects late in cytokinesis; mutant spermatocytes form cleavage furrows that ingress, yet the constricted furrows are unstable and later regress, resulting in failure of cytokinesis.

Cellular functions of PI4Kβ have been characterized in yeast and mammalian cells, where the kinase and its lipid product PI4P are required for Golgi integrity and secretion (Balla and Balla, 2006). Budding yeast Pik1 also plays an important but less well understood role in endocytosis (Walch-Solimena and Novick, 1999). To carry out these functions, PI4Kβs bind and interact with several conserved regulators and effectors. The small Ca^{2+}-binding protein Frq1 acts as an essential regulatory subunit of yeast Pik1 (Hendricks et al., 1999). Localization and activity of mammalian PI4Kβ depend on Arf1 and the Frq1-related protein Neuronal calcium sensor-1 (Ncs-1), and the interaction with Ncs-1 promotes both regulated secretion and endocytic recycling (Zheng et al., 2005; Kapp-Barnea et al., 2006). Notably, mammalian PI4Kβ binds and recruits Rab11 to the Golgi, where Rab11 plays a role in post-Golgi secretory trafficking (Chen et al., 1998; de Graaf et al., 2004). Based on these observations in other systems, we hypothesized that Fwd, together with one or more binding partners, acts at the Golgi to regulate membrane addition during cytokinesis.

Here we show that Fwd localizes to the Golgi and is required for accumulation of PI4P on Golgi membranes and for localization of PI4P on organelles at the midzone in late stages of cytokinesis. A catalytically inactive version of Fwd (Fwd^{KD}) shows partial function in vivo, suggesting that, independent of its kinase activity, Fwd may bind and regulate other proteins involved in this process. Importantly, Rab11 binds Fwd and Fwd^{KD} in yeast two-hybrid and coimmunoprecipitation (coIP) experiments and colocalizes with Fwd in vivo. As Rab11 also colocalizes with PI4P at the midzone, and this localization is Fwd dependent, we postulate that Fwd recruits Rab11 to Golgi membranes, where Rab11 becomes associated with organelles containing PI4P. Our data suggest that PI4Kβ has both catalytic and noncatalytic functions in promoting localization of Rab11 during cytokinesis.

Results

PI4P-containing organelles localize to the midzone during cytokinesis

The requirement for Fwd in spermatocyte cytokinesis suggested that PI4P and secretory trafficking likely play critical roles in this process. To determine if PI4P associates with secretory cargo at the midzone, we examined dividing cells coexpressing an RFP fusion to the PI4P marker PH-FAPP (RFP-PH-FAPP; Dowler et al., 2000; Wei et al., 2008) and a secreted GFP (sGFP; Pfeiffer et al., 2000) in real-time imaging experiments (Wong et al., 2005) (Fig. 1). RFP-PH-FAPP and sGFP were concentrated at the poles of the cell in early telophase and were also associated with parafusorial membranes that extend along the length of the meiotic spindle (Fig. 1; t = 00:00, arrowheads). In later stages of telophase, sGFP and RFP-PH-FAPP continued to localize to the poles of the cell, but also colocalized in a small number of puncta at the midzone (Fig. 1; arrows, t = 05:00). The association of these markers at the midzone appeared to increase as cleavage progressed (Fig. 1; t = 11:00–18:00). Colocalization of PI4P and secretory cargo at the midzone suggested that Fwd catalytic activity is required for cytokinesis.

PI 4-kinase activity is required to restore fertility to *fwd* mutant flies

To determine if catalytic activity of Fwd, that is, the conversion of PI to PI4P, is crucial for cytokinesis and male fertility, we generated transgenic flies expressing GFP fused to either

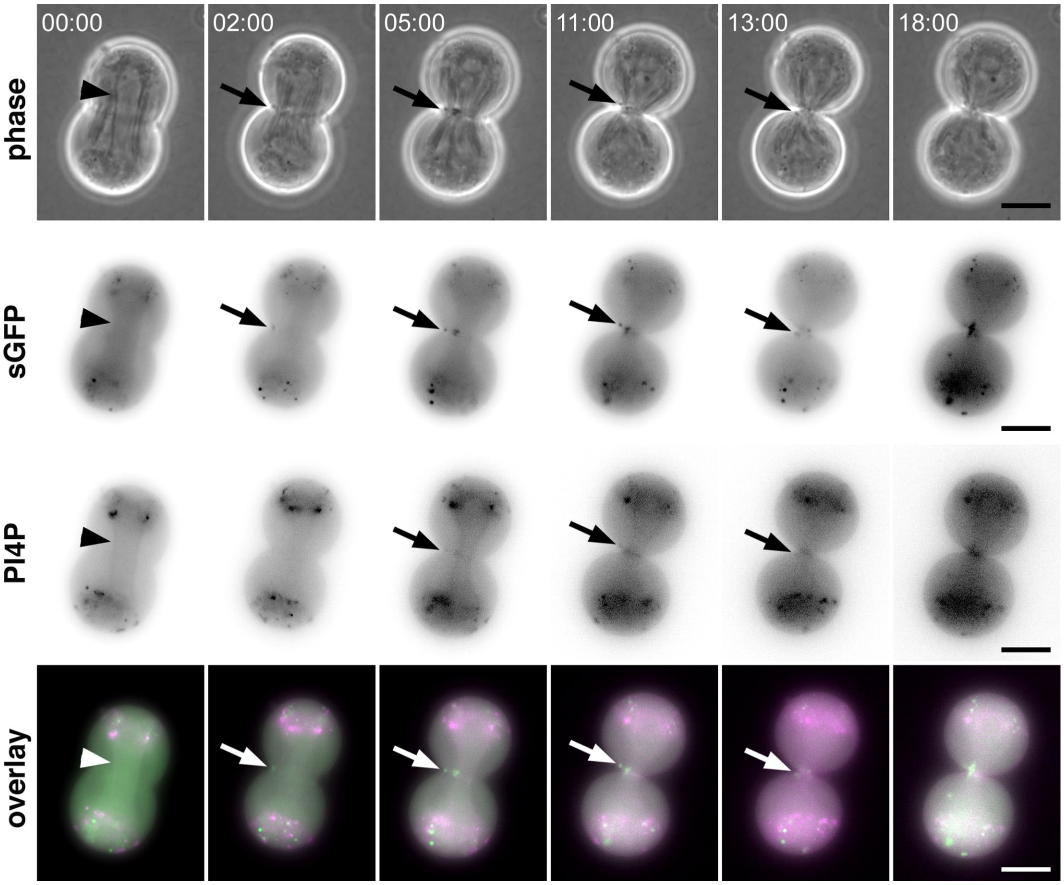


Figure 1. **PI4P-containing organelles localize at the midzone during cytokinesis.** Phase–contrast (phase) and corresponding fluorescence time-lapse images of a dividing spermatocyte expressing RFP-PH-FAPP as a marker for PI4P and sGFP as a secretory marker. Fluorescence micrographs are shown as inverted images for clarity. Times are in min:sec. Arrowheads (t = 00:00) indicate parafusorial membranes. Arrows indicate the midzone. Colocalization of sGFP (green) and PI4P (magenta) appears white (overlay). Bar, 10 µm.

wild-type Fwd (GFP-Fwd) or a kinase-dead (KD) version of the protein (GFP-Fwd^{KD}). The latter carries an amino acid substitution (D1191A) analogous to a mutation (D656A) that abolishes catalytic function of the human and bovine enzymes in vitro (Godi et al., 1999; see Fig. S1). Examination of live squashed preparations of wild-type early round spermatids revealed a 1:1 correspondence of haploid nuclei to mitochondrial derivatives of similar size (Fig. 2, A and B; Fuller, 1993). In contrast, *fwd* mutant testes exhibited multinucleate spermatids containing either two or four haploid nuclei accompanied by an enlarged mitochondrial derivative (Fig. 2 A, arrowheads), indicating a failure of meiotic cytokinesis (Fig. 2, A and B; Brill et al., 2000). Importantly, GFP-Fwd completely rescued the cytokinesis defects and infertility of *fwd* null males (Fig. 2, A and B; and not depicted). Surprisingly, expression of GFP-Fwd^{KD} partially rescued the cytokinesis defect of *fwd* mutant males, causing an increase in the frequency of normal haploid spermatids (Fig. 2, A and B).

