COPI-mediated membrane trafficking is required for cytokinesis in *Drosophila* male meiotic divisions

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Summary

The coatomer protein complex, COPI, mediates retrograde vesicle transport from the Golgi apparatus to the ER. Here, we investigated the meiotic phenotype of *Drosophila melanogaster* spermatocytes expressing dsRNA of 52 genes encoding membrane-trafficking-related factors. We identified COPI as an essential factor for male meiosis. In *Drosophila* male meiotic divisions, COPI is localized in the ER–Golgi intermediate compartment of tER–Golgi units scattered throughout the spermatocyte cytoplasm. Prior to chromosome segregation, the vesicles assemble at the spindle pole periphery through a poleward movement, mediated by minus-end motor dynein along astral microtubules. At the end of each meiotic division, COPI-containing vesicles are equally partitioned between two daughter cells. Our present data strongly suggest that spermatocytes possess a regulatory mechanism for equal inheritance of several types of membrane vesicles. Using testis-specific knockdown of COPI subunits or the small GTPase Arf or mutations of the *γCOP* gene, we examined the role of COPI in male meiosis. COPI depletion resulted in the failure of cytokinesis, through disrupted accumulation of essential proteins and lipid components at the cleavage furrow region. Furthermore, it caused a reduction in the number of overlapping central spindle microtubules, which are essential for cytokinesis. *Drosophila* spermatocytes construct ER-based intracellular structures associated with astral and spindle microtubules. COPI depletion resulted in severe disruption of these ER-based structures. Thus, we propose that COPI plays an important role in *Drosophila* male meiosis, not only through vesicle transport to the cleavage furrow region, but also through the formation of ER-based structures.

Key words: Cell division, COPI, Cytokinesis, *Drosophila melanogaster*, Meiosis

Introduction

Cytokinesis is the final step of cell division, and results from the constriction of a contractile ring comprising F-actin and myosin fibers (Satterwhite and Pollard, 1992). Microtubule bundles known as the central spindle help position the actomyosin ring at the equator (Glotzer, 2001; Cao and Wang, 1996; Inoue et al., 2004). A protein complex called centralspindlin plays a key role in initiating contractile ring formation (D'Avino et al., 2005; Mishima and Glotzer, 2003). Completion of cytokinesis is dependent not only on contractile ring formation, but also on vesicle transport (Glotzer, 2001; Albertson et al., 2005; Montagnac et al., 2008; Prekeris and Gould, 2008).

The delivery of membrane components to the cleavage furrow region of the equatorial plasma membrane is thought to involve two different vesicle transport pathways: the secretory pathway and the endocytic pathway. The *Drosophila melanogaster* secretory pathway involves several transport proteins. Syntaxin 5 (one of the SNAREs required for membrane fusion) is necessary for male meiotic divisions (Xu et al., 2002). Lva (a *Drosophila* ortholog of golgin) is essential for cellularization, which is a cognate cytokinesis process in early embryos (Sisson et al., 2000). An *fws*-encoded *Drosophila* Cog5 plays a role in intra-Golgi vesicle transport, and is essential for cytokinesis in male meiotic divisions (Farkas et al., 2003). Using proteomic analysis, Skop and colleagues revealed that one-third of proteins accumulated in the mammalian midbody were secretory or membrane-trafficking proteins. RNAi experiments in *Caenorhabditis elegans* confirmed the dependence of cytokinesis on these proteins (Skop et al., 2004).

The endocytic pathway involves intracellular transport through early and recycling endosomes (Boucrot and Kirchhausen, 2007). The small GTPase, Rab11, is important in regulating membrane trafficking through recycling endosomes (Giansanti et al., 2007; Prekeris and Gould, 2008). Giansanti and colleagues reported the accumulation of Golgi-derived vesicles at the midzone in *rab11* mutant spermatocytes, and the perturbation of membrane vesicle insertion into the plasma membrane. Recruitment of Rab11 to the cleavage furrow is dependent on the regulatory factors, Giotto and Fwd (Giansanti et al., 2007). Nuf, a Rab11 effector, is required for targeting of endosomal vesicles and F-actin through the central spindle and furrow microtubules (Albertson et al., 2008; Cao et al., 2008). Furthermore, in cell abscission, the Rab11 interacting proteins, Fip3/Nuf and Cep55, are responsible for targeting of recycling endosomes to the midbody (Simon et al., 2008; Zhao et al., 2006). These recycling endosomes are rich in Arf6 GTPase, which interacts with Rab11-mediated membrane addition, but is not required for central spindle formation, contractile ring assembly, or targeting of recycling endosomes to the central spindle (Dyer et al., 2007). The *Drosophila bru* gene encodes TRAPP (membrane-trafficking transport protein particle) II complex, which is required for Rab11 localization at the cleavage furrow, and ingression in cytokinesis (Robinett et al., 2009).

To identify other cytokinesis factors, two independent large RNAi screens were previously performed in S2 cultured cells (Echard et al., 2004; Eggert et al., 2004). Several subunits of a coatomer protein complex, COPI, were commonly identified. The first screen identified five out of seven subunits as essential cytokinesis factors. Consistent with these results, brefeldin A (which inhibits coatomer assembly) disrupted the completion of cytokinesis (Sisson et al., 2000; Skop et al., 2004).

Transported vesicles are coated with one of three types of coatomer protein complex (COPI, COPII, or clathrin), according to cellular transport steps. COPI is a protein complex consisting of seven subunits (α, β, β′, γ, δ, ε, and ζ). COPI-containing vesicles return membrane components and selected proteins from the *cis* end of the Golgi apparatus to the ER (Kondylis and Rabouille, 2009). In budding yeast, all COPI subunits except for ζ were shown to be essential genes. The seven subunits are reported to be ubiquitously expressed in somatic and germline cells during *Drosophila* development (Grieder et al., 2005). Mutants for *γCOP* or *δCOP* were lethal at the embryonic stages. The *γCOP* mutant displayed marked morphogenetic defects of the epithelia and tracheal tubes (Grieder et al., 2008; Jayaram et al., 2008). Immunolocalization of the βCOP subunit in rat cells by electron microscopy revealed the localization of COPI over the Golgi apparatus, mainly at the *cis* side (Oprins et al., 1993). In *Drosophila* cells, βCOP and γCOP colocalize with the Golgi stacks (Jayaram et al., 2008; Ripoche et al., 1994). COPI is involved in the formation and/or maintenance of the Golgi apparatus (Kamena et al., 2008; Lippincott-Schwartz et al., 1998). A large-scale RNAi screen revealed that COPI is involved not only in retrograde vesicle transport, but also in the regulation of lipid homeostasis (Beller et al., 2008). The screen identified several genes encoding subunits of COPI, but not of COPII or clathrin, as essential regulators of lipid droplets. These lipid droplets are ubiquitous lipid storage organelles, and may be important suppliers of vesicle components (Cermelli et al., 2006). Thus, COPI may mediate the transport of lipid droplets out of the internal stores. Mutants for the COG family, which regulates COPI-mediated transport through the Golgi cisternaes, demonstrated cytokinesis defects (Farkas et al., 2003). Thus, it appears that COPI may be involved in cytokinesis. However, its precise role remains unclear. In the present study, we investigated the cytokinesis role of COPI in *Drosophila* male meiosis.

The *Drosophila* spermatocyte has frequently been used to characterize vesicle transport-related factors in cytokinesis (Fuller, 1993). The cell is the largest diploid cell to undergo cell division. A cyst, which is a unit of 16 spermatocytes, simultaneously gives rise to 64 spermatids following two successive meiotic divisions. Each onion-stage spermatid in a cyst contains an equal-sized nucleus and mitochondrial-derived Nebenkern, achieved through correct chromosome segregation and cytokinesis in germline cells, and by equal partitioning of mitochondria (Fuller, 1993; Maines and Wasserman, 1997). Phase-contrast microscopy facilitates the identification of subtle defects in meiotic divisions (Castrillon et al., 1993; Ichihara et al., 2007). The *Drosophila* spermatocyte possesses well-developed membranous cellular structures, and therefore constitutes a suitable model for investigating the involvement of membrane trafficking in cell divisions (Bobinnec et al., 2003). Primary spermatocytes posses particularly long astral microtubules (Fuller, 1993; Inoue et al., 2004; Inoue et al., 2011). By contrast, the spindle microtubules form within a nuclear space, surrounded by a multilayer of parafusorial membranes (Fuller, 1993; Tates, 1971). Thus, it is easy to distinguish spindle microtubules from asters. Inoue and colleagues reported that a microtubule-associated protein, Orbit/CLASP, accumulated on spindle microtubules, but not on asters (Inoue et al., 2004). Thus, it appears that each type of microtubule has a different biochemical feature.

