Rab11 Is Required for Membrane Trafficking and Actomyosin Ring Constriction in Meiotic Cytokinesis of *Drosophila* Males

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Rab11 is a small GTPase that regulates several aspects of vesicular trafficking. Here, we show that Rab11 accumulates at the cleavage furrow of *Drosophila* spermatocytes and that it is essential for cytokinesis. Mutant spermatocytes form regular actomyosin rings, but these rings fail to constrict to completion, leading to cytokinesis failures. *rab11* spermatocytes also exhibit an abnormal accumulation of Golgi-derived vesicles at the telophase equator, suggesting a defect in membrane-vesicle fusion. These cytokinesis phenotypes are identical to those elicited by mutations in *giotto* (*gio*) and *four wheel drive* (*fwd*) that encode a phosphatidylinositol transfer protein and a phosphatidylinositol 4-kinase, respectively. Double mutant analysis and immunostaining for Gio and Rab11 indicated that *gio*, *fwd*, and *rab11* function in the same cytokinetic pathway, with Gio and Fwd acting upstream of Rab11. We propose that Gio and Fwd mediate Rab11 recruitment at the cleavage furrow and that Rab11 facilitates targeted membrane delivery to the advancing furrow.

INTRODUCTION

Cytokinesis is the process by which two daughter cells separate at the end of mitosis or meiosis. In animal cells, cytokinesis is mediated by an actomyosin-based contractile ring that assembles just beneath the equatorial cortex of the dividing cell. Ring constriction leads to the formation of a furrow in the plasma membrane, which invaginates until the two daughter cells remain connected by a thin cytoplasmic bridge, called the midbody. This bridge is ultimately cleaved during the final step of cytokinesis, named abscission, which results in the complete separation of daughter cells (Glotzer, 2001; Schweitzer and D’Souza-Schorey, 2004). Recent work has shown that both cleavage furrow ingression and abscission require substantial membrane remodeling. Studies in a variety of organisms indicate that cleavage furrow invagination is accompanied by targeted membrane addition from internal membrane stores (Strickland and Burgess, 2004; Albertson *et al.*, 2005; Matheson *et al.*, 2005). There is also evidence that formation of new furrow membrane requires vesicle delivery through both the secretory and the endocytic pathways. The secretory pathway involves vesicle transport from the endoplasmic reticulum (ER) to the Golgi and then to the plasma membrane. In the endocytic pathway, plasma membrane-derived vesicles proceed through

the early endosome and the recycling endosome (RE), which directs them back to the plasma membrane (Strickland and Burgess, 2004; Albertson *et al.*, 2005).

In *Drosophila*, Golgi-based vesicle delivery is crucial for both cytokinesis and cellularization of syncytial embryonic nuclei, a process with features equivalent to those that occur during cleavage furrow ingression (Albertson *et al.*, 2005). The Golgi-associated proteins required for cellularization include the Lava Lamp (Lva) golgin (Sisson *et al.*, 2000) and the integral membrane protein called Strabismus (Lee *et al.*, 2003). In vivo studies have shown that Lva is enriched at vesicles that move toward the apex of the advancing cellularization furrow, suggesting that they are a source of new membrane for furrow progression (Sisson *et al.*, 2000). Golgi-associated proteins have been also implicated in cytokinesis of *Drosophila* spermatocytes. Four way stop (Fws), a protein homologous to the Cog5 subunit of the conserved oligomeric Golgi complex, is enriched at the Golgi stacks and Golgi-derived vesicles and it is required for cytokinesis (Farkas *et al.*, 2003). Another protein required for meiotic cytokinesis of males is the *Drosophila* orthologue of Syntaxin 5, a conserved Golgi-associated soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) (Xu *et al.*, 2002). Golgi-associated proteins involved in cytokinesis have been also identified in mammals and *Caenorhabditis elegans*. A proteomic analysis of purified mammalian midbodies revealed that approximately a quarter of the midbody components are Golgi-derived proteins; RNA interference (RNAi) experiments showed that the homologues of many of these proteins are required for *C. elegans* cytokinesis (Skop *et al.*, 2004).

Successful cytokinesis also relies on the endocytic pathway. Cleavage furrow-specific endocytosis has been observed in zebra fish embryos from early to late stages of cytokinesis (Feng *et al.*, 2002). In HeLa cells, endocytic ves-

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icles internalized from the polar regions during anaphase are subsequently trafficked to the midbody where they are thought to contribute to completion of cytokinesis (Schweitzer *et al.*, 2005). Clathrin and dynamin, two proteins that promote endocytic vesicle budding from the plasma membrane have been implicated in cytokinesis in several systems, including *Dictyostelium discoideum*, *C. elegans*, zebra fish, and mammalian cells (Niswonger and O'Halloran, 1997; Gerald *et al.*, 2001; Thompson *et al.*, 2002; Schweitzer *et al.*, 2005). There is also evidence that *Drosophila* dynamin encoded by the *shibire* gene is required for cellularization (Swanson and Poodry, 1980; Pelissier *et al.*, 2003). Furthermore, recent studies have shown that Rab35, which is enriched at endocytic clathrin-coated pits and vesicles, is essential for cytokinesis in both *Drosophila* and human cells (Kouranti *et al.*, 2006). Another protein involved in the endocytic pathway and required for cytokinesis is Rab11, a small GTPase that regulates membrane trafficking through the RE (Ullrich *et al.*, 1996; Matheson *et al.*, 2005). An involvement of Rab11 in cytokinesis was first demonstrated in *C. elegans* (Skop *et al.*, 2001). Subsequent studies showed that Rab11 and its interacting partner Nuclear-fallout (Nuf) are both required for cellularization of *Drosophila* embryos (Pelissier *et al.*, 2003; Riggs *et al.*, 2003). In mammalian cells, Rab11 and its binding partners FIP3/Arf1 and FIP4/Arf2, which share homology with Nuf, mediate the delivery of endosomes to the cleavage furrow and they are essential for completion of cytokinesis (Fielding *et al.*, 2005; Wilson *et al.*, 2005). These results suggest that Rab11 and Nuf/FIP3/FIP4 work in concert to play a conserved function required for cytokinesis.

Targeted vesicle fusion at the advancing cytokinetic furrow is likely to be regulated by the unique lipid composition of the cleavage furrow membrane. The outer leaflet of the plasma membrane at the equator of dividing mammalian cells is enriched in phosphatidylethanolamine (Emoto and Umeda, 2000). In sea urchin embryos, the equatorial membrane domain is enriched in ganglioside G_{M1} and cholesterol (Ng *et al.*, 2005). Sterol-rich membrane domains essential for cytokinesis have been also found at the *Schizosaccharomyces pombe* cleavage site (Wachtler *et al.*, 2003; Takeda *et al.*, 2004). Another relevant component of the furrow membrane is phosphatidylinositol 4,5 biphosphate [PtdIns(4,5)P₂], which is synthesized from phosphatidylinositol (PtdIns) molecules through sequential phosphorylation events mediated by PtdIns-4-kinases (Fwd in *Drosophila*) and PtdIns(4)P-5-kinases. Recent work has shown that at least in some species PtdIns(4,5)P₂ and the kinases involved in its generation are enriched at the furrow membrane and required for cytokinesis (Emoto *et al.*, 2005; Field *et al.*, 2005; Wong *et al.*, 2005; for review, see Logan and Mandato, 2006).

Here, we have analyzed the role of Rab11 during cytokinesis of *Drosophila* spermatocytes. We show that Rab11 is enriched at the equator of ana-telophase cells and required for cleavage furrow ingression. Our results strongly suggest that Rab11 mediates membrane addition at the advancing furrow and that this process is essential for actin ring constriction. In addition, we show that Rab11 functions in a common pathway with Gio (phosphatidylinositol transfer protein; PTP) and Fwd (PtdIns 4-kinase) to control formation of new membrane during *Drosophila* cytokinesis.