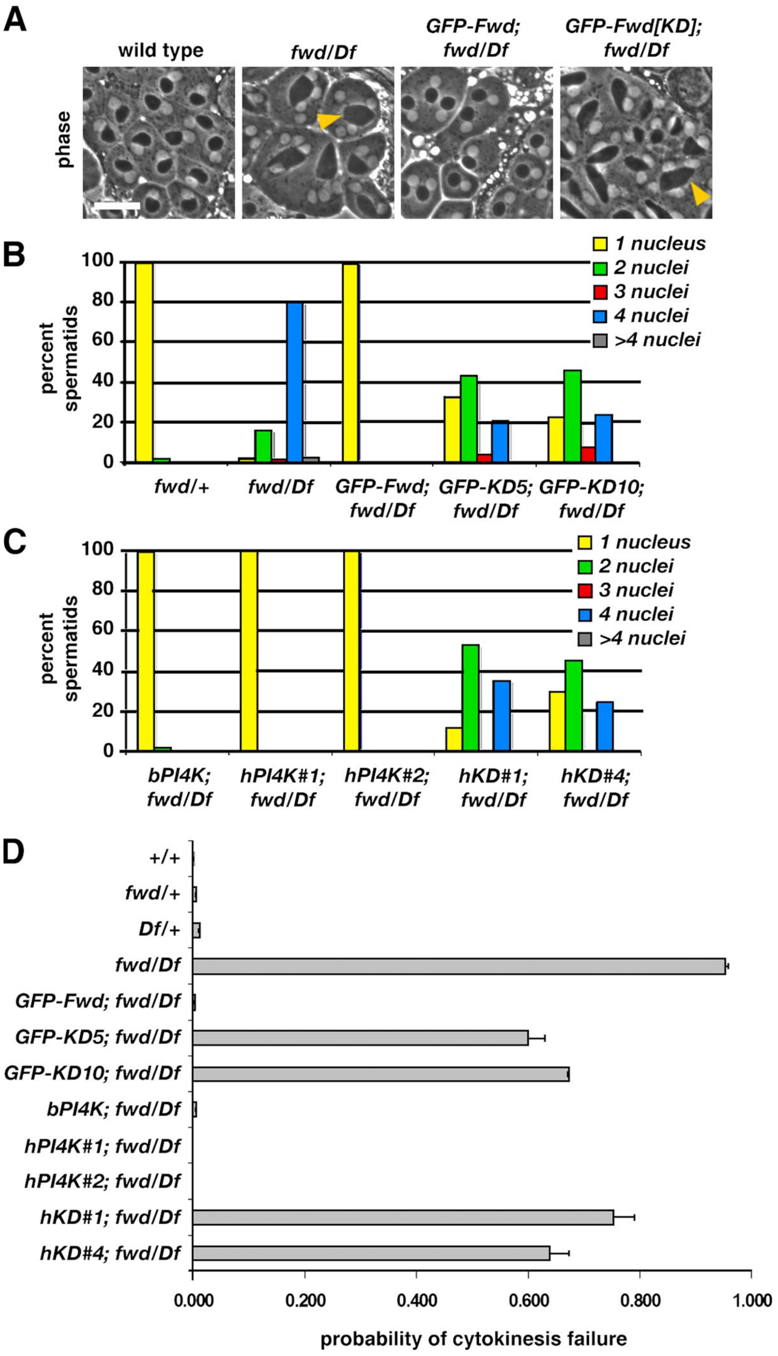
To determine if mammalian PI4Kβ can substitute for Fwd, we generated flies expressing either bovine or hemagglutinin (HA)-tagged human PI4Kβ (bPI4K or hPI4K). Remarkably, the mammalian enzymes fully rescued the cytokinesis defects and infertility of *fwd* mutant males (Fig. 2 C), suggesting that the role of Fwd has been evolutionarily conserved. In parallel, we also expressed a KD version of hPI4K (Godi et al., 1999). As with GFP-Fwd^{KD}, expression of hPI4K^{KD} partially rescued the *fwd* cytokinesis defect. Both the fly and human KD constructs decreased the probability of cytokinesis failure by roughly 40% (Fig. 2 D), indicating that PI4Kβ may have noncatalytic as well as catalytic functions in cytokinesis. However, because neither Fwd^{KD} nor hPI4K^{KD} rescued the infertility of *fwd* mutant males, the enzyme's catalytic activity and hence, PI4P, is required for male germ cell development.

Fwd is required for Golgi accumulation of PI4P

To characterize the subcellular distribution of PI4P, we examined spermatocytes coexpressing RFP-PH-FAPP with Fws-GFP, a known Golgi marker (Farkas et al., 2003). In wild type, 82% of RFP-PH-FAPP puncta (n = 361) colocalized with Fws-GFP (Fig. 3 A), indicating that PI4P is found on Golgi membranes. Interestingly, the RFP-PH-FAPP signal was often both overlapping and adjacent to Fws-GFP, indicating that Fws and PI4P may be concentrated in distinct regions of the Golgi.

To test whether Fwd was required for Golgi morphology or for PI4P accumulation on Golgi membranes, we examined Fws-GFP, Lva (Sisson et al., 2000), and RFP-PH-FAPP. The distribution

Figure 2. ***Drosophila* Fwd and mammalian PI4Kβ rescue the cytokinesis defect of *fwd* mutant males.** (A) Phase–contrast micrographs of early round haploid spermatids from wild-type, *fwd* mutant (*fwd/Df*), and *fwd* mutant flies expressing GFP-Fwd or GFP-Fwd^{KD}. Nuclei appear as light-colored discs, mitochondrial derivatives as dark-colored organelles. Multinucleate spermatids containing four haploid nuclei accompanied by an enlarged mitochondrial derivative (arrowheads) indicate failure of cytokinesis during meiosis I and II. Note that cells with multiple mitochondrial derivatives are a common artifact of squashing spermatocytes with a coverslip (Cenci et al., 1994). Bar, 20 µm. (B and C) Quantification of spermatids after successful cytokinesis (1 nucleus/mitochondrial derivative) versus cytokinesis failure (>1 nucleus/mitochondrial derivative) for *fwd* mutant and rescued flies. A minimum of 400 cells derived from at least 10 males was counted for each genotype. (B) Rescue of *fwd/Df* with GFP-Fwd or GFP-Fwd^{KD} (GFP-KD5, GFP-KD10). (C) Rescue of *fwd/Df* with bovine PI4Kβ (bPI4K) or with wild-type (hPI4K#1, hPI4K#2) or kinase-dead (hKD#1, hKD#4) human PI4Kβ. (D) Quantification of the probability of meiotic cytokinesis failure of *fwd* mutant and rescued flies. Error bars show the least sum of squares difference between the observed proportion of cells with 1, 2, or 4 nuclei/mitochondrial derivative and the proportion predicted from the model, based on the calculated probability (Dyer et al., 2007). Because the error bars are small, the model appears to accurately reflect the probability of cytokinesis failure. The probability of cytokinesis failure is negligible in control flies (*+/+*, *fwd/+*, *Df/+*) and nearly 1 (100%) in *fwd* mutant flies (*fwd/Df*), which are completely or partially rescued by wild-type or KD transgenes, respectively.



of Fws-GFP (Fig. 3 A) and Lva (not depicted) appeared unaltered in *fwd* mutant spermatocytes, suggesting Golgi morphology was normal. However, the distribution of RFP-PH-FAPP was severely affected by loss of *fwd*. RFP-PH-FAPP appeared cytoplasmic and diffuse, although occasional bright puncta colocalized with Fws-GFP (Fig. 3 A, arrows). Note that we do not expect RFP-PH-FAPP to become completely diffuse because PH-FAPP binds Golgi-localized Arf1 (Lemmon, 2008) and may also bind PI4P synthesized by other PI4Ks. Thus, Fwd is required for accumulation of normal levels of PI4P on Golgi membranes.