In the present study, we initially performed an RNAi screen to identify essential genes for male meiosis among genes encoding membrane trafficking-related factors. We identified COPI subunits and regulatory proteins as essential cytokinesis factors. Next, we examined the dynamics of COPI-containing vesicles during the progression of cell division in *Drosophila* male meiosis. Prior to chromosome segregation, the vesicles assemble at the spindle pole periphery through a poleward movement, mediated by minus-ended motor dynein along astral microtubules. At the end of each meiotic division, the vesicles are equally partitioned between two daughter cells. We revealed that COPI depletion resulted in the failure of cytokinesis, through disrupted accumulation of essential proteins and lipid components at the cleavage furrow. Furthermore, it caused a reduction in the number of overlapping central spindle microtubules, which are essential for cytokinesis. *Drosophila* spermatocytes construct ER-based intracellular structures associated with astral and spindle microtubules. COPI depletion resulted in severe disruption of these ER-based structures. Thus, we believe that COPI plays an important role in male meiosis, not only through vesicle transport to the cleavage furrow region, but also via the formation of intracellular structures.

Results

Identification of COPI subunits as essential factors for *Drosophila* male meiosis

Whole genome screen of *Drosophila* S2 cells previously identified genes encoding certain subunits of the COPI complex as essential for cytokinesis (Echard et al., 2004; Eggert et al., 2004). Here, we investigated the meiotic phenotype of *Drosophila* spermatocytes, by performing knockdown of 52 genes for membrane trafficking-related factors (including COPI subunits). Using the *Drosophila*-targeted gene expression system, Gal4/UAS, we performed spermatocyte-specific knockdown of genes for the seven COPI subunits, five *arf* genes encoding COPI assembly factors (supplementary material Table S1), and 40 membrane trafficking-related genes. For induction of dsRNA for these genes, we mainly used *bam–Gal4* driver and UAS-RNAi stocks from the Vienna Drosophila RNAi Center. We observed that each wild-type, onion-stage spermatid contained an equal-sized nucleus and mitochondrial-derived Nebenkern (Fig. 1A,B). Knockdown males of COPI α, β, γ, and ζ subunits exhibited abnormal spermatids, with a higher ratio of nucleus to Nebenkern (Fig. 1A,C,D). Our genetic data indicate the incorrect execution of cytokinesis. Spermatids from knockdown males of all four subunits exhibited similar meiotic phenotypes.

To confirm our observations, we examined male meiotic phenotypes of mutants for the γCOP subunit (Fig. 1A,E). Hypomorphic γCOP mutants died before the third instar larval stage, because of the viability requirement for COP. We therefore provided *γCOP* expression by using an *arm-Gal4* driver, which induces ubiquitous UAS-dependent gene expression in somatic cells rather than in germline cells (Kirchner et al., 2008). We generated *arm-Gal4/+;γCop{XP}*^{d06498} flies, with an *XP* element

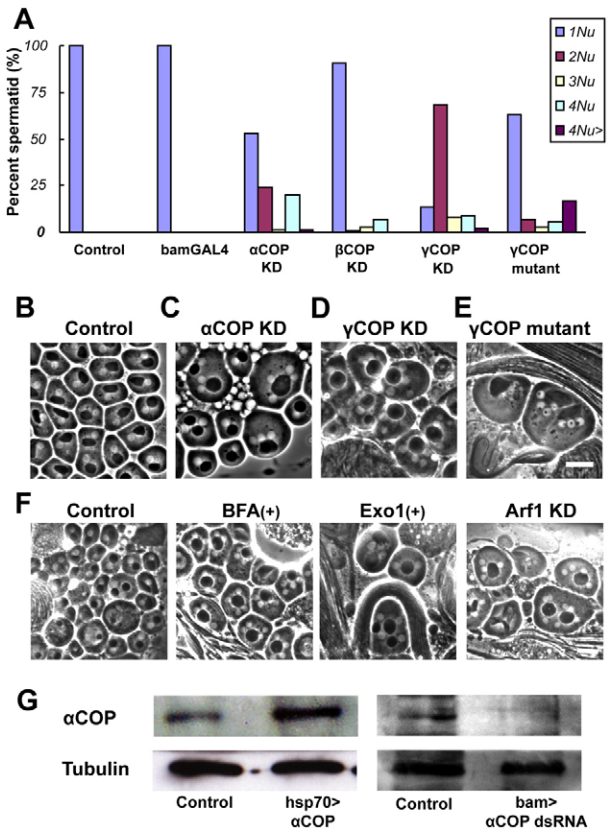

Fig. 1. Loss of COPI subunits or inhibition of COPI assembly results in the failure of cytokinesis in *Drosophila* male meiotic divisions.
(**A**) Quantification of cytokinesis defects in onion-stage spermatids from control and *bam–Gal4* males, knockdown males for COPI subunits and *γCop* mutant males with the genotype *arm-Gal4;γcop{XP}*^{d06498}*/P{SUPor-P}*^{G06383}. Testes from 15 males of each genotype were examined. (**B–E**) Phase-contrast micrographs of onion-stage spermatids derived from control males (B), *αCop* knockdown males (C), *γCop* knockdown males (D) and *γCop* mutant males with the genotype *arm-Gal4;γcop{XP}*^{d06498}*/P{SUPor-P}*^{G06383} (E) Scale bar: 10 μm. (**F**) Effects of two Arf1 inhibitors, brefeldin A (BFA) and Exo1, on wild-type spermatids. Cells underwent meiotic divisions in the presence or absence of the inhibitors. Spermatids from *arf72A* knockdown male are also shown. (**G**) Western blots of the *Drosophila* αCOP subunit. The antibody against αCOP recognized a single polypeptide of 138 kDa, which corresponded to the predicted molecular mass of αCOP in western blot analysis using testis extracts prepared from wild-type adult males. Overexpression of the *αCop* gene in *hsp-Gal4>UAS-αCop* males resulted in accumulation of the polypeptide. Conversely, extracts prepared from *bam-Gal4>UAS-αCop* IR males expressing *αCop* dsRNA had less polypeptide.

integrated into the *γCop* locus. Somatic cells transcribed *γCop* mRNA from the *XP* element, in a manner that was dependent on *arm-Gal4*, whereas germ cells transcribed *γCop* mRNA less abundantly. The *arm-Gal4/+;γCop{XP}*^{d06498} males displayed meiotic defects in cytokinesis at a low frequency (Table 1). We used a different insertional mutation, induced by a UAS-lacking P element, *P{SUPor-P}*, as one of the *γCop* alleles (Table 1). This mutation increased the frequency of abnormal cells exhibiting cytokinesis phenotypes. Our genetic data from testis-specific knockdown of COPI subunits, and the male meiotic phenotype of *γCop* mutants, consistently indicated the requirement of COPI for cytokinesis.

Furthermore, testis-specific knockdown of five Arf1 family members revealed the most severe cytokinesis phenotype in onion-stage spermatids (Fig. 1F, 98%; $n=820$) from *arf72A* knockdown males containing multiple normal-sized nuclei. In *arf79F* knockdown males, cytokinesis defects were also observed repeatedly, but less frequently (12%, $n=2412$). Treatment of meiotic cells with the Arf1 inhibitors, brefeldin A or Exo1, influenced cytokinesis in meiotic divisions (Fig. 1F). Taken together, our results indicate that the COPI complex assembled by Arf1 is required for cytokinesis in *Drosophila* male meiotic divisions. We raised antibody against αCOP and confirmed that it recognizes single polypeptides in testis extracts, which increased in amount after overexpression of *αCOP* and significantly reduced by expression of its dsRNA (Fig. 1G).