MATERIALS AND METHODS

Fly Strains and Genetics

The P element insertion *rab11^{EP3017}* and the ethyl methanesulfonate (EMS)-induced *rab11^{93Bi}* mutant alleles were obtained from the Bloomington Stock Center (Indiana University, Bloomington, IN). We sequenced the *rab11^{93Bi}*

mutant allele; it carries an Arg→Trp missense mutation at amino acid 104, a conserved residue between species. The EMS-induced *rab11^{ET011}* and *rab11^{ET03}* mutant alleles, and *Df(3R)e-R1* that removes *rab11⁺*, were kindly provided by M. Erdélyi (Hungarian Academy of Sciences Szeged, Hungary; Jankovics *et al.*, 2001). The strain expressing Rab11-green fluorescent protein (GFP) was a gift of R. S. Cohen (University of Kansas, Lawrence; Dollar *et al.*, 2002). The flies expressing β -tubulin-enhanced GFP (EGFP) were kindly provided by M. Savoian and D. Glover (University of Cambridge, UK; Inoue *et al.*, 2004). The *gio^{EP513}*, *fwd³*, and *fwd^{Z0453}* mutant alleles have been described previously (Brill *et al.*, 2000; Giansanti *et al.*, 2004, 2006). Previous studies have shown that the *fwd³* allele carries an early nonsense mutation, resulting in a truncated protein of 309 amino acids; the wild-type Fwd protein contain 1338 amino acids (Brill *et al.*, 2000). We have sequenced the *fwd^{Z0453}* mutant allele; it carries a Ser→Leu missense mutation at amino acid 1318, a conserved residue of the kinase catalytic domain (our unpublished results). In addition, we compared *fwd^{Z0453}/fwd^{Z0453}*, *fwd³/fwd^{Z0453}*, and *fwd^{Z0453}/Df(3L)7C* males for the frequencies of aberrant spermatids, and we found no significant differences. Thus, both the *fwd^{Z0453}* allele and the *fwd³/fwd^{Z0453}* heteroallelic combination are genetically null. Double mutants were generated by recombination by using standard methods. All mutations were maintained over the *TM6B* third chromosome balancer, and mutant larvae were identified based on their non-Tubby phenotype.

The strain expressing GFP-protein disulfide isomerase (Pdi) (*P(PTT-GA)Pdi^{G00198}*) was obtained from the Bloomington Stock Center. The flies of this strain contain a GFP sequence inserted into the single intron of the protein disulfide isomerase-coding gene (CG6988), and they express a GFP-Pdi chimera that can be detected by fluorescence microscopy (Bobinnec *et al.*, 2003). Genetic markers and special chromosomes are described in FlyBase (<http://www.flybase.org/>).

Immunostaining and Microscopy

Spermatid morphology in live material was examined in testes from third instars as described by Giansanti *et al.* (2004). Fixed cytological preparations were made with third instar larvae testes, which were dissected in testis buffer (183 mM KCl, 47 mM NaCl, and 10 mM Tris-HCl, pH 6.8), gently squashed in the same buffer, and frozen in liquid nitrogen. After removal of the coverslip, preparations were fixed with either of the following procedures. For visualization of the Rab11-GFP fluorescence, simultaneous detection of Rab11-GFP and Lva or Dsas-4 immunostaining, and actin and tubulin double staining, testes were fixed with methanol-free formaldehyde (Polysciences, Warrington, PA) as described by Gunsalus *et al.*, (1995). However, this fixation procedure does not work well for immunostaining with anti-Rab11 antibodies. Thus, for all the other immunostaining procedures with multiple antibodies, testes were fixed with 3.7% formaldehyde (containing 10% methanol) in 1× phosphate-buffered saline (PBS) and then squashed in 60% acetic acid according to Giansanti *et al.* (1999). This procedure, which does not preserve GFP fluorescence, is henceforth referred to as methanol/formaldehyde fixation. After this type of fixation, preparations were generally incubated with PBT (1× PBS containing 0.1% Triton-X 100) for 30 min. However, in double immunostaining experiments for Rab11 and anillin, Rab11 and Nuf, Rab11 and Gio and for staining with fluorescent wheat germ agglutinin (WGA), preparations were treated with PBT for a maximum of 5 min. The short incubation in PBT helps to visualize the plasma membrane-associated proteins but not the structures in the interior of the cell, such as the Golgi-derived fragments.

For immunostaining with rabbit and rat primary antibodies, testis preparations were incubated overnight at 4°C with the antibodies diluted in 1× PBS. For tubulin immunostaining, testis preparations were incubated for 1 h at room temperature with an anti- α tubulin monoclonal (Sigma-Aldrich, St. Louis, MO) diluted in 1× PBS. The following dilutions were used: anti- α tubulin (Sigma-Aldrich), 1:300; anti-anillin (gift from C. Field, Harvard University, Boston, MA; Field and Alberts, 1995), 1:200; anti-Lva (gift from J. Sisson, University of Texas, Austin; Sisson *et al.*, 2000), 1:500; anti-Giotto (Giansanti *et al.*, 2006), 1:2000; anti-Nuf (gift from W. Sullivan, University of California, Santa Cruz; Rothwell *et al.*, 1998), 1:200; rabbit anti-Cnn (gift from T. Megraw, University of Texas, Dallas; Megraw *et al.*, 2001), 1:300; rabbit anti-Dsas-4 (gift from R. Basto and J. Raff, Gurdon Institute, Cambridge, UK; Basto *et al.*, 2006), 1:300; rabbit anti-Rab11 (gift from M. Gaitan, Max-Planck Institute, Dresden, Germany; Emery *et al.*, 2005), 1:25; and rat anti-Rab11 (gift from R. S. Cohen, University of Kansas, Lawrence; Dollar *et al.*, 2002), 1:300. Primary antibodies were detected by incubation for 1 h at room temperature with secondary antibodies obtained by Jackson ImmunoResearch Laboratories (West Grove, PA), diluted according to the supplier's instructions. F-actin was stained with rhodamine-labeled phalloidin (Invitrogen, Carlsbad, CA) diluted 1:2 in 1× PBS (Gunsalus *et al.*, 1995). For staining with fluorescein-conjugated WGA (Invitrogen) spermatocytes immunostained with anti-Rab11 antibodies were incubated for 2 h at room temperature with fluorescent WGA diluted 1:10 in PBS. In all cases, slides were mounted in Vectashield medium H-1200 with 4,6 diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA) to stain DNA and reduce fluorescence fading. It should be noted that the rat and rabbit anti-Rab11 antibodies produced identical staining patterns in *Drosophila* testes (data not shown). Thus, in double immunofluorescence experiments, the choice of the anti-Rab11 antibody depended on the other primary antibody used for immunostaining.

Figure 1. Rab11 dynamic behavior during the first meiotic division of *Drosophila* spermatocytes. (A–C) Selected frames from three time lapses of primary spermatocytes expressing Rab11-GFP. The numbers at the bottom of each frame indicate the minutes elapsed from the beginning of imaging. (A) Frames from Supplemental Movie S1 showing a spermatocyte undergoing prophase (0), prometaphase (12, 18) metaphase (24), and anaphase (32). Note the Rab11 concentration at the Golgi stacks and at the nuclear envelope from prophase to metaphase, and its subsequent accumulation at the cell poles and the spindle envelope. (B) Frames from Supplemental Movie S2 showing a spermatocyte from early telophase (0) through mid- (6, 9) and late telophase (17); Rab11 starts to accumulate to the cleavage furrow during mid-telophase (6, arrowhead). (C) Frames from Supplemental Movie S3 showing a spermatocyte undergoing telophase. At mid-telophase, Rab11 starts to concentrate at the cell equator (2). During mid/late telophase Rab11-containing vesicles (arrow) travel from the poles to the middle of the cell and seem to reach the Rab11-enriched equatorial band (7–9). (D) Prophase (Pro), metaphase (Meta), anaphase (Ana) mid-telophase (Midtelo), and late telophase (Late telo) figures from primary spermatocytes expressing GFP-Pdi. Note that Pdi localizes to the cell

poles and the spindle envelope like Rab11. However, Pdi does not concentrate at the Golgi stacks of prophase spermatocytes and at the equatorial region of telophase figures. Bars, 10 μ m. (E–G) Diagrams of primary spermatocytes undergoing metaphase (E), early telophase (F), and late telophase (G) based on electron microscopy studies by A. D. Bates (Bates, 1971; also see Fuller, 1993) (printed with permission of the author). The diagrams show an entire metaphase cell (E) and only one of the two presumptive daughter cells that comprise a telophase figure (F and G). Major structures include the centrioles (C), astral membranes (AM), parafusorial membranes (P), and mitochondria (M). I indicates the cleavage site. Note the absence of parafusorial membranes in late telophase cells.