To examine localization of Fwd, we compared the distribution of GFP-Fwd with that of PI4P. In wild-type (not depicted) and *fwd* mutant spermatocytes, GFP-Fwd colocalized with RFP-PH-FAPP on Golgi membranes (Fig. 3 B, arrows); 93% of GFP-Fwd puncta colocalized with RFP-PH-FAPP (*n* = 212) and 86% of RFP-PH-FAPP puncta colocalized with GFP-Fwd (*n* = 228). GFP-Fwd also colocalized with RFP-PH-FAPP at the poles of dividing cells in telophase but, unlike PI4P (Fig. 1), appeared to be absent from the midzone in squashed preparations (Fig. 3 C, arrowheads). Indeed, time-lapse imaging of dividing wild-type spermatocytes expressing GFP-Fwd confirmed this result (Fig. 3 D). Thus, Fwd colocalizes with its lipid product on Golgi membranes but not at the midzone during cytokinesis.

To determine if Fwd catalytic activity influences the distribution of Fwd or PI4P, we examined colocalization of GFP-Fwd^{KD} and RFP-PH-FAPP in wild-type versus *fwd* mutant cells

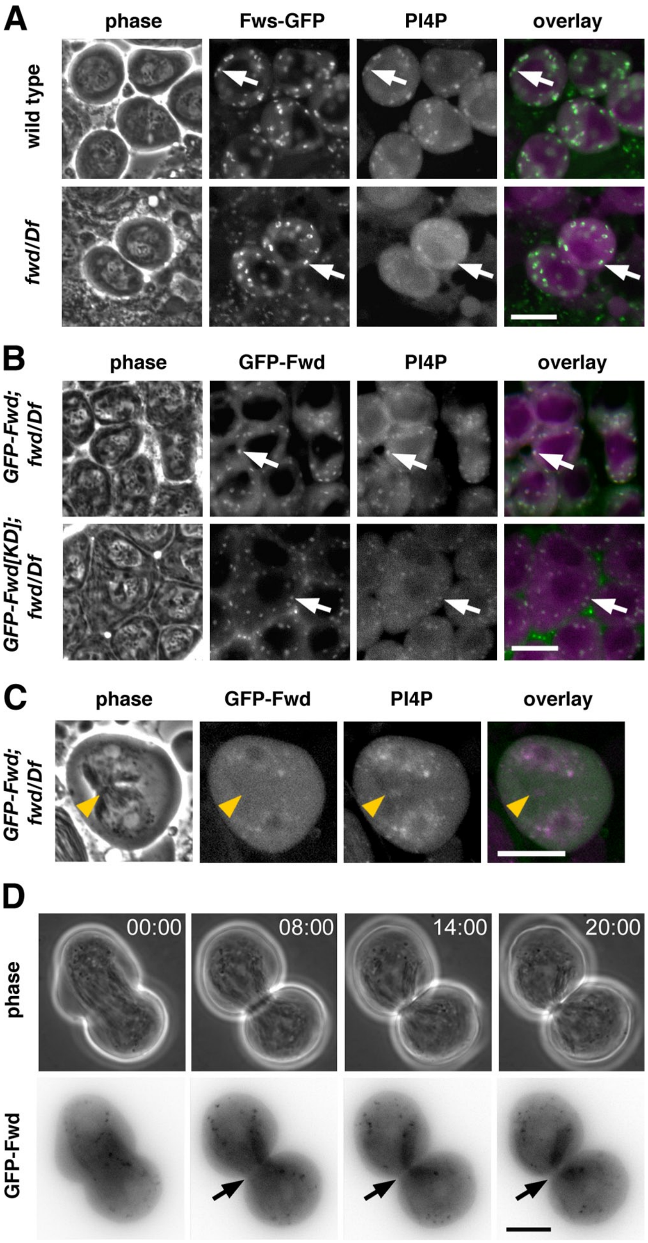


Figure 3. **PI4P and Fwd colocalize on Golgi membranes.** (A and B) Phase–contrast (phase) and corresponding fluorescence micrographs of live squashed wild-type or *fwd* mutant (*fwd/Df*) spermatocytes expressing RFP-PH-FAPP (PI4P) with Fws-GFP (A) or with GFP-Fwd or GFP-Fwd^{KD} (B). Colocalization (arrows) of Fws-GFP, GFP-Fwd, or GFP-Fwd^{KD} (green) and RFP-PH-FAPP (magenta) appears white (overlay). Bar, 20 µm. Note the diffuse RFP-PH-FAPP signal in *fwd/Df* (A) and *GFP-Fwd^{KD}; fwd/Df* (B) spermatocytes (bottom panels). (C) Phase–contrast (phase) and corresponding fluorescence micrographs of a squashed dividing spermatocyte coexpressing GFP-Fwd and RFP-PH-FAPP (PI4P). Note the presence of PI4P and absence of GFP-Fwd at the midzone (arrowheads). Bar, 20 µm. (D) Phase–contrast (phase) and corresponding fluorescence micrographs showing time-lapse images of a dividing spermatocyte expressing GFP-Fwd. Fluorescence micrographs are inverted for clarity. Times are in min:sec. GFP-Fwd is primarily in puncta at the poles (t = 08:00–20:00) and fails to accumulate at the midzone (arrows). Note background mitochondrial autofluorescence (elongated dark structures) due to long exposure times. Bar, 10 µm.

(Fig. 3 B). In wild type, GFP-Fwd^{KD} localization was indistinguishable from that of GFP-Fwd, and RFP-PH-FAPP appeared unaffected, indicating that GFP-Fwd^{KD} does not interfere with PI4P biosynthesis (not depicted). Localization of GFP-Fwd^{KD} appeared normal in *fwd* mutant spermatocytes. However, the intensity of RFP-PH-FAPP-containing puncta appeared reduced (Fig. 3 B, arrows), similar to that observed in *fwd* mutant spermatocytes lacking Fwd^{KD} (Fig. 3 A). Because GFP-Fwd and GFP-Fwd^{KD} were expressed at similar levels (not depicted), Fwd kinase activity promotes normal accumulation of PI4P.

Fwd is required for colocalization of PI4P and sGFP at the midzone

Based on the phenotype of *fwd* mutant spermatocytes, we predicted that Fwd is required for midzone localization of PI4P. To test this, we examined live squashed preparations of dividing spermatocytes for localization of RFP-PH-FAPP and sGFP (Fig. 4 A). A majority of wild-type cells showed RFP-PH-FAPP and sGFP at the midzone in late telophase (43/45 cells). In contrast, few telophase *fwd* spermatocytes showed midzone RFP-PH-FAPP or sGFP and, when present, their localization appeared diffuse (5/19 cells) (Fig. 4 A, arrows). In addition, the numbers of RFP-PH-FAPP and sGFP-positive puncta were substantially reduced in *fwd* mutant cells. This was particularly obvious in highly squashed cells in which the plasma membrane was no longer associated with the midzone (Fig. 4 B, arrows). In wild type, RFP-PH-FAPP and sGFP precisely colocalized in multiple puncta in the cleavage plane, as well as in individual puncta in other regions of the cytoplasm. In *fwd*, the few sGFP-containing puncta at the midzone failed to colocalize with RFP-PH-FAPP and sGFP rarely colocalized with RFP-PH-FAPP in other regions of the cell. Thus, Fwd is required for formation and localization of PI4P-containing secretory organelles during late stages of cytokinesis.

The failure of PI4P and sGFP to localize properly in dividing *fwd* mutant cells suggested a potential defect in distribution of Golgi membranes during cytokinesis. To test this, we examined localization of the Golgi proteins in dividing cells. Fws-GFP and Lva localized to the poles in both wild-type and *fwd* mutant spermatocytes (Fig. 4 C). However, in *fwd* mutant cells, Fws-GFP appeared less punctate and more diffuse, and Lva-containing puncta appeared smaller. In addition, as previously reported (Giansanti et al., 2006), Lva was also found in the midzone of *fwd* mutant cells (Fig. 4 C, arrows). Thus, proper Golgi organization during cytokinesis appears to require Fwd.