COPI localization in an ER–Golgi intermediate compartment

Prior to further investigation of the biological role of COPI in male meiosis, we investigated the cellular distribution of the COPI complex in primary spermatocytes. Immunostaining of premeiotic spermatocytes with antibody against αCOP showed that the antibody recognized 20–40 foci in the cells (supplementary material Fig. S1). Moreover, αCOP knockdown spermatocytes exhibited considerably fewer foci (42% of normal control, $n=51$) and reduced whole signal intensity (supplementary material Fig. S2). The αCOP foci perfectly overlapped with the mRFP–γCOP foci in spermatocytes (Fig. 2A). Thus, it is likely that *Drosophila* αCOP forms part of the COPI complex with additional subunits, as in other organisms (Hosobuchi et al., 1992; Duden et al., 1998). Hereafter, we consider foci stained with αCOP antibody to indicate the presence of COPI.

In mammalian cultured cells, COPI co-localizes with the Golgi apparatus (Griffiths et al., 1995). We therefore examined whether *Drosophila* COPI continuously co-localizes with several Golgi markers in premeiotic spermatocytes. In *Drosophila*, as in yeast or worms, the Golgi apparatus exists as multiple stacks in the cytoplasm. In premeiotic spermatocytes, these Golgi stacks appeared as 20–40 horseshoe-shaped foci (Inoue et al., 2011). We investigated the co-localization of COPI by using several Golgi markers: GM130 for the *cis* side and Rab11 for the *trans* side of the Golgi stacks; KDEL for the ER–Golgi intermediate compartment (ERGIC); and Sec31 for the transitional ER (tER) sites (Fig. 2B). We observed that the COPI distribution perfectly overlapped with the ERGIC marker in premeiotic spermatocytes, but was not identical to foci visualized using the other Golgi markers (Fig. 2C–F). Given that COPI may play an important role in vesicle transport between the ER and Golgi membranes, it is likely that most of the COPI is distributed in the ERGIC of the Golgi stacks.

Astral microtubules are required for equal partitioning of COPI-containing vesicles

Using the αCOP antibody (Fig. 1G), we examined the cellular localization of COPI-containing vesicles during male meiotic divisions (supplementary material Fig. S3). We observed that spermatocytes entering meiosis contained a twofold higher number of COPI vesicles than did premeiotic spermatocytes (supplementary material Figs S1, S3). Moreover, each vesicle diminished in size, implying their fragmentation into smaller pieces during prophase to prometaphase. At each meiotic division, the vesicles were equally partitioned between two daughter cells (supplementary material Figs S3, S4). At prophase, they were uniformly distributed throughout

Table 1. Quantification of meiotic defects in spermatids from *γCOP* mutant males with somatic rescue induced by *arm–Gal4*

Genotype	No. of onion-stage spermatids scored	Nebenkern-to-nuclei ratio (% cells)							Macro/micronuclei (% cells)
			Abnormal						
		Normal	1:0	1:2	1:3	1:4	1:>5	Total	
arm-GAL4	2820	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
*γCop{XP}^{d06498}/+**	2721	100.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
arm-Gal4/+;γCop{XP}^{d06498}	821	99.5	0.0	0.5	0.0	0.0	0.0	0.5	2.6
arm-Gal4/+;γCop{XP}^{d06498}/P{SUPor-P}γCop^{KG06383}	362	65.2	2.2	6.9	3.0	5.5	17.1	34.8	3.6

*$\gamma Cop\{XP\}^{d06498}$ homozygotes are lethal.
Testes from 15 males were scored per genotype.

the cytoplasm. Prior to chromosome segregation, they were assembled into two groups, each containing similar numbers of vesicles. The vesicles were localized on astral microtubules, and became comprehensively assembled at the spindle pole periphery prior to metaphase. It appears that the vesicles are kept away from the midzone, in order to achieve equal partitioning between two daughter cells. During cytokinesis, they are redistributed throughout the cytoplasm.

During the second meiotic division, we observed the same distribution of vesicles. On completion of the meiotic divisions, the COPI vesicles in each spermatid were assembled into a single spherical structure, called an acroblast (supplementary material Fig. S3F). Immunostaining of male meiotic cells with γCOP antibody revealed the same distribution (supplementary material Fig. S4). This characteristic distribution of COPI vesicles in male meiotic cells was not observed during mitosis of *Drosophila* S2 cells or female meiotic cells (Fig. 3A). Such a characteristic COPI distribution seems to be closely associated with astral microtubules in male meiotic cells.

Drosophila spermatocytes possess particularly long astral microtubules. By contrast, spindle microtubules form within a nuclear space, surrounded by a multilayer of parafusorial membranes (Fuller, 1993; Tates, 1971) (see supplementary material Figs S3, S5). Thus, it is easy to distinguish spindle microtubules from asters. We subsequently investigated the influence of several mutations affecting microtubule structures, on the distribution of COPI vesicles (Fig. 3B,C). Meiotic cells from $\beta Tub85D^{D}$/+ males exhibited disrupted formation of astral and spindle microtubules. The characteristic COPI distribution was no longer present, and the vesicles were uniformly distributed throughout the cytoplasm. Our results suggest that COPI vesicle distribution is dependent on microtubule structures.

Meiotic cells from *asl* males may lack astral microtubules, because *asl* is required for spindle pole formation (Bonaccorsi et al., 1998). Fig. 3B shows the distribution of COPI vesicles throughout the cytoplasm of *asl* mutants, including the central region of metaphase-I-like cells. By contrast, the *orbit*[7] mutation influences central spindles and not astral microtubules (Inoue

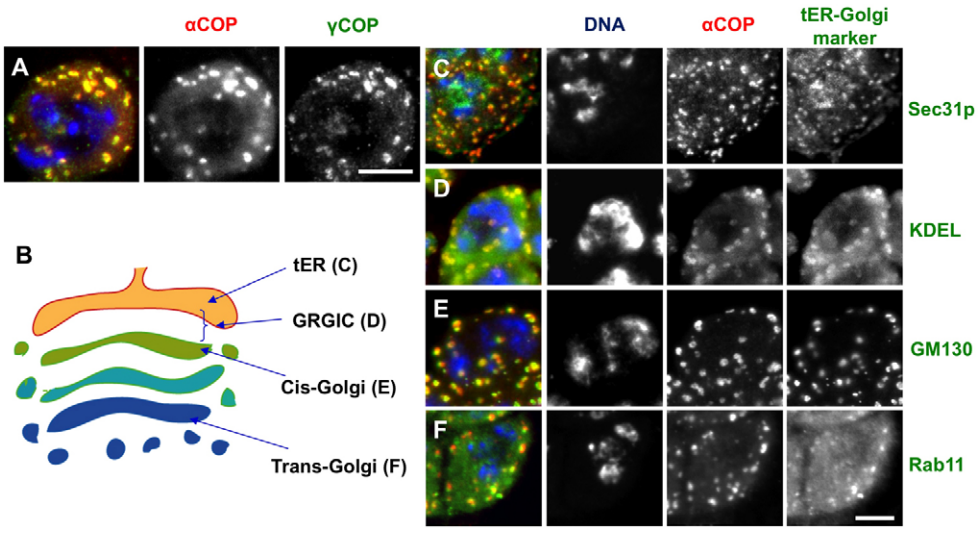

Fig. 2. Immunodetection of the COPI complex and simultaneous localization of COPI with tER–Golgi marker proteins. (**A**) Simultaneous immunostaining of αCOP and fluorescence of mRFP–γCOP. In the merged image (left panel), αCOP and γCOP are displayed in red and green, respectively. DNA is shown in blue. Colocalization of αCOP with γCOP appears as yellow spots. (**B**) Schematic presentation of tER–Golgi components and their intracellular localization. (**C–F**) Simultaneous immunostaining of premeiotic spermatocytes with the αCOP antibody and tER–Golgi markers at the S5–S6 stage of the growth phase. Developmental stages of spermatocytes were determined by DAPI staining and phase-contrast observation, according to a previous study (Bonaccorsi et al., 2000). αCOP immunofluorescence is shown in the middle column, and displayed in red in the left column. Immunofluorescence of the Golgi–tER site marker Sec31 (C), ER–Golgi intermediate compartment (ERGIC) marker KDEL (**D**), *cis*-side marker GM130 (**E**), and *trans*-side marker Rab11 (**F**) is displayed in the left column, and visualized in green in the merged images. Scale bars: 10 μm.