Images were captured using a CoolSnap HQ charge-coupled device camera (Photometrics; Tucson, AZ) connected to a Zeiss Axioplan fluorescence microscope equipped with an HBO 100-W mercury lamp as described previously (Giansanti *et al.*, 2004, 2006). Gray scale digital images were collected separately, converted to Photoshop format, pseudocolored, and merged.

Time-Lapse Imaging

Time-lapse imaging of living spermatocytes was carried out according to the protocol described by Inoue *et al.* (2004). Testes isolated from third instars were dissected under 10S voltalef oil (Elf Atochem North America, Inc., Philadelphia, PA) onto a clean coverslip attached to the underside of an aluminum slide. In most cases (except Supplemental Movie S4 generated using confocal microscopy), cells were examined with a Zeiss Axiovert 20 microscope equipped with a 63 $\times$ objective and a filter wheel combination (Chroma Technology, Brattleboro, VT). Images were acquired with a CoolSnap HQ camera (Photometrics) by using a 2 $\times$ 2 bin. Image acquisition was controlled through a MetaMorph software package (Molecular Devices, Sunnyvale, CA). Images were collected at one-minute intervals; 10 (for β -tubulin-EGFP), or 14 (for Rab11-GFP) fluorescence optical sections were captured at 1- μ m z-steps. Movies were created using the MetaMorph software; each fluorescent image shown is the maximum-intensity projection of all the sections.

Confocal time lapse imaging (Supplemental Movie S4 and Figure 2A) was carried out using a BD CarvII spinning disk confocal system (BD Biosciences, Rockville, MD) attached to a Nikon Eclipse TE 2000 S inverted microscope, using a 63 $\times$ lens and 2 $\times$ 2 bin. The movie was created with the MetaMorph software using maximum-intensity projections of z-series recorded at 1-min intervals.

RESULTS

The Dynamic Behavior of Rab11 during *Drosophila* Male Meiosis

To determine Rab11 behavior during male meiosis, we examined spermatocytes expressing Rab11-GFP. These cells were obtained from flies bearing one copy of a *rab11-GFP*

transgene with an endogenous *rab11* promoter (Dollar *et al.*, 2002) and two copies of the wild-type *rab11* gene. This *rab11-GFP* transgene rescues the lethality and the meiotic phenotype associated with *rab11* mutations (see below), indicating that the Rab11-GFP protein is functional.

To avoid possible GFP photobleaching problems, we imaged spermatocytes from late prophase to mid-anaphase and from mid-anaphase to the end of cell division (Figure 1, A–C, and Supplemental Movies S1–S3). Time-lapse imaging revealed a highly dynamic behavior of the protein. During late prophase and prometaphase I, Rab11 is in part diffuse in the cytoplasm and in part concentrated at both the Golgi stacks and the nuclear envelope (Figure 1A; see below for Golgi stacks identification). At prometaphase/metaphase I, the cytoplasmic pool of Rab11 and the Golgi stacks progressively concentrate at the cell poles. When the Golgi stacks disassemble at metaphase (Farkas *et al.*, 2003; Giansanti *et al.*, 2006), the resulting Rab11-containing breakdown products accumulate at the cell poles, which remain enriched in Rab11 throughout anaphase and telophase (Figure 1, A–C, and Supplemental Movies S1–S3). At early telophase I, Rab11 starts to concentrate at the cleavage furrow (Figure 1, B and C, and Supplemental Movies S2 and S3). This process does not seem to be mediated by vesicle fusion events at the furrow membrane, because we do not see evident Rab11-enriched vesicles moving toward the cell equator at this stage of meiotic division. However, a clear movement of Rab11-containing vesicles does occur in mid/late telophase (Figure 1C and Supplemental Movie S3). We followed the movement of 50 of these vesicles; 12% of them displayed a poleward or an oscillatory movement, whereas the remain-

Figure 2. Rab11 localization in ana-telophase primary spermatocytes. (A) Selected frames from confocal images (Supplemental Movie S4) of primary spermatocytes expressing Rab11-GFP. The numbers at the bottom of each frame indicate the minutes elapsed from the beginning of imaging. Note that Rab11-GFP accumulates at both the spindle envelope (in all cells) and the equatorial plasma membrane (in 2 cells; arrows). (B) Oregon-R (wild-type control) and Rab11-GFP-expressing spermatocytes fixed with methanol/formaldehyde and immunostained for Rab11, anillin (red), and centrosomin (blue). (C) An Oregon-R spermatocyte fixed with methanol/formaldehyde and stained with anti-Rab11 antibodies and fluorescent WGA (red). Note that Rab11 colocalizes with both WGA and anillin at the cell equator. (D–F) spermatocytes expressing Rab11-GFP (D and E) and Oregon-R spermatocytes (F) stained for tubulin (green) and DNA (blue). Rab11-GFP-expressing spermatocytes were either fixed with methanol-free formaldehyde (D) and examined for GFP fluorescence (red), or they were fixed with methanol/formaldehyde (E) and immunostained for Rab11 (red). (F) Oregon-R spermatocytes fixed with methanol/formaldehyde and immunostained for Rab11 (red). Note that the Rab11-GFP signals are fully comparable to the signals produced by Rab11 immunostaining. Bars, 10 μm.

ing 88% traveled from the poles to the cell equator. We speculate that the latter vesicles are transported along microtubule tracks to the cleavage site, where they fuse with the furrow membrane, resulting in both membrane addition and further local enrichment in Rab11.

In addition to the cleavage site and the spindle poles, Rab11 also accumulates at the “spindle envelope” (Figure 1A; also see Figure 2A, Supplemental Figure S1, and Supplemental Movie S4), an elliptical structure that encircles the meiotic chromosomes from metaphase to mid-telophase and disintegrates during late telophase (Tates, 1971; Fuller, 1993; Wolf, 1995; Inoue *et al.*, 2004; Giansanti *et al.*, 2006). According to Tates’ ultrastructural analysis, the spindle envelope consists of a series of parafusorial membranes that surround the chromosomes (Figure 1, E–G). He also suggested that these parafusorial membranes and the so-called astral membranes at the cell poles correspond to the spermatocyte ER (Figure 1, E–G; Tates, 1971; Fuller, 1993). Thus, Rab11 localization in dividing spermatocytes seems to overlap the distribution of the ER membranes. Consistent with this idea, most spermatocyte structures enriched in Rab11 also express the ER marker Pdi fused with GFP (Figure 1D; Bobinnec *et al.*, 2003; Giansanti *et al.*, 2006). However, there are some interesting differences between Rab11 and Pdi localization. In prophase spermatocytes, GFP-Pdi does not concentrate at the Golgi stacks but decorates a typical ER network (Figure

1, A and D). In metaphase and ana-telophase cells, GFP-Pdi accumulates at the cells poles and the spindle envelope like Rab11, but it fails to concentrate at the cell equator during mid-telophase (Figure 1, C and D). In late telophases, whereas the Rab11 signal extends across the entire equatorial region of the cells, a weak GFP-Pdi signal is limited to the inner part of this region (Figure 1, C and D; also see Giansanti *et al.*, 2006).