Fwd binds and colocalizes with Rab11

Because Fwd^{KD} and hPI4K^{KD} partially rescued the *fwd* mutant phenotype, we hypothesized that Fwd might directly bind other proteins as part of its function in cytokinesis. To identify Fwd binding partners, we performed yeast two-hybrid tests on *Drosophila* homologues of proteins previously reported to bind yeast or mammalian PI4Kβ. As bait, we used full-length and truncated versions of Fwd. As prey, we tested Frq (the homologue of *S. cerevisiae* Frq1p), Mlc-c (myosin essential light chain, the homologue of *Schizosaccharomyces pombe* Cdc4p), and Rab11, together with several related proteins. For the Frq

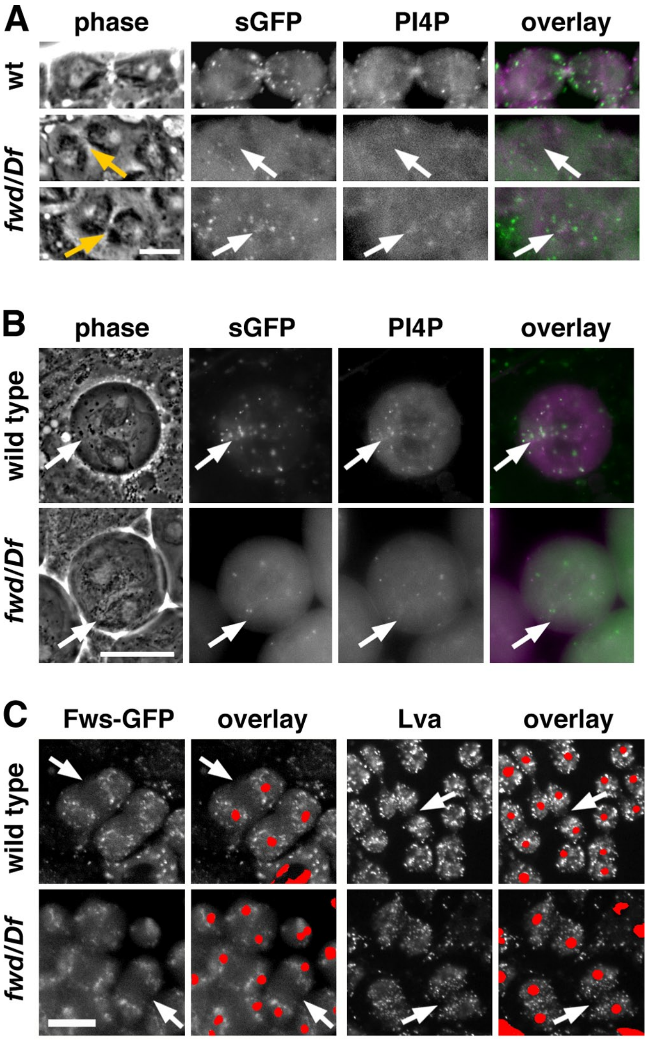


Figure 4. ***fwd* is required for midzone accumulation of PI4P and sGFP.** (A and B) Phase–contrast (phase) and corresponding fluorescence micrographs of live preparations of dividing wild-type and *fwd* mutant (*fwd/Df*) spermatocytes coexpressing sGFP and RFP-PH-FAPP (PI4P). Colocalization of sGFP (green) and RFP-PH-FAPP (magenta) appears white (overlay). (Arrows) Midzones of late telophase cells that have been squashed (B) or in which the cleavage furrow has regressed (*fwd/Df*) (A). Bars, 20 µm. (A) sGFP and PI4P colocalize at the midzone in wild type (wt; top panels), but not in *fwd* mutant (middle and bottom panels). (B) In squashed wild-type cells, sGFP and PI4P colocalize at the midzone (top panels). The few sGFP-positive puncta present at the midzone in *fwd* mutant cells (bottom panels) fail to colocalize with RFP-PH-FAPP. (C) Golgi proteins localize to the poles of dividing *fwd* mutant cells. Fluorescence micrographs of wild-type and *fwd* mutant cells expressing Fws-GFP (left panels) or stained for Lva (right panels). Distribution of Fws-GFP or Lva relative to nuclei (red) is shown (overlay). (Arrows) Midzones of representative dividing cells. Bar, 20 µm.

experiments, we also used as bait a stretch of amino acids from yeast Pik1 that binds mammalian Ncs-1 (Strahl et al., 2003). Frq and the related protein Nca strongly bound this portion of Pik1, but not full-length or truncated versions of Fwd (unpublished data). Fwd also did not interact with Mlc-c or the related myosin regulatory light chain Sqh (unpublished data). In contrast, Rab11 showed a strong two-hybrid interaction with Fwd (Fig. 5, A and B). The binding was specific to Rab11, as Rab5 and Rab7 failed to interact in the same assay.

To determine if the interaction between Fwd and Rab11 depended on either the catalytic activity of Fwd or the GTP-binding state of Rab11, we tested mutated versions of Fwd and Rab11. Both Fwd and Fwd^{KD} interacted with wild-type Rab11, Rab11^{Q70L} (GTP-bound), and Rab11^{S25N} (GDP-bound). Wild-type Fwd exhibited stronger binding interactions with Rab11 than did Fwd^{KD} (Fig. 5 B), but their interactions showed similar trends: both Fwd and Fwd^{KD} appeared to bind slightly better to Rab11^{Q70L} than to Rab11^{S25N}, and the weakest binding in each case was with wild-type Rab11. Importantly, the lack of strong preferential binding of Fwd to activated (GTP-bound) Rab11 suggested that Fwd, rather than being a Rab11 effector, might regulate Rab11 in vivo.

To verify the binding of Fwd to Rab11, we performed coIP experiments on fly proteins expressed in mammalian tissue culture cells (Fig. 5 C). Flag-tagged Rab11 and HA-tagged Fwd or Fwd^{KD} were expressed in COS-7 cells, alone or in combination. IPs with anti-Flag antibody yielded HA-Fwd or HA-Fwd^{KD} only in the presence of Flag-Rab11. Reciprocally, IPs with anti-HA antibody yielded Flag-Rab11 only in the presence of HA-Fwd or HA-Fwd^{KD}. Thus, *Drosophila* Fwd, like mammalian PI4Kβ, binds the recycling endosome regulator Rab11.

The binding of Fwd to Rab11 suggested the two proteins might colocalize in vivo. To test this, we examined developing male germ cells expressing CFP and YFP fusions to Fwd (CFP-Fwd) and Rab11 (YFP-Rab11). Rab11 and Fwd colocalized at the Golgi in spermatocytes (Fig. 5 D, arrows) and at the poles of dividing cells (not depicted). In spermatocytes, 91.9% of CFP-Fwd puncta (n = 280) colocalized with YFP-Rab11 and 82.3% of YFP-Rab11 puncta (n = 318) colocalized with CFP-Fwd. In dividing cells, Rab11 also localized to structures where Fwd was not evident, including parafusorial membranes and puncta at the midzone (see next section). Thus, Fwd localizes to a subset of Rab11-positive structures before and during meiotic cytokinesis.

Rab11 colocalizes with PI4P and its localization depends on Fwd

Because the distribution of Rab11 appeared similar to that of PI4P, we examined flies coexpressing GFP-Rab11 with RFP-PH-FAPP. In spermatocytes, GFP-Rab11 tightly colocalized with RFP-PH-FAPP on Golgi membranes (Fig. 6 A, top panels, arrows). 98.2% of GFP-Rab11 puncta (n = 171) colocalized with RFP-PH-FAPP and 77.4% of RFP-PH-FAPP–positive puncta (n = 217) were positive for GFP-Rab11. Strikingly, GFP-Rab11 and RFP-PH-FAPP also colocalized to parafusorial membranes and at the midzone of dividing cells (Fig. 6 A, bottom panels, arrows). Thus, Rab11 is found primarily on PI4P-containing organelles, including those found in the midzone during cytokinesis.