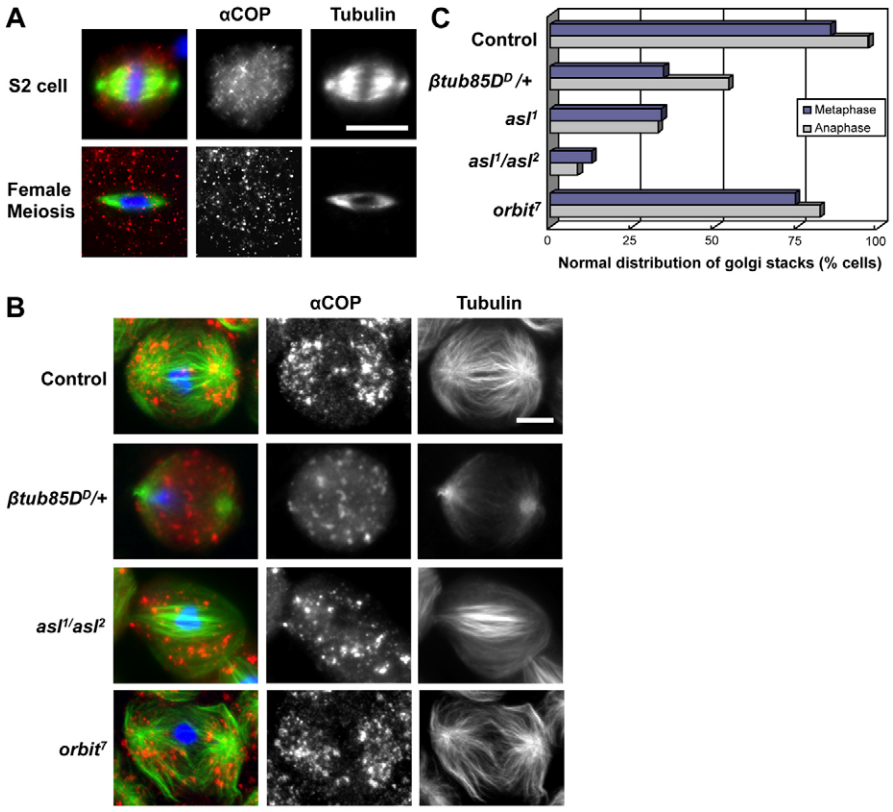

Fig. 3. Distribution of COPI-containing vesicles during male meiosis is affected in flies mutants for astral microtubules rather than for spindle microtubules. (**A**) Immunolocalization of COPI vesicles in S2 cultured cells at metaphase and in metaphase I cells from wild-type females. Anti-tubulin immunostaining is shown in green, anti-αCop immunostaining in red and DNA in blue. Note the random distribution of COPI vesicles throughout the cytoplasm of dividing cells. (**B**) Immunofluorescence of the COPI vesicles (red) in metaphase I cells from wild-type and mutant males with abnormal microtubule structures. In wild-type cells, the COPI vesicles appear to be localized on astral microtubules around two spindle poles. In a metaphase-I-like cell from a $\beta Tub85D^{D}$/+ male, the COPI vesicles are distributed as far as the central region of the cell. In a metaphase-like cell from an asl^{1}/asl^{2} male, with few or incorrectly oriented astral microtubules, the distribution of COPI vesicles is compromised, and a subset of vesicles remains in the center of the cell. In the metaphase-I-like cell from an $orbit^{7}$ male, in which spindle microtubules fail to overlap each other at the plus ends, COPI vesicles accumulate around a spindle pole. Scale bars: 10 μm. (**C**) Frequency of cells displaying normal distribution of COPI vesicles at metaphase I (M) or anaphase I (A). Spermatocytes from wild-type [M, 85.1% (n=141); A, 96.4% (n=111)], $\beta Tub85D^{D}$/+ [M, 34.4% (n=96); A, 54.1% (n=185)], asl^{1} [M, 33.8% (n=139); A, 32.7% (n=87)], asl^{1}/asl^{2} [M, 12.5% (n=96); A, 8.2% (n=107)], and $orbit^{7}$ [M, 74.3% (n=74); A, 81.8% (n=110)].

et al., 2004). Thus, COPI distribution was maintained in an $orbit^{7}$ metaphase-I-like cell (Fig. 3B). In comparison with the $orbit^{7}$ mutants, the *asl* mutants contained markedly fewer cells displaying normal COPI distribution (Fig. 3C).

Thus, our results indicate that microtubule structures are essential for the distribution of COPI vesicles during meiosis. Moreover, the equal partitioning of COPI vesicles is dependent on well-developed astral microtubules in male meiotic cells, rather than on spindle microtubules.

Requirement of dynein for COPI vesicle transport along astral microtubules towards the spindle pole periphery

To understand the inheritance of COPI vesicles during male meiosis, we investigated the movement of mRFP–γCop-labeled vesicles in a living spermatocyte by means of a time-lapse experiment (Fig. 4A; supplementary material Movie 1). The ubiquitously distributed vesicles moved towards each spindle pole during prometaphase to metaphase. We observed the movement of several COPI foci from the plus ends of asters towards a spindle pole, along the microtubules. [All foci examined (n=50 foci in 26 cells) performed the movement. Arrowhead in Fig. 4A]. These foci eventually converged around the spindle poles on astral microtubules, until metaphase.

Sisson and colleagues proposed minus-ended motor dynein as a candidate for the transport of COPI vesicles (Sisson et al., 2000). We observed the enrichment of dynein around the spindle poles (Fig. 4B). Moreover, COPI vesicles were distributed on and over a dynein-accumulated region. In mutants for *Dhc64C* encoding the dynein heavy chain, or *Dhc64C* knockdown spermatocytes, the characteristic COPI distribution was not present (67% abnormality, n=21 mutant cells, Fig. 4C). Furthermore, we observed Lva-containing vesicles, which may correspond to Golgi stacks (Giansanti et al., 2007; Papoulas et al., 2005), dispersed throughout the cytoplasm. Our results suggest that dynein is required for the transport of COPI and Lva-containing vesicles, along astral microtubules towards the spindle poles.

Defective accumulation of cleavage furrow proteins and lipid droplets in COPI-depleted spermatocytes

To understand the primary cytokinesis defects in COPI-depleted spermatocytes, we investigated perturbations in the accumulation

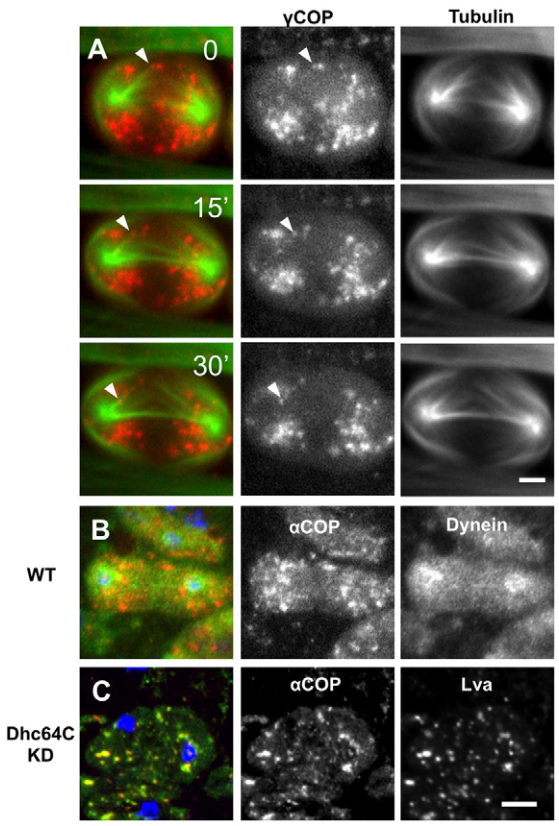

Fig. 4. Time-lapse observation of COPI vesicle transport along astral microtubules toward spindle poles, and its disruption by depletion of dynein heavy chain. (**A**) Spermatocytes derived from males in which β-tubulin–GFP and mRFP–γCOP are simultaneously expressed were used to trace the single COPI foci from early prometaphase I (t=0 min) to metaphase I (t=30 min). A single COPI focus indicated by an arrowhead is moving along astral microtubules in the minus-ended direction. (**B**,**C**) Simultaneous immunostaining of anaphase I cells from wild-type (B) or knockdown males for dynein heavy chain (C) with αCop antibody (red) and dynein heavy chain antibody (green in B) or Lva antibody (green in C). DNA is shown in blue. Scale bars: 10 μm.

of cleavage furrow components. In wild-type cells, we observed the accumulation of three vesicle transport-related proteins (Fig. 5A–C). Rab11 is highly enriched at the cleavage furrow region during telophase (92% accumulation; n=12) (Giansanti et al., 2007). Nuf, an effector of Rab11, is designated as a midzone-concentrated protein (91% accumulation; n=11) (Polevoy et al., 2009). Clathrin, another coatomer protein complex, is enriched at the midzone region during telophase [100% accumulation (n=10), Fig. 5C]. COPI-depleted cells failed to accumulate these three proteins at the midzone region [29% abnormal Rab11 accumulation (n=14), Fig. 5D; 42% abnormal Nuf accumulation (n=12), Fig. 5E; 37% abnormal Clh accumulation (n=11), Fig. 5F].