To confirm that Rab11 accumulates at both the spindle envelope and the equatorial plasma membrane, we imaged Rab11-GFP-expressing spermatocytes by using confocal microscopy. As shown in Figure 2A and Supplemental Movie S4, Rab11 is enriched at the spindle envelope from metaphase to early telophase. However, at late anaphase the protein starts to concentrate at the equatorial region of the cell and it seems to localize at the plasma membrane. It should be noted that at this stage of meiotic division, the central spindle lies both outside and inside the spindle envelope and it exhibits little or no pinching in its midzone (Supplemental Figure 1). When the cleavage furrow constricts and the spindle envelope disassembles, Rab11 concentration at the cleavage site increases and its signal extends across the entire equatorial region of the cell (Figure 1).

To demonstrate that Rab11 colocalizes with the furrow membrane, we stained dividing spermatocytes with anti-Rab11 antibodies and either anti-anillin antibodies or fluo-

rescent WGA. Anillin is a well-known contractile ring component that binds both actin and myosin, and interacts with the plasma membrane (Field and Alberts, 1995; Field *et al.*, 2005a); WGA is a good marker for equatorial plasma membrane (Ng *et al.*, 2005). We found that both anillin and WGA colocalize with Rab11 at the equator of telophases (Figure 2, B and C), supporting the conclusion that Rab11 is enriched at the furrow membrane. In summary, Rab11 is concentrated in the Golgi stacks and the nuclear envelope during prophase/prometaphase, it becomes enriched at the ER compartment and at some, but not all (see below), Golgi-derived vesicles during metaphase and ana-telophase, and accumulates at the cleavage furrow of telophase cells.

Our initial experiments with anti-Rab11 antibodies showed that fixation with methanol-free formaldehyde, which preserves GFP fluorescence, does not work well for Rab11 immunostaining. Conversely, the methanol/formaldehyde fixation, which allows efficient immunostaining with anti-Rab11 antibodies, disrupts GFP fluorescence. Thus, we wondered whether antibody staining and direct observation of Rab11-GFP result in identical localization patterns. To address this question, we examined the staining patterns of Rab11-GFP-expressing spermatocytes that were either fixed with methanol-free formaldehyde and scored for GFP fluorescence, or fixed with methanol/formaldehyde and immunostained for Rab11. These patterns were fully comparable and very similar to the staining pattern of wild-type spermatocytes immunostained with anti-Rab11 antibodies after methanol/formaldehyde fixation (Figure 2, D–F).

We next examined the Rab11 localization pattern in fixed spermatocytes and compared it with that of Lva, a Golgi marker that shares homology with mammalian golgins (Sisson *et al.*, 2000). Testes expressing Rab11-GFP were fixed with methanol-free formaldehyde, and then they were immunostained for Lva. As shown in Figure 3A, Lva and Rab11 are both enriched at the Golgi stacks of prophase primary spermatocytes. However, although Rab11 and Lva largely colocalize, their staining patterns do not completely overlap, suggesting a differential accumulation of the proteins within subcompartments of the Golgi apparatus (Figure 3A). Rab11-GFP localization in fixed metaphase and ana-telophase figures does not coincide with Lva distribution. In metaphase and early anaphase, Rab11 is concentrated at the polar regions of the cell and at the spindle envelope, whereas most Lva-enriched vesicles are just outside these regions (Figure 3B). In telophase figures, Rab11 is still concentrated at the cell poles, but it is also enriched at vesicles that are distant from the poles. Cytological analysis of ana-telophases revealed three types of Golgi breakdown products: those that contain either Rab11 or Lva and those that contain both proteins. In addition, this analysis showed that Rab11 is highly enriched at cleavage furrow, whereas Lva is excluded from this site (Figure 3C; data not shown).

To determine the relationship between Rab11 distribution and centrioles localization, Rab11-GFP-expressing testes fixed with methanol-free formaldehyde were immunostained for DSas-4, a marker for *Drosophila* centrioles (Basto *et al.*, 2006). We found that Rab11 does not concentrate around the centrioles throughout the spermatocyte growth phase and the meiotic division (Figure 3D; data not shown). These observations indicate that in *Drosophila* spermatocytes Rab11 is not enriched at a pericentriolar recycling endosome, but it is instead concentrated in the Golgi units.

To further characterize the subcellular distribution of Rab11, fixed spermatocytes were immunostained for Rab11 and either Nuf or Giotto. Rab11 and Nuf colocalized in the Golgi stacks of prophase and prometaphase spermatocytes

Figure 3. Rab11-GFP localization in fixed *Drosophila* spermatocytes stained for either Lva or DSas-4. (A–C) Primary spermatocytes expressing Rab11-GFP stained for Lva. In prophase cells (A), Rab11 (green) largely colocalizes with Lva (red) in the Golgi stacks; the dark areas correspond to the nuclei. In metaphase (B) and telophase (C) cells, Rab11 and Lva colocalize only in a fraction of the Golgi-derived vesicles. (D) An early prophase primary spermatocyte expressing Rab11-GFP (green) immunostained for DSas-4 (red; arrow). Note that Rab11 does not accumulate around the centrioles. Bars, 10 μ m.

(data not shown) and showed very similar distributions in cells undergoing metaphase and ana-telophase (Figure 4, A and B; data not shown), consistent with the idea that Rab11 and Nuf function as a complex (Riggs *et al.*, 2003). Gio and Rab11 displayed comparable subcellular localizations in ana-telophase spermatocytes (Figure 4, C and D), consistent with our previous observations on Gio distribution (Giansanti *et al.*, 2006).

rab11 Is Required for Spermatocyte Cytokinesis

The *rab11* gene is identified by several mutations. Homozygotes for most of these mutations die in early stages of development and are not amenable for cytological analysis of spermatocytes. However, some *rab11* mutations are semi-

Figure 4. Localization Rab11, Nuf and Gio in *Drosophila* primary spermatocytes. (A–D) Wild-type primary spermatocytes in early anaphase (A and C) and telophase (B and D) stained for Rab11 (green) and either Nuf (red) or Gio (red). Note that Rab11 colocalizes with both Nuf and Gio at the cell equator. Bar, 10 μ m.

lethal and produce a few escapers; male escapers are invariably sterile (Figure 5A). Examination of testes from *rab11^{93Bi}/rab11^{93Bi}* larvae, or from larvae carrying the *rab11^{93Bi}* allele in combination with either *rab11^{EP3017}*, *rab11^{E(To)3}*, or *rab11^{E(To)11}*, revealed the presence of multinucleated spermatids, a phenotype diagnostic of failures in meiotic cytokinesis (Figure 5, B–F). In wild type, each onion stage spermatid comprises a phase-clear nucleus and a phase-dense mitochondrial derivative of similar size called the nebenkern (Figure 5B). Failure of cytokinesis abrogates proper mitochondria partitioning between the daughter cells, resulting in spermatids composed of an abnormally large nebenkern associated with multiple nuclei of regular size (Figure 5, C–E; Fuller, 1993). The highest frequencies of abnormal spermatids were observed in testes from *rab11^{93Bi}/rab11^{E(To)3}* and *rab11^{93Bi}/rab11^{E(To)11}* third instars, which displayed 27 and 42% multinucleated spermatids, respectively (Figure 5F). Flies bearing a *rab11*-GFP transgene and either heteroallelic mutant combination were fully viable, and they did not exhibit abnormal spermatids, indicating that the product of this transgene has the same activity of the wild type Rab11 protein.