To test whether Fwd regulates Rab11 localization, we examined GFP-Rab11 in wild-type versus *fwd* mutant flies. In *fwd* mutant spermatocytes, GFP-Rab11 localized to puncta resembling those found in wild-type cells (compare Fig. 6, A and B, top panels, arrows), and showed partial colocalization with residual RFP-PH-FAPP. However, in dividing spermatocytes and elongating spermatids, the distribution of GFP-Rab11 was

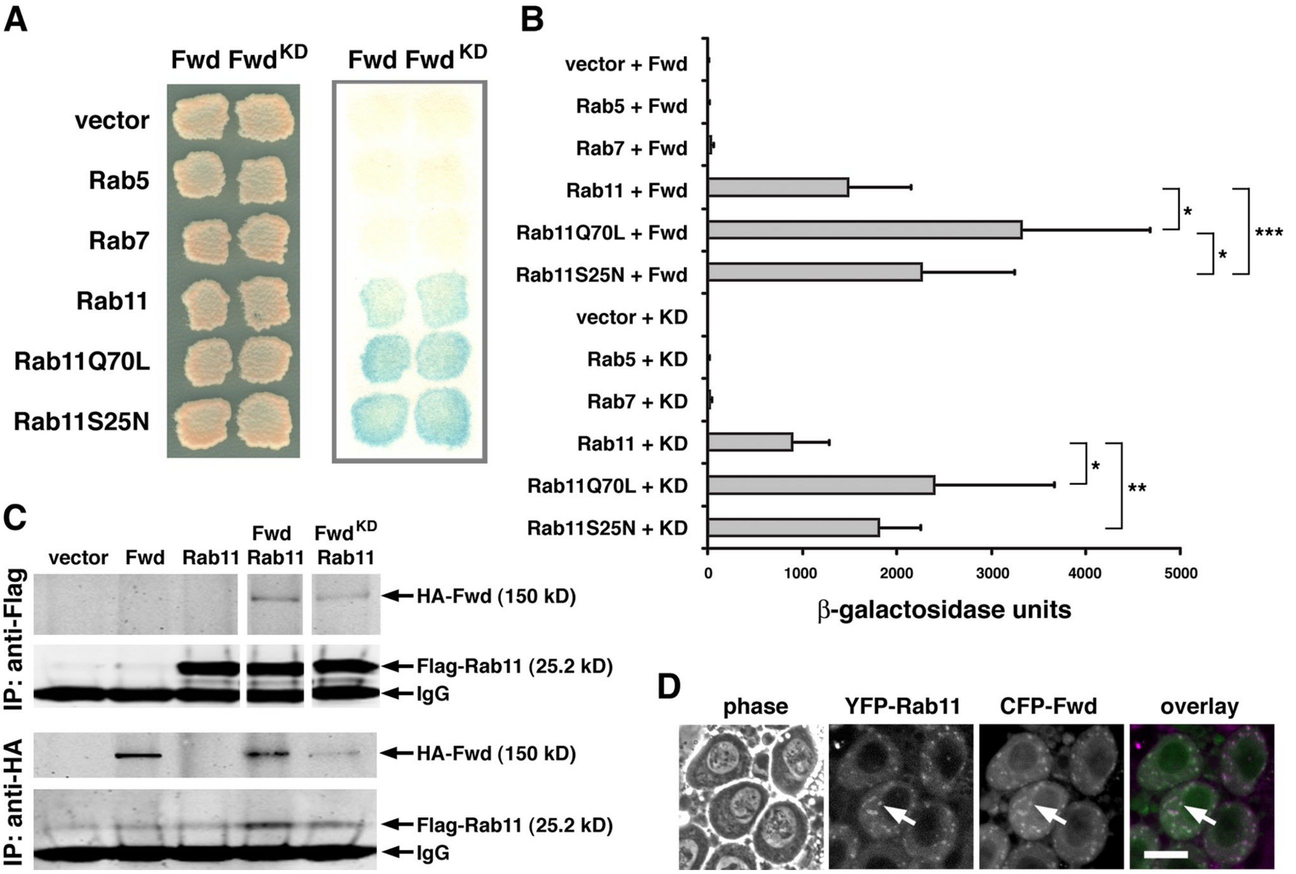


Figure 5. **Fwd binds and colocalizes with the recycling endosome regulator Rab11.** (A) Yeast two-hybrid assays. (Left) Patches of yeast cells cotransformed with Fwd or Fwd^{KD} bait plasmids together with one of several prey plasmids (vector alone, Rab5, Rab7, Rab11, Rab11^{Q70L}, and Rab11^{S25N}). (Right) Filter X-gal assays performed on replicates of these patches. Blue color indicates a positive interaction. (B) Quantification of yeast two-hybrid results. LacZ reporter expression (β-galactosidase units) induced by various combinations of bait and prey plasmids (as in A). Statistically significant differences are: (single asterisk) $P < 0.001$, (double asterisk) $P < 0.05$, (triple asterisk) $P < 0.01$. (C) Co-immunoprecipitation (coIP) of Rab11 with Fwd and Fwd^{KD} expressed in COS-7 cells. Immunoblots were probed with anti-HA or anti-Flag, as indicated on the right. IP with anti-Flag antibody pulls down HA-Fwd and HA-Fwd^{KD} only when coexpressed with Flag-Rab11 (top panels). IP with anti-HA pulls down Flag-Rab11 only when coexpressed with HA-Fwd or HA-Fwd^{KD} (bottom panels). Note that a nonspecific protein of slightly lower mobility than Flag-Rab11 is present in all lanes of the anti-HA IP experiment. (D) Phase–contrast (phase) and corresponding fluorescence micrographs of spermatocytes coexpressing YFP-Rab11 and CFP-Fwd. Colocalization (arrows) of YFP-Rab11 (magenta) and CFP-Fwd (green) appears white (overlay). Bar, 20 µm.

clearly aberrant (compare Fig. 6, A and B, bottom panels, arrows). In contrast to wild type, GFP-Rab11 appeared completely delocalized during cytokinesis in *fwd* mutant cells. Moreover, at elongating stages, GFP-Rab11 concentrated in unusual linear elements along the elongating spermatid tails rather than the uniform distribution seen in wild type (Fig. 6 C, arrowheads). Thus, Fwd is required for proper localization of Rab11 during and after cytokinesis.

To further characterize the effect of Fwd on Rab11 activity during cytokinesis, we examined telophase cells from wild-type and *fwd* mutant flies for localization of Rab11 and its effector Nuf using specific antibodies (Fig. 7 A). In wild type ($n = 85$), Rab11 (62.3%) and Nuf (94.1%) localized in puncta at the midzone (Fig. 7 A, top panels, arrows). However, in *fwd* mutant cells, localization of Rab11 and Nuf was more variable (Fig. 7 A, middle panels, arrows). Occasionally, Rab11 (17.7%) and Nuf (19.1%) were concentrated at the midzone ($n = 68$ cells). However, in the majority of cells, Rab11 was undetectable (82.3%) and Nuf was either undetectable (76.5% cells) or localized to only a portion of the midzone (4.4%; not depicted). Thus, Fwd is required for localization of Rab11 and its effector Nuf in dividing cells.

Rab11 acts downstream of Fwd during cytokinesis

If Rab11 acts downstream of Fwd, overexpression of activated Rab11 should suppress the cytokinesis defect caused by mutations in *fwd*. Indeed, overexpression of Rab11^{Q70L}, but not wild-type Rab11 or dominant-negative Rab11 (Rab11^{S25N}), partially suppressed the *fwd* cytokinesis defect (Fig. 7 B; Rab11^{S25N}, not depicted). Moreover, overexpression of activated Rab11 (Rab11^{Q70L}) restored Nuf localization to the midzone in 82.6% of dividing cells ($n = 23$) (Fig. 7 A, bottom panels, arrows). Thus, activated Rab11 can partially compensate for loss of *fwd*.