In wild-type male meiotic cells, we observed the accumulation of lipid droplets (Fig. 5G–I). Membranous intracellular structures known as astral membranes that are consisted of ER sheets and enriched in several ER markers are closely associated with astral microtubules in early embryos (Bobinnec et al., 2003) as well as in primary spermatocytes (Fig. 7A; supplementary material Fig. S7). These lipid droplets were localized on the astral membranes until metaphase (100% accumulation; n=10). Fibrous distribution connecting each astral membrane was evident at metaphase, indicating that the lipid droplets may be distributed along spindle microtubules, or associated with ER-based spindle envelopes. We observed prominent accumulation at the central region until early anaphase (100% accumulation; n=11; Fig. 5H). It appears that the lipid droplets facilitate *de novo* membrane synthesis during subsequent cell elongation at late anaphase. At telophase, lipid droplets were generally less abundant, and accumulation on the astral membranes was no longer present, even in wild-type cells. The droplets were locally distributed along the edge of the central spindles, and on the invaginated plasma membrane contacting the central spindles [100% accumulation (n=15), closed arrowheads in Fig. 5I; 100% accumulation (n=8), supplementary material Fig. S6A]. By contrast, in COPI-depleted cells, we observed no distinct midzone accumulation during metaphase to anaphase. However, the distribution around the astral membranes remained [71% abnormal accumulation (n=17), Fig. 5J; 71% abnormal accumulation (n=17), Fig. 5K]. Plasma membrane ingression failed to occur effectively; where it did occur, the invaginated membrane did not contact lipid droplets on the central spindle [30% without contact (n=10), open arrowheads in Fig. 5L; 65% without contact (n=23), supplementary material Fig. S6B].

Taken together, our results indicate that disruption of COPI-mediated membrane trafficking results in defective accumulation of vesicle components, such as cleavage furrow proteins and lipid droplets, at the cleavage furrow site.

Effect of COPI depletion on the formation of central spindle microtubules

To elucidate the mechanism of disruption of central spindles and astral microtubules in COPI-depleted cells, we investigated the microtubule structures. In wild-type metaphase I cells, we observed large umbrella-shaped astral microtubules emanating from each spindle pole (Fig. 3B; supplementary material Fig. S5). The central spindles comprise bundles of interdigitated microtubules formed between the separating homologous chromosomes, and play a key role in contractile ring positioning [Fig. 6A (n>50) and 6C (n=26); see supplementary material Movies 2, 3] (Goldberg et al., 1998; Inoue et al., 2004). We occasionally observed abnormal cells, in which the direction of microtubule elongation or orientation was perturbed [42% abnormal microtubule formation (n=52); Fig. 6B].

During anaphase, knockdown cells often lack robust central spindle microtubules. Using a time-lapse experiment, we investigated microtubule structures in the COPI-depleted spermatocytes (Fig. 6D; supplementary material Movies 4, 5). Following breakdown of the nuclear membrane, markedly fewer astral or spindle microtubules were constructed, although the bipolar spindle structure was initially formed. Following anaphase, the spindle microtubule structures elongated in an aberrant direction. The interdigitated spindle microtubules from opposite poles failed to overlap around their plus termini. These microtubule structures became bent and eventually degraded. At 80 min after onset, COPI-depleted cells exhibited no central spindles or astral microtubules (Fig. 6D). The primary spermatocytes did not elongate along the spindle axis after anaphase, and no cleavage furrow ingression occurred.

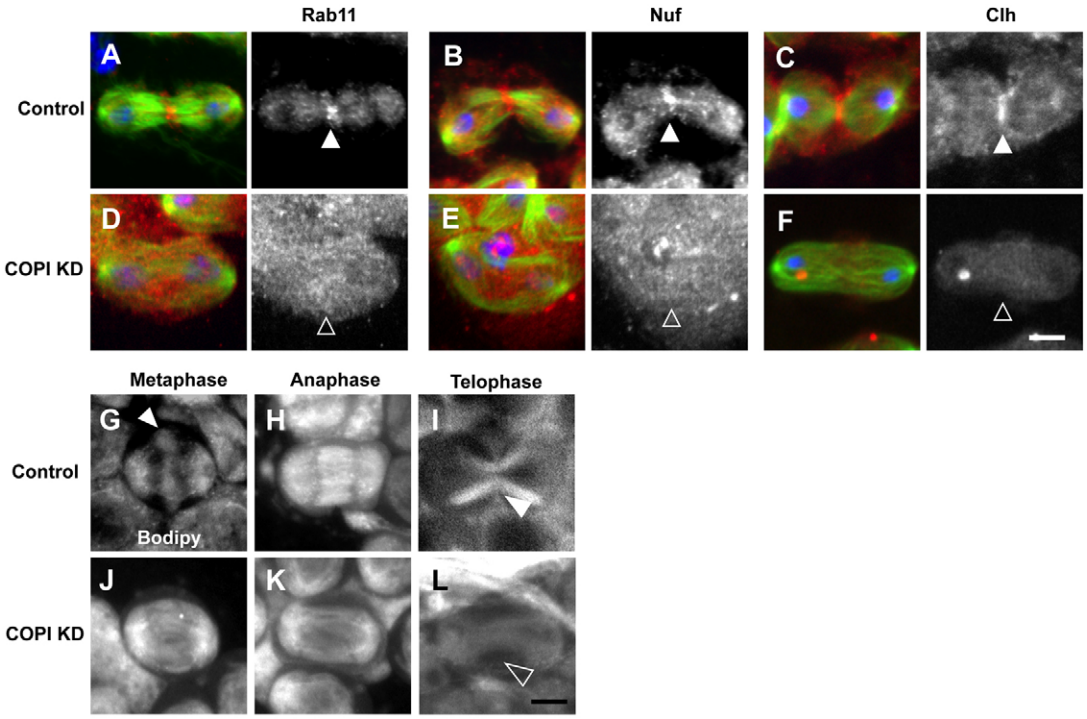

Fig. 5. COPI depletion results in failure to accumulate vesicle components at the midzone of male meiotic cells. (**A–F**) Immunolocalization of three vesicle-related proteins (Rab11, Nuf and clathrin), which are essential for membrane trafficking in cytokinesis. Wild-type (A–C) and *αCop* knockdown (D–F) spermatocytes were examined. DNA is shown in blue, microtubules in green and immunofluorescence of the three proteins in red. Note that each protein accumulated at the midzone of early telophase I cells (filled arrowheads) and around the centrosomes. COPI-depleted cells failed to accumulate each protein at the midzone (open arrowheads). (**G–L**) Intracellular distribution of lipid droplets in living primary spermatocytes stained with Bodipy 493/503. In wild-type cells, the Bodipy fluorescence can be seen on the ER-based astral membranes, and at the midzone of cells (arrowheads) during metaphase (G) to anaphase (H). The accumulation on ribbon-like structures corresponding to ER-based structures is prominent at telophase; the midzone of the plasma membrane is budding inside, so as to contact the cellular pool of lipid droplets before cytokinesis (I). In COPI-depleted cells, central accumulation at metaphase to anaphase is not present (J,K); budding of the plasma membrane towards the internal lipid storage is initiated, but membrane ingression fails to contact the storage organelles (open arrowhead in L). Scale bars: 10 μm.