To determine the primary defect leading to the formation of aberrant spermatids we focused on *rab11^{93Bi}/rab11^{E(To)11}*

Figure 5. *rab11* mutations disrupt spermatocyte cytokinesis. (A) Complementation analysis among *rab11* mutant alleles. V, viable; SL, semilethal; EL, early lethal; and DS, defective spermatids. (B–E) Abnormal spermatids observed in living testes of *rab11* mutant. (B) Wild-type spermatids with nuclei (n) and nebenkern (nk) of similar sizes. (C–E) multinucleated spermatids from *rab11* males containing two (C), three (D), or four nuclei (E) of similar sizes associated with a single large nebenkern. Bar, 10 μ m. (F) Frequencies (% $\pm$ SE) of spermatids with 2 (2nu, gray), 3 (3nu, black), and 4 (4nu, dark gray) nuclei observed in *rab11* mutant males. Mutant males also exhibit very low frequencies of multinucleated spermatids with differently sized nuclei (ir, empty columns). In wild type, the frequency of abnormal spermatids is virtually zero.

mutants. Primary spermatocytes stained for DNA, tubulin and F-actin were examined for both spindle and actin ring organization; early, mid-, and late telophases were identified based on spindle morphology and the degree of separation of the sister centrioles associated with the daughter nuclei (Giansanti *et al.*, 2004). In *rab11^{93Bi}/rab11^{E(To)11}* mutant spermatocytes, early telophases are completely normal and they exhibit regular actin rings and central spindles ($n = 28$; data not shown). However, mid- and late telophases ($n = 49$) displayed actin rings that were not fully constricted (18%; Figure 6B) or poorly constricted and broken into irregular pieces (33%; Figure 6C). Moreover, in four of the seven

Figure 6. *rab11* mutations affect ring constriction (A–D) Primary spermatocyte telophases stained for tubulin (green), actin (red) and DNA (blue). (A) Wild-type late telophase with a constricted actin ring. (B–D) Mutant late telophases displaying an incompletely constricted ring (B), a broken ring (C), or a fully constricted but abnormally thick ring (D). (E and F) Primary spermatocyte late telophases stained for tubulin (green), anillin (red), and DNA (blue). (E) Wild-type telophase with a fully constricted ring. (F) Mutant telophase with an incompletely constricted ring and a defective central spindle. Bars, 10 μ m.

mutant telophases displaying a high degree of ring constriction, the F-actin rings were morphologically abnormal and seemed thicker than in wild-type telophases at the same stage (Figure 6D). In ~80% of telophases with unconstricted or broken actin rings the central spindles were less dense than in wild type. However, central spindles of mutant telophases with normally constricted actin rings were morphologically normal. Similar observations were made on *rab11* mutant testes stained for anillin, an actin-binding protein that colocalizes with the contractile ring (Field and Alberts, 1995; Giansanti *et al.*, 1999). 62% ($n = 63$) of *rab11^{93Bi}/rab11^{E(To)11}* late telophases contained poorly constricted anillin rings and defective central spindles (Figure 6F).

Collectively, these observations strongly suggest that *rab11* spermatocytes are primarily defective in ring constriction and furrow ingression. A similar cytokinetic phenotype has been previously observed in eight different mutants, including *gio*, *fvd*, and *fvs* (Brill *et al.*, 2000; Farkas *et al.*, 2003; Giansanti *et al.*, 2004, 2006). In all these mutants, the degree of central spindle abnormality correlated with the degree of actin ring defect (Giansanti *et al.*, 2006), supporting the view that the central spindle and the contractile ring are mutually dependent structures (Giansanti *et al.*, 1998). To further substantiate this hypothesis, we analyzed living testes of *rab11^{93Bi}/rab11^{E(To)11}* mutants expressing EGFP-tagged β -tubulin. In *rab11* primary spermatocytes that successfully completed cytokinesis ($n = 4$), the central spindle structure and behavior were indistinguishable from wild type ($n = 8$) (Figure 7, A and B, and Supplemental Movies S5 and S6). In mutant primary spermatocytes that failed to complete cytokinesis ($n = 7$), the furrow ingressed to a limited extent and

Figure 7. Spindle formation and behavior during the first meiotic division in *rab11^{93Bi}/rab11^{E(To)11}* males expressing β -tubulin-EGFP. Time, in minutes, is relative to the initiation of cleavage furrow ingression (0). In the wild-type cell (A; Supplemental Movie S5) the central spindle midzone reaches a maximum compaction at 30 min (arrow). In the mutant cell (B; Supplemental Movie S6) that undergoes a successful cytokinesis the maximum compaction of the central spindle midzone (arrow) is the same as in the wild-type telophases and occurs at 33 min. In the mutant cell (C; Supplemental Movie S7), furrowing halts at 15 min; at this time, the central spindle midzone still exhibits a regular compaction, but it decreases in microtubule density during furrow regression; at 39 min, the central spindle has completely disappeared. Bar, 10 μ m.

then regressed. In these cells, the central spindle was normal until the furrow advanced, but it started to disassemble when the furrow began to regress (Figure 7C and Supplemental Movie S7). These findings suggest that the stability of central spindle microtubules requires continuous interactions with elements of the contractile ring (Giansanti *et al.*, 1998, 2004).

To further characterize the cytokinetic phenotype of *rab11* mutant spermatocytes, we examined the Golgi behavior in cells stained for Lva, tubulin, and DNA. Wild-type premeiotic spermatocytes contain multiple Golgi units; we call them Golgi stacks because electron microscopy showed that

Figure 8. Mutations in *rab11* affect Golgi-derived vesicle behavior during primary spermatocyte telophase. Wild-type (A, C, and E) and *rab11* mutant (B, D, and F) spermatocytes undergoing prophase (A and B), anaphase (C and D), and late telophase (E and F) were stained for Lva (red), tubulin (green), and DNA (blue). Note that *rab11* mutations only affect vesicle localization in telophase cells; vesicles are excluded from the cell equator in wild-type spermatocytes (E) but they accumulate at the center of *rab11* mutant cells (F). (G and H) Wild-type (G) and *rab11*^{93Bi}/*rab11*^{E(To)11} mutant (H) telophases immunostained with anti-Rab11 and anti-Lva (red) antibodies. Note that the mutant protein produced by the *rab11*^{93Bi} missense mutation is detected by anti-Rab11 antibodies, and it is enriched at structures that are partially coincident with the Lva-positive vesicles accumulated at the cell equator. Bars, 10 μ m.

each unit is a stack of Golgi cisternae (Figure 8A; Bates, 1971; Fuller 1993; Farkas *et al.*, 2003). During metaphase I, the Golgi stacks breakdown into smaller structures that we call Golgi-derived vesicles. These Golgi breakdown products do not reassemble into large Golgi units (similar to those of premeiotic spermatocytes) throughout meiotic division (Figure 8, C and E; data not shown). However, at the end of meiosis II, the Golgi-derived vesicles coalesce and fuse to form the acroblast (Bates, 1971; Fuller 1993; Farkas *et al.*, 2003; Giansanti *et al.*, 2006; see below). Mutations in *rab11* did not affect the number and the morphology of Golgi stacks in premeiotic spermatocytes (Figure 8B). At metaphase, the Golgi stacks of *rab11* mutant cells fragmented into small structures just as in wild-type cells. *rab11* primary spermatocytes undergoing anaphase were not substantially different from their wild-type counterparts in the distribution of the Golgi-derived vesicles (Figure 8D). However, vesicle distribution was affected in mutant spermatocytes undergoing telophase. In 96% ($n = 73$) of wild-type telophases, Lva-positive vesicles were concentrated near the poles and excluded from the central region of the cell (Figure 8E). In contrast, 48% of *rab11*^{93Bi}/*rab11*^{E(To)11} telophases ($n = 65$) displayed an abnormal localization of vesicle-like structures at the cell equator (Figure 8F). Accumulation of Golgi fragments at the equator of telophase figures has been previously observed in *gio* and *fwd* mutants, and it is not a general consequence of cytokinesis failures. The most likely explanation for this phenotype is that vesicles accumulate in the middle of the cell because they are unable to fuse with the furrow membrane (Giansanti *et al.*, 2006).

The *rab11*^{93Bi} missense mutation produces a protein that can be detected by antibody staining. This gave us the opportunity to ask whether this protein associates with the Golgi-derived vesicles that accumulate the equator of *rab11* mutant spermatocytes. Thus, we immunostained *rab11*^{E(To)11}/*rab11*^{93Bi} mutant testes with both anti-Rab11 and anti-Lva antibodies. Although the Rab11 signal was fainter than in wild-type spermatocytes (Figure 8, G and H), we were able to see that many of the Lva-positive vesicles near the cleavage furrow coincide with Rab11-positive structures (Figure 8H). This finding suggests that the Golgi-derived fragments that fail to fuse with the equatorial membrane are those enriched in Rab11.