Discussion

The discovery that *Drosophila* PI4Kβ Fwd and fission yeast PI4P 5-kinase Its3 are required for cytokinesis provided the first genetic evidence that phosphoinositides play a critical role in this

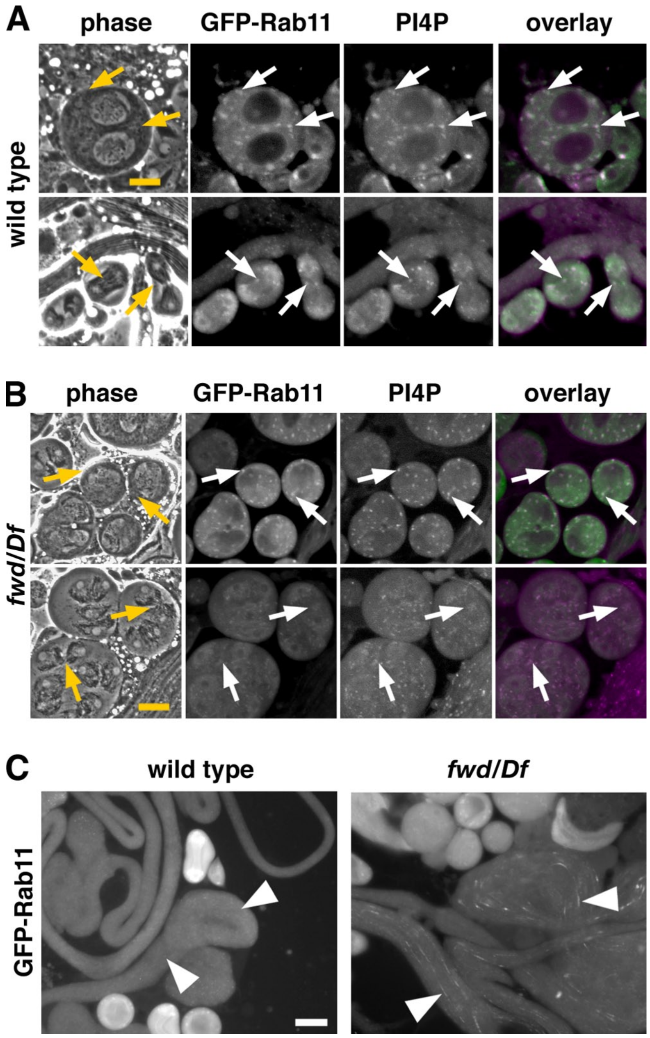


Figure 6. **Rab11 localizes to PI4P-containing organelles and its localization during cytokinesis requires *fwd*.** (A and B) Phase–contrast (phase) and corresponding fluorescence micrographs of live squashed spermatocytes expressing GFP-Rab11 and RFP-PH-FAPP. Colocalization (arrows) of GFP-Rab11 (green) and RFP-PH-FAPP (magenta) appears white (overlay). Arrows indicate the midzone. Bars, 20 µm. (A) GFP-Rab11 and RFP-PH-FAPP (PI4P) colocalize in spermatocytes (top panels) and in dividing cells (bottom panels). Rab11 and RFP-PH-FAPP colocalize on organelles near the midzone of dividing cells (bottom panels). (B) In *fwd* mutant cells (*fwd/Df*), GFP-Rab11 and RFP-PH-FAPP colocalize to small puncta in spermatocytes (top panels) and show diffuse localization during cytokinesis (bottom panels). Note that pictures in A and B were taken on different days and the images in B were adjusted for brightness and contrast to reveal weak signals in *fwd* mutant. (C) Fluorescence micrographs of wild-type and *fwd* mutant (*fwd/Df*) male germ cells expressing GFP-Rab11. Arrowheads indicate elongating spermatids. GFP-Rab11 is uniformly distributed in wild type (left), but localizes to linear structures in *fwd* mutant (right). Bar, 20 µm.

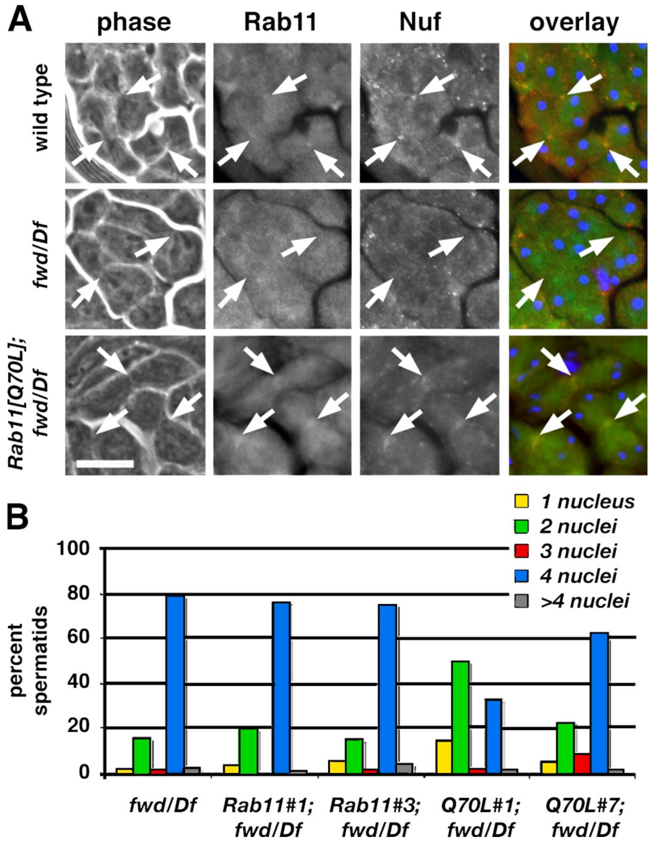


Figure 7. **Rab11 acts downstream of *fwd* to promote completion of cytokinesis.** (A) Phase–contrast (phase) and fluorescence images of dividing spermatocytes stained for Rab11 (green), Nuf (red), and DNA (blue). Arrows indicate the midzone. Colocalization of Rab11 and Nuf is yellow (overlay). Endogenous Rab11 and Nuf colocalize at the midzone in wild type (top panels). Neither Rab11 nor Nuf accumulate in the midzone in *fwd* mutant cells (middle panels). Localization of Rab11 and Nuf is restored upon expression of Rab11^{Q70L} (bottom panels). Bar, 20 µm. (B) Quantification of the number of spermatids resulting from successful cytokinesis versus cytokinesis failure (as in Fig. 2, B and C). A minimum of 300 cells derived from at least 10 males was counted for each genotype. *fwd* mutant testes (*fwd/Df*) show a large proportion of cells with 2 or 4 nuclei. Overexpression of activated Rab11^{Q70L} (Q70L#1, Q70L#3), but not wild-type Rab11 (Rab11#1, Rab11#3), partially rescues the *fwd* cytokinesis defect, producing increased numbers of cells with 1, 2, or 3 nuclei.

process (Brill et al., 2000; Zhang et al., 2000). Consistent with this, the PITPs Gio and Nir2 are also required for cytokinesis (Litvak et al., 2002; Gatt and Glover, 2006; Giansanti et al., 2006), and may serve in part to provide the PI precursor for PI4P. In addition, the pool of PI4P synthesized by PI4Kβ may serve as a precursor to PIP$_2$, which is also required for cytokinesis (Emoto et al., 2005; Field et al., 2005; Wong et al., 2005). Nonetheless, individual phosphoinositides and their regulatory enzymes likely play unique roles, regulating distinct steps of the process. Importantly, a role for PI4Kβ—and therefore PI4P—in cytokinesis appears conserved (Garcia-Bustos et al., 1994; Desautels et al., 2001; Rodgers et al., 2007; Park et al., 2009).

Our experiments reveal that Fwd is required for synthesis of PI4P on Golgi membranes and for formation of PI4P- and Rab11-associated secretory organelles at the midzone. On the surface, this result appears at odds with previous observations suggesting that Fwd and Gio function at a later step to promote fusion of Lva-containing Golgi-derived vesicles with the cleavage furrow (Giansanti et al., 2006, 2007). However, because Lva serves as a Golgi scaffold (Sisson et al., 2000), accumulation of Lva at the midzone in *fwd* and *gio* mutant cells may reveal a defect in segregation of a subset of Golgi membranes to the poles of the cell rather than a defect in vesicle fusion.