Next, we examined the formation of the anillin rings (known as pre-contractile rings) in the knockdown spermatocytes (Fig. 6E,F). The formation of anillin rings was frequently incomplete (Fig. 6F) or absent (36% abnormal ring formation; $n=36$). Taken together, our results suggest that COPI depletion affects the microtubule structures essential for cytokinesis.

Influence of COPI on central spindle microtubules, through the formation of ER-based cellular structures

To understand the mechanism by which COPI depletion results in the formation of abnormal microtubule structures, we investigated ER-based cellular structures. COPI mediates membrane trafficking between the ER and the Golgi apparatus. In *Drosophila* meiotic spermatocytes, the ER, recycling endosomes, and lipid droplets are frequently associated with the astral membranes and spindle envelopes (Bobinnec et al., 2003; Dorogova et al., 2009; Fuller, 1993; Tates, 1971). Our time-lapse experimental data confirmed the overlapping of astral microtubules with ER-based astral membranes during meiotic division (Fig. 7A). Furthermore, the Rtnl1–GFP signal overlapped with the phase-dense intracellular structures observed using phase-contrast microscope. The astral membranes at the spindle pole periphery were covered by astral microtubules, thereby supporting the microtubule structures from inside. The central spindle microtubules were contiguous with the spindle envelope surrounding the nuclear space in which chromosome segregation occurs; some overlap with the spindle envelope was evident [similar results were obtained ($n=18$), Fig. 7A; see supplementary material Movies 6, 7, 8; supplementary material Fig. S7].

We subsequently obtained genetic evidence for a close relationship between ER-based and microtubule structures in spermatocytes. A dominant mutation for a testis-specific tubulin, $\beta Tub85D^{D}$, inhibits microtubule polymerization (Kemphues et al., 1980). We observed the disruption of ER-based astral membranes and spindle envelopes in the $\beta Tub85D^{D}/+$ cells, but no distribution of COPI vesicles around the centrosomes [63% distinctly abnormal ER-based structures ($n=54$), supplementary material Fig. S8C]. Our findings suggest that ER-based and microtubule structures interact considerably during male meiotic divisions.

Using a time-lapse experiment, we investigated the way in which aberrant ER-based structures are constructed in COPI-depleted cells (39% distinctly abnormal ER-based structures ($n=18$; Fig. 7B; supplementary material Movies 9, 10, 11). In normal spermatocytes, the ER formed concentric circles on the outside of the nuclear membrane at prophase. The ER network consisted of multi-layers of membranous sheets, visible as phase-dense structures around the nuclear space. The reticular ER structure was elongated and transformed into multiple layers of membranes (known as spindle envelopes) surrounding the

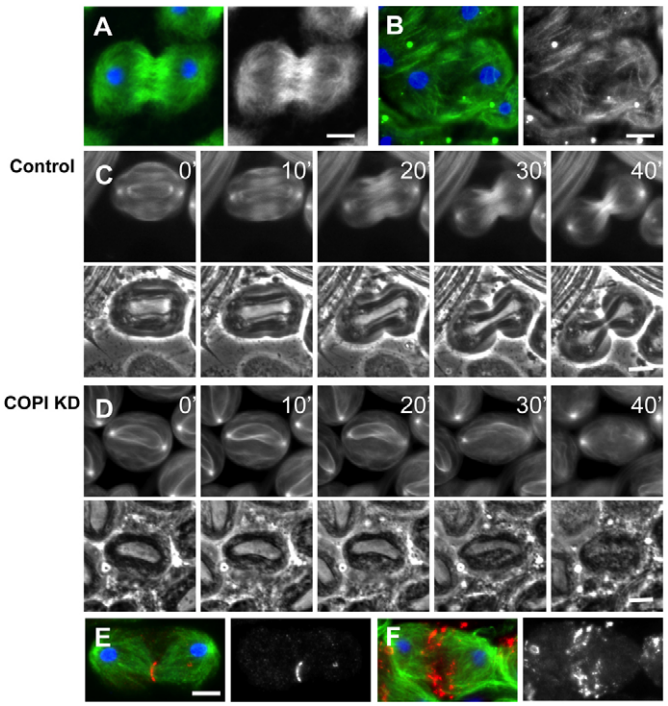

Fig. 6. Abnormal meiotic microtubule structures required for cytokinesis, and loss of contractile rings, in COPI-depleted spermatocytes. (**A**,**B**) Fixed meiotic cells from wild-type (A) or *αCop*-depleted testes (B), Wild-type spermatocytes exhibit robust microtubule bundles, known as central spindle microtubules, in the middle of anaphase cells. Microtubules are visualized in green; DNA is shown in blue. (**C**) Time-lapse observation of meiotic microtubule structures, and phase-contrast images of living wild-type spermatocytes. Microtubules were visualized by expression of GFP–tubulin. The time-lapse images were continuously collected from anaphase I, in which interior microtubules emanating from each spindle pole made contact at the equator (t=0 min), to the end of meiosis I (t=40 min). (**D**) Time-lapse observation of COPI-depleted spermatocytes. (**E**,**F**) A contractile ring (visualized in red by anillin immunostaining) is evident in telophase I cells from wild-type males (E) and *αCop* knockdown males (F). Microtubules are shown in green, and DNA in blue. In the knockdown spermatocytes, elongation of microtubules or their orientation seems to be disturbed. In anaphase-I-like cells, the central spindle microtubules are missing, whereas in non-constricted cells within a telophase cyst, a disconnected contractile ring is formed. Scale bars: 10 μm.

nuclear space. In COPI-depleted cells, the ER-based structure was affected (Fig. 7B; supplementary material Fig. S8C). Initially, the astral membranes and spindle envelopes were indistinguishable from those of wild-type cells. However, as the cells elongated at anaphase, the spindle envelopes became curved in the middle and disconnected (arrowheads in Fig. 7B; supplementary material Fig. S8).

Simultaneous observation confirmed the interaction of microtubules and ER-based structures (Fig. 7A,B). In COPI-depleted cells, microtubules exhibited aberrant organization (Fig. 7B,C). At anaphase, spindle microtubules and ER-based structures aligned side by side. In normal cells, characteristic microtubule structures (termed exterior central spindles) are constructed alongside the spindle envelope (Inoue et al., 2004). In COPI-depleted cells, the orientation of the elongating central spindle was lost, resulting in the generation of disorganized microtubule structures. Interestingly, the disorganized microtubules overlapped with the aberrant ER-based spindle envelopes visualized as phase-dense structures (arrowheads in Fig. 7B). Moreover, no cleavage furrow ingression occurred.

Taken together, our results suggest that COPI depletion influences the formation of ER-based spindle envelopes, which mutually interact with the central spindle microtubules essential for cytokinesis.

Discussion

In the present study, our initial RNAi screen identified several COPI subunits and assembly regulators as essential factors for cytokinesis in *Drosophila* male meiosis. We subsequently elucidated the cellular function of COPI during *Drosophila* male meiosis. We observed the localization of COPI in the ER–Golgi intermediate compartment of tER–Golgi units scattered throughout the spermatocyte cytoplasm. Prior to metaphase, COPI-containing vesicles assemble at the spindle pole periphery through a poleward movement, mediated by astral microtubules and minus-ended motor dynein. At the end of each meiotic division, the vesicles are equally partitioned between two daughter cells. Our findings strongly suggest that spermatocytes possess a regulatory mechanism, to fulfill equal inheritance of several types of membrane vesicles. Testis-specific knockdown of COPI, or mutations of the *γCOP* gene, resulted in defective cytokinesis during male meiosis. COPI depletion disrupted the accumulation of vesicle components, such as cleavage furrow proteins and lipid droplets, at the cleavage furrow zone. Furthermore, we observed aberrant central spindle microtubules. In COPI-depleted cells, the astral membrane and spindle envelope (associated with astral and spindle microtubules, respectively) were severely disrupted. We propose that these ER-based structures are required as a structural foundation, to facilitate the formation of meiotic microtubule structures. Thus, we believe that COPI plays an important role in *Drosophila* male meiosis, not only through vesicle transport to the cleavage furrow region, but also via the formation of intracellular structures.