We also examined larval brain squash preparations from *rab11* mutants for the presence of polyploid cells. Highly polyploid mitotic figures are commonly observed in mu-

tants defective in mitotic cytokinesis, such as those in the *citron kinase*, *diaphanous*, or *twinstar* genes (Castrillon and Wasserman, 1994; Gunsalus *et al.*, 1995; D'Avino *et al.*, 2004; Naim *et al.*, 2004; Shandala *et al.*, 2004). However, *rab11*^{93Bi}/*rab11*^{E(To)3} and *rab11*^{93Bi}/*rab11*^{E(To)11} mutant brains did not exhibit polyploid mitoses ($n = 500$), suggesting that Rab11 is either not required for cytokinesis or residual Rab11 activity is sufficient to mediate cytokinesis in these cells.

Functional Relationships among Rab11, Gio, and Fwd

Mutations in *rab11* cause cytokinesis defects that are virtually identical to those observed in *fwd* and *gio* mutants (Brill *et al.*, 2000; Giansanti *et al.*, 2004, 2006; Gatt and Glover, 2006). These defects include incomplete constriction of the actin and anillin rings, central spindle disorganization in late telophases, and accumulation of Golgi-derived vesicle at the cell equator. This prompted us to construct and analyze *rab11 fwd*, *rab11 gio*, and *fwd gio* double mutants to ask whether the three genes function in the same pathway. Pathway analysis can be reliably performed when both mutations are null, or when one mutation is null and the other hypomorphic; the two genes belong to the same epistasis group if the phenotype of the double mutant is the same as the phenotype of the strongest mutant. When pathway analysis is performed with two hypomorphic mutations, there are two possibilities. If the double mutant has the same phenotype as the strongest hypomorphic mutant, then the genes are likely to be in the same epistasis group. If the double mutant has a phenotype stronger than either single mutant, it cannot be established whether the genes function in the same linear pathway or in two different pathways. For pathway analysis, we used the null *fwd*³/*fwd*^{Z0453} heteroallelic combination, the *gio*^{EP513} hypomorphic allele and the *rab11*^{93Bi}/*rab11*^{E(To)11} hypomorphic combination (see *Materials and Methods*). We found that in each double mutant the cytokinesis defect was qualitatively and quantitatively identical to that observed in animals homozygous for the stronger mutation used to construct the double mutant (Figure 9, A–C; data not shown). Specifically, we found that *fwd*³/*fwd*^{Z0453} single mutants are not significantly different from *rab11*^{93Bi} *fwd*³/*rab11*^{E(To)11} *fwd*^{Z0453} and *fwd*³ *gio*^{EP513}/*fwd*^{Z0453} *gio*^{EP513} double mutants for the frequency of aberrant spermatids (Figure 9, A and C). Comparable frequencies of aberrant spermatids were also observed in *gio*^{EP513}/*gio*^{EP513} single mutants and in *rab11*^{93Bi} *gio*^{EP513}/*rab11*^{E(To)11} *gio*^{EP513} double mutants (Figure 9B). Collectively, these results indicate

Figure 9. Functional relationships between Rab11, Nuf, Gio, and Fwd. (A–C) Frequencies (%) of multinucleated spermatids, containing two (2nu), three (3nu), four (4nu), or more than four (>4nu) nuclei, observed in *rab11^{93Bi}/rab11^{E(To)11}* and *fwd³/fwd^{Z0453}* single mutants and in *rab11^{93Bi} fwd³/rab11^{E(To)11} fwd^{Z0453}* double mutants (A); in *rab11^{93Bi}/rab11^{E(To)11}* and *gio^{EP315}/gio^{EP315}* single mutants and *rab11^{93Bi} gio^{EP313}/rab11^{E(To)11} gio^{EP313}* double mutants (B); and in *gio^{EP513}/gio^{EP513}* and *fwd³/fwd^{Z0453}* single mutants and in *fwd³ gio^{EP513}/fwd^{Z0453} gio^{EP513}* double mutants (C). (D) Rab11 fails to concentrate at both the cell poles and the cleavage furrow of primary spermatocyte telophases from *gio^{EP513}/gio^{EP513}* and *fwd³/Df(3L)7C* mutants. Telophases were also stained for Cnn (red) and DNA. Rab11 staining at the cell poles and the cleavage site was detected in all wild-type telophases (n = 128), none of the *fwd* mutant telophases (n = 56) and 4% of the *gio* mutant telophases (n = 83). (E) Gio localization in telophases I from *rab11^{93Bi}/rab11^{E(To)11}* mutants is the same as in wild type. The merges show only tubulin (green) and DNA staining (blue). Gio staining at the cell poles and the cleavage site was detected in all wild-type (n = 70) and *rab11* mutant (n = 71) telophases. (F) Nuf localization in wild-type and mutant telophases. In wild type telophases, Nuf concentrates at the cell poles and the cleavage site (we observed this staining pattern in all telophases examined; n = 50). This localization is disrupted in all the *rab11^{93Bi}/rab11^{E(To)11}* (n = 60), *fwd³/Df(3L)7C* (n = 36) and *gio^{EP513}/gio^{EP513}* (n = 38) mutant telophases. In the merges, Nuf is red, tubulin green and DNA blue. Bars, 10 μ m.

that *fwd*, *gio* and *rab11* belong the same epistasis group. This suggests that *fwd*, *gio*, and *rab11* function in the same linear

pathway controlling membrane addition at the cleavage furrow and ring constriction during spermatocyte cytokinesis.

To determine the hierarchy of *fwd*, *gio*, and *rab11* in the pathway, we immunostained *gio* and *fwd* mutants for Rab11 and Nuf, and *rab11* mutants for Gio and Nuf. In *fwd* and *gio* mutant ana-telophases, Rab11 staining at the cell poles and at the cleavage site were both abolished (Figure 9D). *fwd*, *gio*, and *rab11* mutant anatelephases failed to show localized Nuf signals after immunostaining for this protein (Figure 9F), confirming Nuf dependence on Rab11 (Riggs *et al.*, 2003). In contrast, the analysis of *fwd* and *rab11* anatelephases immunostained for Gio revealed a normal localization of this P1TP at both the cell poles and at the cleavage furrow (Figure 9E; Giansanti *et al.*, 2006). These results indicate that *rab11* is not required for Gio localization, whereas *gio* and *fwd* are essential for Rab11 localization. Thus, *gio* and *fwd* must be placed upstream of *rab11* in the cytokinetic pathway.

Rab11 Is Required for Acroblast Formation

Immunostaining of testis preparations revealed that Rab11 accumulates to a conical structure at the anterior side of spermatid nuclei, resembling the acroblast (Figure 10A). *Drosophila* acroblasts are Golgi-derived structures enriched in the Lva and Fws proteins (Farkas *et al.*, 2003; Giansanti *et al.*, 2006). Double immunostaining of wild type spermatids for either Rab11 and Lva or Rab11 and Nuf revealed exten-

sive colocalization of the three proteins, demonstrating that Rab11 and Nuf are both enriched at the acroblast (Figure 10, A and B).

To determine whether Rab11 is required for acroblast assembly, we immunostained wild-type and *rab11* mutant testes for Lva. In wild-type testes, both early (52/52) and elongating spermatids (70/70) displayed a compact acroblast (Figure 10C). In 11% of the early (round) spermatids ($n = 72$) from *rab11*^{93Bi}/*rab11*^{E(TO)11} mutants, Lva staining did not detect an organized acroblast but only multiple cytoplasmic vesicles. The same phenotype was seen in most elongating spermatids (97%; $n = 86$), which instead of an acroblast showed numerous vesicular structures dispersed along the developing sperm tail (Figure 10D). These observations indicate that Rab11 is required for acroblast assembly and maintenance during spermiogenesis. Failure in acroblast formation due to defective Golgi vesicle fusion has been previously observed in *Drosophila* males carrying mutations in the *gio* and *fws* genes and in mice lacking the Golgi-associated protein GOPC (Yao *et al.*, 2002). Collectively, these results indicate that acroblast assembly requires multiple functions involved in membrane trafficking.