Although Rab11 has been shown to traffic to the midzone during cytokinesis (Prekeris and Gould, 2008), the membrane composition of Rab11-containing organelles was previously unknown. Our finding that PI4P is present on these organelles is consistent with proteomic analyses demonstrating an enrichment of Rab11 and PI4Kβ on PI4P-containing liposomes (Baust et al., 2006). Interestingly, these liposomes were also enriched in actin regulatory factors such as Rac1 and Wave/Scar. As actin is transported on vesicles to the midzone in *Drosophila* embryos, and the Rab11 effector Nuf promotes actin polymerization at the furrow (Albertson et al., 2008; Cao et al., 2008), PI4P-dependent organelles may concentrate or recruit factors such as Nuf that contribute to maintenance of F-actin in the contractile ring. Consistent with this idea, mutations in *fwd*, like mutations in *nuf* and *rab11*, are associated with failure to maintain proper actin organization during cytokinesis (Brill et al., 2000; Giansanti et al., 2007; Cao et al., 2008).

The regulatory relationship between Fwd and Rab11 is evolutionarily conserved. In budding yeast, the Rab11 homologues Ypt31/32 act downstream of Pik1 to regulate post-Golgi trafficking (Sciorra et al., 2005). The two *Arabidopsis thaliana* PI4Kβs, PI-4Kβ1 and PI-4Kβ2, show genetic interactions with the Rab11 homologue Rab4Ab in root hair development and colocalize with RabA4b on root hair tip-associated membranes, and PI-4Kβ1 binds GTP-bound RabA4b in vitro. Moreover, RabA4b-containing membranes exhibit altered morphology in PI-4Kβ1/β2 double mutants (Preuss et al., 2006), suggesting RabA4b may act downstream of PI4Kβs in this process. Mammalian PI4Kβ binds activated Rab11, and is thought to recruit Rab11 to Golgi membranes to promote post-Golgi secretory trafficking (de Graaf et al., 2004). Our results demonstrate that Fwd acts upstream of Rab11 during cytokinesis, and that bovine and human PI4Kβ can fully substitute for Fwd in vivo.

PI4Kβ and PI4P participate in vesicular and nonvesicular trafficking of cellular membranes and their lipid constituents (D'Angelo et al., 2008), suggesting that, in addition to its role in formation of secretory organelles, Fwd may direct other trafficking pathways. For example, several conserved lipid transport proteins bind PI4P and depend on PI4Kβ for their localization and function in yeast and mammalian cells. PI4P is also found at ER exit sites (also called transitional ER, or tER) (Blumental-Perry et al., 2006; Peretti et al., 2008). Intriguingly, tER was recently shown to accumulate at the midzone of dividing *S. pombe* cells (Vjestica et al., 2008), and normal ER morphology in dividing *Caenorhabditis elegans* embryos was found to require Rab11 (Zhang et al., 2008). Future experiments will be required to determine if Fwd-dependent tER or nonvesicular trafficking pathways actively participate in cytokinesis.

Despite strong parallels between cytokinesis in mammalian cells and in *Drosophila*, the mechanism by which Rab11 affects completion of cytokinesis is not entirely conserved. In mammalian cells (Prekeris and Gould, 2008), Rab11 associates indirectly with the plasma membrane regulator Arf6 via FIP3, a Rab11-binding protein with homology to Nuf. Both Rab11 and Arf6 bind members of the exocyst complex, which in turn mediates targeting of endosomes to the midzone. In contrast, in *Drosophila*, Arf6 and Rab11 appear to function in separate pathways. Nuf binds and colocalizes with Rab11, yet fails to bind Arf6 (Hickson et al., 2003; Riggs et al., 2003). Consistent with this, Rab11 is essential and has specific functions at multiple stages of development, whereas Arf6 is required only for spermatocyte cytokinesis (Dyer et al., 2007; Giansanti et al., 2007; Li et al., 2007). Even in spermatocytes, Arf6 promotes trafficking of Rab4-positive but not Rab11-positive vesicles (Dyer et al., 2007). Thus, in spermatocytes, Arf6/Rab4 and Fwd/Rab11 appear to constitute nonredundant membrane trafficking pathways required for completion of meiotic cytokinesis.

Despite its vital role in spermatocyte cytokinesis, Fwd is dispensable for normal development and female fertility. *Drosophila* tissue culture cells show only a weak requirement for *fwd* during cytokinesis, with knockdown of *fwd* by RNAi resulting in a small increase in binucleate cells (Eggert et al., 2004). This is particularly surprising given that yeast *PIK1* is required for post-Golgi secretory trafficking and endocytosis (Balla and Balla, 2006). As secretion and endocytosis are essential processes, we hypothesize that *fwd* is redundant with other genes for carrying out these functions outside of the male germline. Future investigations will determine the identity of these *fwd*-interacting genes.

Materials and methods

Molecular cloning

For P element vectors, we used CaSpeR4 (C4) (Pirrotta, 1988), pCaSpeR-hs83 (hs83), which contains the *hsp83* promoter (from J. Horabin via K. Miller; Washington University, St. Louis, MO; Hicks et al., 1999), and tv3, which contains the spermatocyte-specific β2-tubulin promoter (Wong et al., 2005). For yeast two-hybrid vectors, we used pGADT7 (Chien et al., 1991) and pGBKT7 (Louvet et al., 1997). *Drosophila* cDNA clones corresponding to *nca*, *mlc-c*, *sqh*, *Rab5*, *Rab7*, and *Rab11* were from Research Genetics or the Canadian *Drosophila* Microarray Centre. *fwd* cDNA and genomic clones (Brill et al., 2000) and bovine PI4Kβ (bPI4K) plasmids were described previously (Balla et al., 1997; Zhao et al., 2000). We obtained HA-tagged human PI4Kβ clones from R. Meyers and L. Cantley (Harvard Medical School, Boston, MA; Meyers and Cantley, 1997), *frq* cDNA (Pongs et al., 1993) from A. Jeromin (Mount Sinai Hospital, Toronto, Ontario, Canada), monomeric EGFP (GFP), ECFP (CFP), and EYFP (YFP) (Zacharias et al., 2002) from E. Snapp (National Institutes of Health, Bethesda, MD), monomeric RFP (Campbell et al., 2002) from R. Tsien (University of California, San Diego, La Jolla, CA), and secreted GFP (sGFP), a fusion of the signal sequence of the wingless protein to EGFP (Pfeiffer et al., 2000), from J.-P. Vincent via G. dos Santos (University of Toronto, Toronto, Ontario, Canada). For IPs, FLAG-tagged *Drosophila* Rab11, HA-tagged Fwd, and HA-Fwd^{KD} were cloned into pCDNA3.1 (from D. Rotin; The Hospital for Sick Children, Toronto, Ontario, Canada).

Standard molecular cloning (Sambrook et al., 1989) was performed with restriction enzymes and T4 DNA ligase (New England Biolabs, Inc.). Oligonucleotides were from Operon, Invitrogen, or The Centre for Applied Genomics (TCAG, SickKids). PCR was performed on a PTC200 thermocycler using Pfusion polymerase (MJ Research). Site-directed mutagenesis was performed using QuikChange or QuikChange XL (Agilent Technologies). Plasmids were confirmed by DNA sequencing (TCAG). Cloning details are available upon request.

Yeast two-hybrid assays

Yeast strain Y190 (James et al., 1996) was cotransformed with bait (pGADT7) and prey (pGBKT7) plasmids using a standard protocol (Clontech PT3247) and transformants were selected on SD–Trp–Leu at 29°C. PIK1 plasmids were from O. Pongs (University of Hamburg, Hamburg, Germany). Expression of HA- or myc-tagged bait and prey proteins was confirmed by immunoblotting with specific antisera (Santa Cruz Biotechnology, Inc.). X-Gal filter assays and β-galactosidase assays on yeast extracts were performed at 30°C as described previously (Brill et al., 1994). For each sample, a total of six independent transformants (three each in two independent experiments) assayed in duplicate was used to calculate average β-galactosidase units and standard error. Statistical significance was determined by one-way ANOVAs using the Newman-Keuls test.