Equal partitioning of membrane vesicles during *Drosophila* male meiosis

Giansanti et al. reported that Golgi-derived vesicles were concentrated around each spindle pole and excluded from the equatorial region (Giansanti et al., 2007). In *gio* or *fwd* mutants, which encode vesicle transport factors, these vesicles continued to be accumulated at the equator. The authors concluded that accumulation of such vesicles was the consequence of failed incorporation into the membrane at the invaginating cleavage furrow. However, this phenotype was not observed in other cytokinesis mutants, such as *fws* and *pbl*, which encode factors for Golgi structure and function, and cleavage furrow formation, respectively (Farkas et al., 2003; Giansanti et al., 2006). Although Golgi stack components are required for *Drosophila* cytokinesis, evidence for the direct fusion of Golgi-derived vesicles with the furrow membrane remains elusive (Albertson et al., 2008; Farkas et al., 2003; Xu et al., 2002). Our present findings indicate the existence of a transport system, which mediates equal partitioning not only of COPI vesicles, but also of vesicles containing Lva (cis-Golgi components), during male meiosis. Thus, we believe that abnormal distribution of Golgi-derived vesicles, and of other membranous organelles, results from the perturbation of vesicle transport.

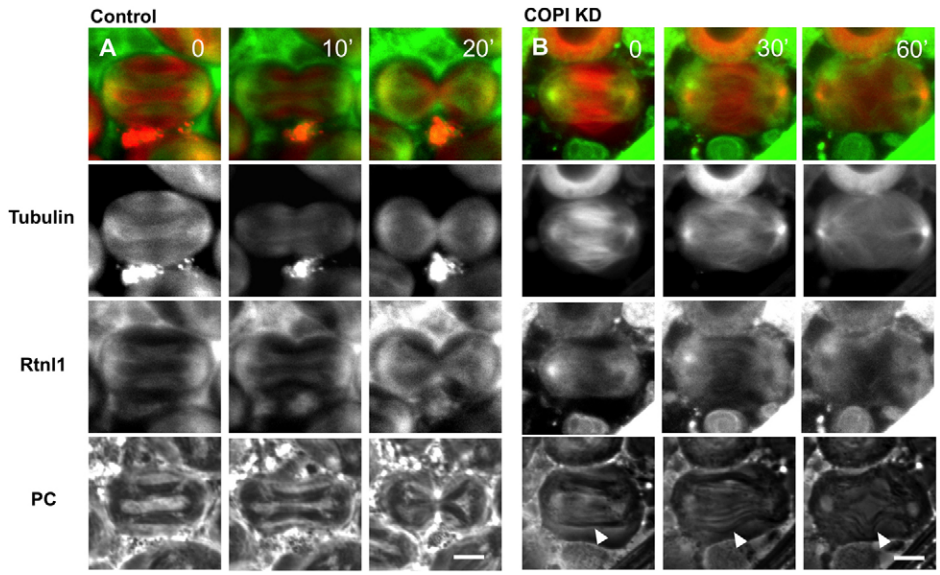

Fig. 7. ER-based intracellular structures associated with astral or spindle microtubules are affected in COPI-depleted spermatocytes. (**A**) Time-lapse observation of microtubules (second row) and ER-based structures (third row) in primary spermatocytes expressing mRFP–tubulin and GFP–Rtnl1 (an ER-localizing enzyme). In merged images (first row), tubulin and Rtnl1 are shown in red and green, respectively. Simultaneous observation by phase-contrast microscopy is shown in the fourth row (PC). The time-lapse images were continuously collected from mid-anaphase I (t=0 min) to the end of meiosis I. Note the association of astral and central spindle microtubules with astral membranes and spindle envelopes, respectively. The ER-based structures labeled by GFP–Rtnl1 overlapped with phase-dense structures. (**B**) Time-lapse observation of COPI-depleted spermatocytes. Bundles of central spindle microtubules were initially constructed, but overlapping microtubules were rapidly degraded, and disappeared by the end of the experiment. Spindle envelopes were less abundant, and became malformed and disappeared, in line with the degradation of microtubule structures (arrowheads). Scale bars: 10 μm.

On completion of meiosis during *Drosophila* spermatogenesis, considerable cell elongation takes place synchronously in 64 spermatids within a cyst (Fuller, 1993; Inoue et al., 2011). It is crucial for each spermatid to obtain sufficient numbers of vesicles. Within spermatids, COPI and other Golgi components comprise a single large Golgi apparatus, called an acroblast (Farkas et al., 2003). A mammalian counterpart known as the acrosome precursor (Moreno et al., 2000) is required for fusion between the sperm and oval membranes. Thus, it is likely that *Drosophila* spermatocytes possess a mechanism to ensure equal partitioning of vesicles during male meiosis.

Dependence of COPI vesicle distribution on astral microtubules and minus-ended motor dynein

Our present findings indicate that vesicle transport towards the spindle pole periphery is mediated by minus-ended motor dynein. Dynein was previously reported to form complexes with Lva-containing vesicles, which may correspond to Golgi stacks (Papoulas et al., 2005). It is reasonable to speculate that other Golgi-derived vesicles are transported along astral microtubules by motor dynein. In budding yeast, the actin cytoskeleton plays an essential role in Golgi transport during mitosis (Arai et al., 2008). We failed to observe co-localization of COPI vesicles with actin networks in male meiotic cells (D.K. and Y.H.I., unpublished data). It is likely that microtubule structures play a key role in the inheritance of vesicles during male meiosis. Ordered vesicle inheritance in mammalian male meiotic divisions has not been demonstrated. In mammalian cultured cells, the Golgi ribbon is fragmented into smaller pieces by the onset of mitosis (Rabouille and Jolo, 2003; Shima et al., 1998). Wei and Seemann reported that COPI vesicles accumulate around the centrosomes prior to anaphase initiation (Wei and Seemann, 2009). Moreover, the vesicles are transferred by the mitotic spindles during mitosis. However, it is difficult to distinguish between spindles and asters. By contrast, in *Drosophila* spermatocytes, the spindle microtubules are formed within a nuclear space, surrounded by a multilayer of parafusorial membranes (Fuller, 1993; Tates, 1971). *Drosophila* spermatocytes possess particularly long astral microtubules, and it is likely that these play an important role in the equal inheritance of membrane vesicles during male meiosis.

Indispensability of Arf1-regulated, COPI-mediated membrane trafficking during *Drosophila* male meiosis

In the present study, we have indicated that not only COPI subunits, but also Arf1, which is a small GTPase required for COPI assembly are essential for correct execution of cytokinesis during *Drosophila* male meiosis. By contrast, in mammalian cultured cells, inactivation of Arf1 is necessary for COPI and clathrin disassembly, which normally occur prior to the initiation of mitosis (Altan-Bonnet et al., 2003). The inhibition of COPI disassembly causes defects not only in chromosome segregation, but also in cleavage furrow ingression. Thus, inactivation of vesicle transport complexes is believed to be indispensable for mitotic entry. However, the meiotic phenotype of the *Drosophila* knockdown cells indicates that the COPI complex plays an active role in the progression of meiotic divisions. The significance of COPI in cell division may be dependent on cell types. For smaller cells to initiate cell division, it is important to shut down cytoplasmic activities, such as protein synthesis and vesicle transport (Hernandez-Verdun, 2011; Hernandez-Verdun et al., 2002). By contrast, in *Drosophila* spermatocytes, membranous cellular structures, such as multi-layers of parafusorial

membranes, are well developed. The ER-based structures on which COPI function depends undergo a massive alteration during meiotic divisions (Fig. 7A). Spermatocytes are much larger than other somatic cells, and may need to maintain higher COPI activity, not only at interphase, but also during cell division. The involvement of membrane trafficking components in cell division was recently reported. The membranous spindle matrix, which has an ER feature, influences spindle morphology during partially open mitosis in *Drosophila* early embryos, and also during open mitosis in vertebrate cells (Zheng, 2010; Liu and Zheng, 2009; Civelekoglu-Scholey et al., 2010). The endocytic adaptor protein, epsin I, was also shown to regulate mitotic membrane organization and spindle morphology (Liu and Zheng, 2009).