DISCUSSION

Rab11 Localizes to Multiple Membrane Compartments of Dividing *Drosophila* Spermatocytes

Studies in mammalian cells have shown that during interphase Rab11 localizes to the Golgi apparatus, the Golgi-derived vesicles and the RE, suggesting an involvement of this GTPase in both the secretory and the endocytic pathway (Ullrich *et al.*, 1996; Chen *et al.*, 1998; for review, see Zerial and McBride, 2001). In addition, Rab11-enriched endosomes accumulate at the cleavage furrow of dividing mammalian cells and they are required for completion of cytokinesis (Horgan *et al.*, 2004; Fielding *et al.*, 2005; Wilson *et al.*, 2005). We have shown that Rab11 exhibits a highly dynamic behavior during *Drosophila* male meiosis. In prophase primary spermatocytes, Rab11 is enriched in a subcompartment of the Golgi stacks, which is likely to correspond to the *trans*-Golgi network (TGN). A preferential localization of Rab11 in this Golgi compartment is suggested by studies indicating that Rab11 associates with the TGN and TGN-derived vesicles in both mammalian cells and *Drosophila* photoreceptors (Urbe *et al.*, 1993; Chen *et al.*, 1998; Satoh *et al.*, 2005). When *Drosophila* spermatocytes enter the first meiotic division and the Golgi stacks disassemble, Rab11 concentrates at the ER and remains associated with this compartment throughout meiotic division. In addition, Rab11 concentrates in several vesicle-like structures that reside at the cell poles during anaphase and early telophase. During mid- and late telophase, these vesicles move toward the cell equator where they seem to fuse with the advancing cleavage furrow.

The precise origin of these Rab11-enriched vesicles is currently unclear. Specifically, it is not clear whether these vesicles are recycling endosomes. In prophase spermatocytes, Rab11 is not concentrated in pericentriolar RE, but it is enriched at the Golgi stacks. In dividing spermatocytes, pericentriolar regions are enriched in both Rab11 and Pdi and they do not appear as distinct REs. It is thus likely that *Drosophila* spermatocytes do not possess a canonical pericentriolar RE. Previous studies have shown that different *Drosophila* cell types differ markedly in the organization and function of Rab11-containing structures. In embryonic cells, Rab-11 is enriched at both the Golgi apparatus and a pericentriolar RE, and membrane traffic to the advancing cellu-

Figure 10. Mutations in *rab11* disrupt acroblast formation. (A) Wild-type spermatids expressing Rab11-GFP (green) stained for Lva (red). Note that the acroblasts are enriched in both Rab11 and Lva. (B) Rab11 (green) colocalizes with Nuf (red) in the acroblasts of early elongating spermatids. (C and D) Spermatids from wild-type (C) and *rab11* mutant (D) males stained for Lva (red), tubulin (green), and DNA (blue). Note that both round (left arrow) and elongating wild-type spermatids (right arrow) display compact acroblasts; elongating spermatids from *rab11* mutants do not exhibit an acroblast at the anterior side of the spermatid nuclei (arrowheads), but they show multiple Golgi vesicles dispersed along the growing sperm tails. Bar, 10 μ m.

larization furrow is mediated by this RE (Pelissier *et al.*, 2003; Riggs *et al.*, 2003). In contrast, in differentiating photoreceptor cells, which do not contain a pericentriolar RE, rhodopsin-containing vesicles move directly from the TGN to the apical membrane to form the rhabdomere (Satoh *et al.*, 2005). Studies on external sensory organ precursor (SOP) cells have shown that the formation of a pericentriolar RE is subject to a striking regulation. The SOP cells divide into a posterior pIIa cell and an anterior pIIb cell, which will give rise to the outer (hair and socket) and inner (neuron and sheath) sensory organ cells, respectively. During mitosis, Rab11 is equally distributed between the pIIa and pIIb daughter cells, but after completion of cytokinesis, only the pIIb cell forms a pericentriolar Rab11-enriched RE. In the pIIa cell, the formation of an RE is inhibited and Rab11 remains dispersed in the cytoplasm (Emery *et al.*, 2005). Collectively, these studies indicate that the formation of a pericentriolar RE is cell specific and tightly controlled. Thus, it is likely that *Drosophila* spermatocytes, like the photoreceptor and the SOP pIIb cells, do not possess a pericentriolar RE. This implies that the Rab11-enriched vesicles generated by mitotic fragmentation of the Golgi apparatus can traffic directly to the equatorial plasma membrane.

The Cytokinetic Phenotype of *rab11* Mutants

Our results have shown that *rab11* mutant spermatocytes display two main cytokinetic defects. First, they exhibit an abnormal accumulation of Lva-enriched vesicles at the equator of telophase cells. Second, although they form regular contractile rings, these rings fail to constrict properly, leading to cytokinesis failures.

Several studies suggest that the presence of Lva-containing vesicles at the cell equator reflects a defect in membrane-vesicle fusion at the cytokinetic furrow. In wild-type spermatocytes undergoing telophase, Lva-enriched vesicles are concentrated at the cell poles and they are excluded from the equatorial region. An aberrant localization of these vesicles near the cleavage furrow has been observed previously in males homozygous for *gio* and *fwd*, two genes that encode proteins involved in membrane traffic (Giansanti *et al.*, 2006). However, this vesicle phenotype is not a general feature of mutants that disrupt spermatocyte cytokinesis; Lva-positive vesicles were not observed at the equator of spermatocyte telophases from *fws* and *pebble* (*pbl*) mutants, which identify the Cog5 subunit of the conserved oligomeric Golgi complex and a Rho GEF, respectively (Farkas *et al.*, 2003; Giansanti *et al.*, 2006). There are several additional examples of abnormal vesicle accumulations caused by inhibition of functions required for vesicular traffic. For example, in Rab11-depleted *Drosophila* photoreceptor cells, rhodopsin-containing vesicles fail to fuse with the rhabdomere and accumulate in the cytoplasm (Satoh *et al.*, 2005). Vesicle accumulation near the cleavage site has been also observed after inactivation of the exocyst, a multiprotein complex that targets Golgi-derived vesicles to the plasma membrane (Hsu *et al.*, 2004). Exocyst disruption results in abnormal secretory vesicle clustering near the cleavage site in both *Saccharomyces cerevisiae* and *S. pombe* (Salminen and Novick, 1989; Wang *et al.*, 2002) and at the midbody of mammalian cells (Gromley *et al.*, 2005). Collectively, these results strongly suggest that in *rab11* mutant spermatocytes a fraction of the Lva-enriched vesicles fails to fuse with the invaginating furrow membrane, resulting in an abnormal accumulation of these structures at the cell equator.

We have shown that disassembly of the Golgi stacks generates Lva-positive vesicles that are either enriched in Rab11 or devoid of this GTPase. In addition, we have found that in *rab11* mutants that carry a missense mutation, the mutant

protein associates with many of the Lva-containing vesicles that accumulate at the telophase equator. These results lead us to suggest that in wild-type telophase spermatocytes Rab11-associated vesicles generated by Golgi disassembly move from either pole toward the cleavage site and fuse with the furrow, simultaneously delivering Rab11 and providing plasma membrane required for the completion of cytokinesis.