Tissue culture, immunoprecipitation, and kinase assays

COS-7 cells were grown in DME with penicillin–streptomycin and 10% fetal bovine serum. For IPs, cells were transfected with pcDNA3.1 plasmid(s) using Lipofectamine 2000 (Invitrogen), lysed in RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.5% NP40, 0.25% Na deoxycholate, and 1 mM EDTA) containing protease inhibitors and DTT, and pelleted to remove debris. Lysates precleared with protein G–Sepharose (GE Healthcare) were incubated with monoclonal antibody against FLAG (M2, Sigma-Aldrich) or HA (Clone 1.11, Covance). Immune complexes bound to protein G–Sepharose were washed in RIPA containing 0.5 M LiCl and 0.5% Triton X-100 and immunoblotted using anti-HA and anti-FLAG M2 antisera.

PI4K assays were performed on IPs of HA-tagged bPI4K or $bPI4K^{KD}$ as described previously (Downing et al., 1996). In brief, reactions were carried in 50 µl kinase buffer (50 mM Tris/HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM EGTA, 1 mM PI, 0.4% Triton X-100, and 0.5 mg/ml BSA) containing γ-$[^{32}P]$-ATP (2 µCi/sample, 0.1 mM final) and terminated by adding $CHCl_3$/MeOH/HCl (200:100:0.75). After phase separation and washing, samples were transferred to scintillation vials, evaporated and counted in Econofluor (5 ml) in a β counter. This procedure eliminates ATP contamination and yields pure $[^{32}P]$-PI4P.

Fly stocks, maintenance, and analysis of male fertility

Flies were raised on standard cornmeal molasses agar at 25°C (Ashburner, 1990). *fwd*^{3} and deletions *Df(3L)17E* and *Df(3L)7C* were described previously (Brill et al., 2000). In brief, *fwd*^{3} contains a stop codon at amino acid 310, which truncates the protein in the middle of the nonconserved N-terminal domain. *Df(3L)17E* and *Df(3L)7C* are overlapping deletions that remove the entire *fwd* coding region. *fwd*^{3} behaves as a null in genetic experiments (Brill et al., 2000). Unless indicated, *Df* refers to *Df(3L)7C*. *Df/Df* refers to *Df(3L)17E/Df(3L)7C*. Transgenes were introduced into embryos as previously described for tv3::RFP-PH-FAPP (Wei et al., 2008). Where independent insertions of the same transgene were examined (Figs. 2 and 7), these were given different isolate numbers (in parentheses): wild-type human PI4Kβ (hPI4K#1, hPI4K#2); kinase-dead human PI4Kβ (hKD#1, hKD#4); wild-type Rab11 (Rab11#1, Rab11#3); activated $Rab11^{Q70L}$ (Q70L#1, Q70L#3). Fws-GFP (Farkas et al., 2003) and α_1-tubulin::YFP-Rab11 (Classen et al., 2005) flies were from M. Fuller (Stanford School of Medicine, Palo Alto, CA) and S. Eaton (Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Male fertility was determined by crossing individual males to groups of five wild-type (w^{1118}) virgin females and scoring for offspring after 10 d at 25°C. w^{1118}, *fwd*^{3}*/TM6B* or *Df(3L)7C/TM6B* flies were used as controls.

Fluorescence microscopy and imaging

Squashed live testis preparations were observed with a 40x phase–contrast objective on an Axioplan 2E or Axiovert microscope (Carl Zeiss, Inc.). Quantification of multinucleate cells was performed as described previously (Brill et al., 2000), except that 200–500 cells rather than nuclei were counted for each genotype. Probability of cytokinesis failure was calculated from the proportion of cells with 1, 2, or 4 nuclei using an algorithm provided by N. Dyer and M. González-Gaitán (University of Geneva, Geneva, Switzerland; Dyer et al., 2007). For DNA staining, testes dissected in testis isolation buffer (TIB; Casal et al., 1990) were cut with tungsten needles in TIB containing 8.3 µg/ml Hoechst 33342 and squashed with a coverslip. Colocalization of fluorescent marker proteins was determined as a fraction of total puncta in 20 spermatocytes.

Real-time imaging was performed on dividing spermatocytes embedded in fibrin clots, as described previously (Wong et al., 2005). Testes were isolated from *Drosophila* third instar larvae in insect Ringer's buffer (0.13 M NaCl, 5 mM KCl, 1 mM $CaCl_2$, and 5 mM KH_2PO_4 and 7 mM $Na_2HPO_4 \cdot 7H_2O$, pH 6.8) and transferred to a drop (~5-6 µl) of 0.5–1% fibrinogen solution (EMD) on a preflamed coverslip. Testes were pierced with a tungsten needle and spread to separate the cells, which were allowed to settle for 30 s. Subsequently, 2–5 µl bovine thrombin (Sigma-Aldrich) was added to form the clot. The coverslip was inverted onto a drop of Ringer's on a second coverslip affixed to the bottom of a brass perfusion chamber sealed with melted wax (1:1:1 mixture of Vaseline, lanolin, and paraffin) or silicon grease (Dow Corning Corp.). Dividing cells were perfused with Ringer's via small aluminum pipes inserted into holes in the side of the chamber and imaged at room temperature.

Immunofluorescence was performed on preparations of germ cells isolated from testes of 0–2-d-old males as described previously (Hime et al., 1996; Wei et al., 2008). Testes were dissected in testis isolation buffer (Casal et al., 1990), transferred to microscope slides pretreated with polylysine (Sigma-Aldrich), squashed under a siliconized coverslip, and frozen in liquid nitrogen. After removal of the coverslip, samples were extracted in chilled 95% ethanol for 10 min, fixed with 4% EM-grade paraformaldehyde in phosphate-buffered saline (PBS) for 7 min at room temperature, permeabilized two times (15 min each) in PBS containing 0.3% Triton X-100 and 0.3% sodium deoxycholate, washed once (for 5 min) in PBS with 0.1% Triton X-100 (PBT), and blocked for at least 30 min in PBT with 3% bovine serum albumin (PBTB) at room temperature or at 37°C. Slides were incubated overnight (~16 h) at 4°C in primary antibodies diluted in PBT, washed four times in PBTB (15 min each), incubated with Alexa 488– or Alexa 568–conjugated secondary antibodies (20 units/ml; Invitrogen), and washed four times for 10 min with PBTB, with 1 µg/ml DAPI (Sigma-Aldrich) included in the second wash. Samples were mounted in 9:1 glycerol/PBS containing 100 mg/ml *p*-phenylenediamine. Anti-Nuf was from B. Riggs and W. Sullivan (University of California, Santa Cruz, Santa Cruz, CA; Riggs et al., 2003), anti-Lva from J. Sisson (University of Texas at Austin, Austin, TX; Sisson et al., 2000), and anti-Rab11 from BD Biosciences, R. Cohen (University of Kansas, Lawrence, KS; Dollar et al., 2002), or D. Ready (Purdue University, West Lafayette, IN; Satoh et al., 2005). Antibodies were used at the published or recommended dilutions (BD Biosciences).

Images were acquired with an Axiocam CCD camera on an Axioplan 2E microscope equipped with phase–contrast objectives (40x plan-Neofluoar 0.75 NA, 63x plan-Apochromat oil immersion 1.4 NA or 100x plan-Apochromat oil immersion 1.4 NA) using Zeiss Axiovision software (all from Carl Zeiss, Inc.) and imported into Adobe Photoshop. Unless indicated, control and experimental samples were imaged under identical conditions and images were adjusted only for contrast and brightness using identical manipulations.

Online supplemental material

Figure S1: Kinase-dead PI4Kβ lacks detectible catalytic activity in vitro. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908107/DC1.

We dedicate this paper to the memory of John Sisson.

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