Role of ER-based structures as a structural foundation to ensure correct formation of the cell division apparatus

Our present findings indicate that meiotic microtubules and ER-based structures mutually interact. Furthermore, the ER-based astral membranes and spindle envelopes may serve as structural foundations, to ensure the correct formation of astral and spindle microtubules, respectively. Rebollo and González observed that colchicine treatment of primary spermatocytes undergoing male meiosis influenced phase-dense structures corresponding to ER-based structures (Rebollo and González, 2000). Barbosa et al. reported that mutants of *dd4* genes encoding spindle pole factors led to abnormalities in spindle microtubules and phase-dense structures during male meiosis (Barbosa et al., 2000). Further studies using live analysis of mutations affecting additional microtubule structures are required to verify our hypothesis concerning the mutual interaction of meiotic microtubules and ER-based structures. The role of ER structures in microtubule elongation may be compared to that of a splint in the regeneration of broken bones. In the COPI knockdown cells, the microtubules may have lacked support for elongation in the correct orientation. Some microtubules can initiate elongation independently of centrosomes (Rebollo et al., 2004). In human, and also *Drosophila* cells, these noncentrosomal microtubules may be dependent on seed proteins for elongation from the surface of other microtubules (Rogers et al., 2008; Uehara et al., 2009). Thus, in *Drosophila* spermatocytes, advanced ER structures may be useful for facilitating continuous and straight elongation of the particularly long astral microtubules.

The microtubule assembly checkpoint in spermatocytes operates less strictly than does the mitotic cycle (Rebollo and González, 2000). Moreover, *Drosophila* spermatocytes are notable for their well-developed membranous structures (Stafstrom and Staehelin, 1984; Tates, 1971). Thus, the ER structure may facilitate correct formation of the cell division apparatus, to compensate for the incomplete checkpoint system.

In summary, we have demonstrated that COPI depletion results in the failure of cytokinesis, through disrupted accumulation of vesicles at the cleavage furrow region. Mutations for a *Drosophila* golgin ortholog, Lva (which mediates Golgi transport), resulted in a similar cytokinesis phenotype (Sisson et al., 2000). The delivery of membrane components to the cleavage furrow region is thought to involve two different pathways, the secretory pathway and the endocytic pathway. COPI is related to Golgi functions, and therefore COPI-mediated membrane trafficking may be required for the delivery of vesicle components required for cytokinesis, via the secretory pathway, to the cleavage furrow zone. Nevertheless, we cannot exclude the possibility that the failure of vesicle component accumulation in COPI-depleted cells is a consequence, rather than a cause of cytokinesis defects. Detailed observations of cleavage furrow proteins in living meiotic cells are required to clarify this issue.

COPI is required for the construction of ER-based spindle envelopes, which appear mutually to interact with the central spindle microtubules. It is well known that these microtubule structures and the contractile ring are interdependent (Giansanti et al., 1998). Thus, it is possible to speculate that COPI depletion eventually influences contractile ring formation, which is essential for the initiation of cytokinesis.

Materials and Methods

Drosophila stocks

y w, or *βtubulin–GFP* stocks that has been previously described (Inoue et al., 2004) were used as a wild-type control for cytological and time-lapse studies. Stocks containing *UAS-RNAi* constructs of 52 genes, including genes for the seven COPI subunits (*αCop*, *βCop*, *β′Cop*, *γCop*, *δCop*, *εCop*, and *ζCop*), five *arf* genes encoding COPI assembly factors (*arf79F, arf102F*, *arf51F, arf72A*, and *arf84F*), and 40 other membrane trafficking-related factors, were obtained from the Vienna Drosophila RNAi Center. We used *UAS-dir2*; *bam-Gal4::VP16* (a gift from T. Noguchi) or *nanos-Gal4::VP16* for spermatocyte-specific gene induction or knockdown. The following stocks were obtained from the Bloomington Stock Center: *P{sqh-EYFP-Golgi}*, *P{sqh-EYFP-ER}*, *nanos-Gal4::VP16*, *P{GAL4-arm.S}4a P{GAL4-arm.S}4b*, and *P{SUPor-P}γCop*^{KG06383}. Protein trap stocks expressing GFP–Rtnl1 (#G00071) and GFP–PDI (#G00198III) were obtained from L. Cooley; *γCop{XP}*^{d06498} was supplied by the Exelixis Collection at Harvard; *asl*^{1} and *asl*^{2} were gifts from S. Bonaccorsi (Rebollo et al., 2004); and *orbit*^{7} was as described previously (Inoue et al., 2004).

αCOP antibody and western blot analysis

A 1.3-kb *Bam*HI–*Eco*RI fragment of *Drosophila* αCOP cDNA was inserted in-frame into expression vector pET24b (Novagen, Madison, WI, USA). The resulting plasmid expresses a fusion protein of a polypeptide corresponding to amino acids 548–972 with a stretch of 14 amino acids at the NH_3 terminus and a stretch of 18 amino acids at the COOH terminus as linkers after the COOH 6×His tag. The recombinant His–αCOP protein was purified by Ni-NTA Spin Kit (Qiagen, Valencia, CA, USA). Antiserum was prepared by injecting a guinea pig. Western blots were incubated with the antiserum diluted 1:5000, followed by incubation with HRP-conjugated anti-guinea pig IgG.

Live cell imaging of primary spermatocytes

To observe cytokinesis in living primary spermatocytes more easily, we added some modifications to the protocol described previously (Inoue et al., 2004). To examine dynamics of COPI in male meiosis, flies expressing mRFP–γCOP (a gift from N. Grieder) were used. Testes from the adult flies were dissected and cells were laid out under mineral oil (Trinity Biotech, Bray, Ireland) in open chambers surrounded by double-faced tape on clean glass coverslips without any pressure. Using this protocol, we have succeeded in continuous observation of primary spermatocytes undergoing proper spindle formation and cytokinesis for at least an hour. For a drug treatment, we carried out short term in vitro culture of primary spermatocytes according to methods described previously (Robinett et al., 2009) with minor modification. A testis complex attached with accessory gland, ejaculatory duct and pomp were dissected from adult males. A living testis complex in which ejaculatory pomp is actively contracting was selected and transferred into a culture medium consisting of modified M3 medium without bicarbonates (Sigma-Aldrich, St Louis, MO, USA) containing 10% fetal calf serum (Sigma-Aldrich) and 50% male cell extracts. To inhibit COPI assembly, αCOPI inhibitor, brefeldin A (Sigma-Aldrich, St. Louis, MO, USA) or Exo1 (Sigma-Aldrich) was directly added to the culture medium. The testis was incubated in the culture medium for 14 hours before isolation of spermatocytes at room temperature. For observation of lipid droplets, Bodipy 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacence; Molecular Probes, Eugene, OR, USA) or Nile red (Wako, Osaka, Japan) was used. Time-lapse imaging was performed on an Olympus IX81 fluorescence microscope (Olympus, Tokyo, Japan) outfitted with excitation, emission filter wheels (Olympus, Tokyo, Japan). Cells were imaged with a 40× lens. At each 30 sec time interval, near-simultaneous GFP and RFP fluorescence images were captured with a CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Image acquisition was controlled through the Metamorph software package running on a PC. Testis squashes to evaluate onion-stage spermatids were made using previous protocols (Inoue et al., 2004) and viewed by phase-contrast microscopy.

Immunofluorescence

Testis cells were fixed according to the method of Inoue and colleagues (Inoue et al., 2004). For immunostaining, anti-αCOP antibody was used at a 1:500 dilution. The following primary antibodies were used: anti-GM130 (Abcam, Cambridge, MA, USA), anti–γCOP (a gift from Wieland) (Jayaram et al., 2008), anti-centrosomin (T. Kaufman), anti-anillin (D. Glover), anti-Lva (W. Sullivan) and anti-Clathrin (S. Kametaka). For anti-Rab11 (R. Choen) staining was carried out by a method described previously (Giansanti et al., 2007). Microtubules were visualized by immunostaining with anti-α-tubulin (DM1A: Sigma-Aldrich) or expression of GFP–β-tubulin (Inoue et al., 2004). ER was visualized by expression of GFP–Rtnl1 residing predominantly in the intracellular structure. All secondary antibodies and DNA stains were commercially obtained. Images were processed and merged in pseudocolor using MetaMorph version 7.6 (Molecular Devices, Sunnyvale, CA, USA).

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