In addition to defects in membrane-vesicle fusion at the cleavage furrow, *rab11* mutant spermatocytes also exhibit an incomplete actin ring constriction. There is precedent indicating that proper membrane trafficking is essential for actomyosin ring remodeling during animal cell cytokinesis. In cellularizing *Drosophila* embryos, mutant for syntaxin1, a target membrane-SNARE involved vesicle targeting, all sections of plasma membrane lack detectable cortical actin and associated furrow canals (Burgess *et al.*, 1997). Consistent with these results, S2 cells depleted of syntaxin1 by RNAi fail to form a regular actin ring (Somma *et al.*, 2002). Similarly, *Dictyostelium* mutants that lack the vesicle-coating protein clathrin fail to assemble a robust actin ring during cytokinesis (Niswonger and O'Halloran, 1997). Furthermore, disruption of the GM1 and cholesterol-enriched domain at the equatorial membrane of sea urchin eggs does not affect actomyosin ring formation but blocks furrow ingression and completion of cytokinesis (Ng *et al.*, 2005). Finally, recent work has shown that PtdIns(4,5)P₂ mediates actomyosin ring formation and stabilization in several systems, including crane fly, *Drosophila* and mammalian cells (Saul *et al.*, 2004; Field *et al.*, 2005b; Wong *et al.*, 2005; for review, see Logan and Mandato). Collectively, these studies indicate that actomyosin ring formation and constriction during animal cell cytokinesis is intimately related with proper membrane traffic. However, the mechanisms underlying the interplay between the equatorial membrane and the contractile ring are still poorly understood. Specifically, it is not clear why failure of membrane-vesicle fusion at the cleavage furrow can block ring constriction in *Drosophila* spermatocytes. One possibility is that lack of vesicle addition to the advancing furrow results in membrane tension that counteracts ring constriction, ultimately leading to actin ring disassembly. Alternatively, the vesicles that normally fuse with the advancing cleavage furrow might include actin-remodeling factors essential for ring constriction and stability. A strong support for the latter alternative comes from recent studies on the control of ring constriction in budding yeast. In yeast exocyst mutants, the actin ring forms but fails to constrict properly and often disassembles, as occurs in *rab11* mutant spermatocytes. This phenotype has been attributed to a failure of vesicle-mediated delivery at the bud neck of Chs2, a chitin synthase that is thought to control both ring stability and septum formation (VerPlank and Li, 2005). Additional insight into the mechanisms underlying defective ring constriction in *rab11* mutant spermatocytes comes from our own phenotypic analysis. In mutant telophases with a high degree of ring constriction, the actin rings are often substantially thicker than in wild-type telophases at the same stage. This phenotype has been previously observed in *fwd* and *gio* mutants (Brill *et al.*, 2000; Giansanti *et al.*, 2004, 2006), and, in an exacerbated form, in mutants in the *twinstar* (*tsr*) gene, which encodes the actin-severing factor cofilin (Gunsalus *et al.*, 1995). Based on these results, we favor the hypothesis that the thick rings observed in *rab11* mutant telophases are caused by a failure of vesicle-mediated delivery of critical actin remodeling factors. However, we cannot exclude the possibility that mutant rings become thicker to overcome membrane tension.

Functional Relationships among *Gio*, *Fwd*, and *Rab11*

We have recently reported that the *Gio* PITP is enriched at the furrow membrane and that it is required for *Drosophila* cytokinesis (Giansanti *et al.*, 2006). Here, we have shown that the furrow membrane is also enriched in Rab11 and that Rab11 localization at the equatorial membrane requires the wild-type activity of both *gio* and *fwd*. In addition, we have shown that the wild-type functions of *gio*, *fwd*, and *rab11* are all required for membrane-vesicle fusion during cytokinesis, because mutations in these genes result in an abnormal accumulation of Golgi-derived vesicles at the equator of telophase cells (Giansanti *et al.*, 2006; this study). Finally, our results strongly suggest that *gio*, *fwd*, and *rab11* function in the same cytokinesis pathway. These observations suggest a model for the mechanisms underlying membrane addition to the cleavage furrow during spermatocyte cytokinesis. We propose that *Gio* mediates transfer of PtdIns monomers to the furrow membrane, causing a local enrichment in PtdIns molecules. The association of *Gio* with this membrane domain may facilitate recruitment of the PtdIns-4-kinase encoded by *fwd*, which would mediate phosphorylation of PtdIns to PtdIns(4)P, allowing their further phosphorylation to PtdIns(4,5)P₂. *Fwd* may also mediate Rab11 recruitment at the cleavage furrow, allowing targeted Rab11-dependent vesicle fusion events necessary for completion of cytokinesis. We realize that this is a rather speculative model. Its major drawback is that the subcellular localization and the molecular interactions of the *Drosophila* *Fwd* protein are currently unknown. However, studies in *S. pombe* have shown that one of the PtdIns-4-kinases present in this organism interacts with Cdc4p, a contractile ring protein essential for cytokinesis (Desautels *et al.*, 2001). This finding indicates that, at least in fission yeast, one of the PtdIns-4-kinases is associated with the cleavage furrow. In addition, a recent study has shown that one of the mammalian PtdIns-4-kinases interacts physically with Rab11 and is required for Rab11 localization in the Golgi complex. The same study has also shown that recruitment of this kinase to the Golgi does not require Rab11 (de Graaf *et al.*, 2004). These results are consistent with our findings, and they lead us to believe that *Gio*, *Fwd*, and *Rab11* are all enriched at cleavage furrow, where they work in concert to ensure proper vesicle docking and fusion.

Is *Rab11* Specifically Required for Meiotic Cytokinesis?

We have shown that mutations in *rab11* cause frequent failures in meiotic cytokinesis of males without affecting cytokinesis of larval brain neuroblasts. The mutations we analyzed are obviously hypomorphic as they cause lethality at the larval and pupal stages, whereas *rab11* null alleles result in embryonic lethality. Thus, it is possible that the *rab11* mutants we analyzed retain a residual Rab11 activity that is sufficient for neuroblast cytokinesis but not meiotic cytokinesis. Alternatively, Rab11 may not be required for mitotic cytokinesis. A strong support for a specific involvement of Rab11 in meiotic cytokinesis comes from recent RNAi screens that have shown that Rab11 has little or no role in S2 cell cytokinesis (Eggert *et al.*, 2004; Echard *et al.*, 2004; Kouranti *et al.*, 2006).

Previous studies have shown that null mutations in *fwd* and *fws* disrupt spermatocyte cytokinesis but that they have no observable effects on larval neuroblast mitosis (Brill *et al.*, 2000; Farkas *et al.*, 2003; Giansanti *et al.*, 2004). Thus, at least three proteins involved in membrane traffic, Rab11, Cog5, and a PtdIns-4-kinase, seem to be specifically required for meiotic cytokinesis. This specificity is unlikely to depend on

the peculiar features of the final steps of spermatocyte cytokinesis. In male meiotic cells, the cytoplasmic bridges generated by ring constriction are not severed by a canonical abscission process, as occurs in larval neuroblasts; they instead persist and are stabilized by the formation of a specialized structure called ring canal (Hime *et al.*, 1996; Giansanti *et al.*, 1999). Mutations in *rab11*, *fws* and *fwd* inhibit ring constriction and furrow ingression during early telophase and block cytokinesis well before the formation of a cytoplasmic bridge. These observations rule out the possibility that the spermatocyte-specific effects of these mutations reflect problems in the final step of cytokinesis when ring canals are assembled.

The specific role of Rab11, Cog5, and *Fwd* in spermatocyte cytokinesis may reflect a specifically high requirement for formation of new membrane at the advancing cleavage furrow. To fulfill this requirement, male meiotic cells may exploit all the extant pathways for membrane addition. These pathways would be redundant in mitotic cell where the requirements for membrane expansion at the advancing furrow are relatively low. Alternatively, the specific requirement of membrane trafficking functions for spermatocyte cytokinesis may reflect the organization of membrane stores within these cells. Spermatocytes contain a large ER that includes astral and parafusorial membranes, and they do not possess a detectable pericentriolar RE. Larval neuroblasts do exhibit a spindle envelope, but, in contrast to spermatocytes, they are devoid of astral membranes and possess pericentriolar REs (Giansanti *et al.*, 2006; Carpenter, personal communication; our unpublished observations). Thus, formation of new membrane during spermatocyte cytokinesis might utilize membrane trafficking activities that are at least in part distinct from those used by mitotic cells, depending on the organization of membrane stores within the two cell types.

Whatever the reason for their specific sensitivity to mutations that disrupt membrane-related functions, *Drosophila* spermatocytes are emerging as an extremely useful model system for studying membrane traffic during animal cell cytokinesis. There is indeed growing evidence that the analysis of mutations that disrupt spermatocyte cytokinesis can reveal membrane-trafficking genes that play redundant cytotkinetic roles in other animal cell systems.

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