Spermatocyte cytokinesis requires rapid membrane addition mediated by ARF6 on central spindle recycling endosomes

Naomi Dyer1,2,*, Elena Rebollo3,*, Paloma Domínguez3, Nadia Elkhatib4, Philippe Chavrier4, Laurent Daviet5, Cayetano González2 and Marcos González-Gaitán1,6,†

The dramatic cell shape changes during cytokinesis require the interplay between microtubules and the actomyosin contractile ring, and addition of membrane to the plasma membrane. Numerous membrane-trafficking components localize to the central spindle during cytokinesis, but it is still unclear how this machinery is targeted there and how membrane trafficking is coordinated with cleavage furrow ingression. Here we use an arf6 null mutant to show that the endosomal GTPase ARF6 is required for cytokinesis in Drosophila spermatocytes. ARF6 is enriched on recycling endosomes at the central spindle, but it is required neither for central spindle nor actomyosin contractile ring assembly, nor for targeting of recycling endosomes to the central spindle. However, in arf6 mutants the cleavage furrow regresses because of a failure in rapid membrane addition to the plasma membrane. We propose that ARF6 promotes rapid recycling of endosomal membrane stores during cytokinesis, which is critical for rapid cleavage furrow ingression.

KEY WORDS: Drosophila, Meiosis, Spermatogenesis, Testis

INTRODUCTION

Cytokinesis is the division of a cell into two following separation of the chromosomes during anaphase. Central spindle microtubules, the actomyosin contractile ring and their regulators, drive dramatic cell shape changes during cytokinesis (Glotzer, 2005). Cytokinesis requires coordination between the central spindle and the actomyosin contractile ring (Adams et al., 1998; Jantsch-Plunger et al., 2000; Somers and Saint, 2003). Recently, membrane trafficking has also been implicated in cytokinesis (Albertson et al., 2005; Glotzer, 2005). Membrane trafficking is necessary for the massive increase in plasma membrane surface area, and can also locally enrich specific components in the cleavage furrow plasma membrane (Bluemink and de Laat, 1973, VerPlank and Li, 2005).

The central spindle complex, composed of Pavarotti (Pav), RaclGAP50C, and the associated Rho guanine nucleotide exchange factor (GEF) Pebble, is essential for communication between central spindle microtubules and the actomyosin contractile ring, probably by regulating the activity of the small GTPase RhoA (Somers and Saint, 2003). Active RhoA regulates actin polymerization, myosin II and citron kinase (Amano et al., 1996; Matsui et al., 1996; Yamashiro et al., 2003). Around 20 highly conserved proteins, including central spindle, actin myosin ring and RhoA pathway machinery are required for cytokinesis in multiple systems (Glotzer, 2005). However, recent RNAi screens have repeatedly identified membrane-trafficking components necessary for cytokinesis (Echard et al., 2004; Eggert et al., 2004; Skop et al., 2004).

The three main classes of trafficking factors implicated in cytokinesis are the class III ADP ribosylation factor, ARF6. A constitutively active, GTPase-defective ARF6 mutant, ARF6Q67L, concentrated at the central spindle and midbody of HeLa cells, and ARF6Q67L overexpression caused late cytokinesis defects (Schweitzer and D’Souza-Schorey, 2002). Knockdown of ARF6 in HeLa cells using siRNA caused a late cytokinesis block (Schweitzer and D’Souza-Schorey, 2005). Possible effectors for ARF6 during cytokinesis are the Rab11/ARF6 binding proteins Rab11FIP3 and Rab11FIP4, which ARF6 recruits to the central spindle in HeLa cells (Fielding et al., 2005; Schweitzer and D’Souza-Schorey, 2002; Schweitzer and D’Souza-Schorey, 2005). ARF6 regulates endocytosis, recycling and actin remodelling (D’Souza-Schorey et al., 1995; Radhakrishna and Donaldson, 1997; Song et al., 1998). Via these mechanisms, ARF6 affects processes such as adherens junction disassembly and cell migration, including the formation of cord-like structures by hepatocytes in the mouse liver in response to hepatocyte growth factor (D’Souza-Schorey and Chavrier, 2006; Suzuki et al., 2006). In Drosophila, the ARF6-GEF Loner/Schizo is necessary for myoblast fusion (Chen et al., 2003; and midline crossing of axons (Onel et al., 2004).

In contrast to the central spindle microtubules and the actomyosin contractile ring, little is known about temporal and spatial coordination of membrane trafficking during cytokinesis. Many membrane-trafficking components are localized to the central

1Max Planck Institute of Molecular Cell Biology and Genetics, Pfotenhauerstrasse 108, D1307 Dresden, Germany. 2Liverpool School of Tropical Medicine, Pembroke Place, Liverpool L3 5QA, UK. 3Institut de Recerca Biomédica (IRB) and Institució Catalana de Recerca i Estudis Avançats (ICREA), Parc Científic Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain. 4CNRS UMR144, Institut Curie, Membrane and Cytoskeleton Dynamics Group, 26 Rue d’Ulm 75005 Paris, France. 5Hybrigenics SA, 3-5 Impasse Reille, 75014 Paris, France. 6Département de biochimie, Sciences II, 30, Quai Ernest Ansermet CH-1211 Genève 4, Switzerland.

*These authors contributed equally to this work

†Author for correspondence (e-mail: marcos.gonzalez@biochem.unige.ch)

Accepted 20 September 2007
Development 134 (24)

spindle (Skop et al., 2004), but the molecular machinery connecting the central spindle to membrane trafficking is unclear. Here we show that cytokinesis requires ARF6 in the Drosophila male germ line. ARF6 localizes to the plasma membrane and a population of early and recycling endosomes. In dividing cells, ARF6 is specifically enriched on recycling endosomes associated with the Pav central spindle. ARF6 is not required to target recycling endosomes to the central spindle, but is required for rapid membrane addition during cytokinesis. We suggest that ARF6 enrichment on recycling endosomes at the central spindle increases the rate of recycling to the plasma membrane, thus coordinating membrane recycling with cytokinesis. We propose that ARF6 enrichment on recycling endosomes at the central spindle localizes, other punctae as ‘non central spindle’. Images were processed for contrast/brightness, levels and ‘dust and scratches’ with Adobe Photoshop 7.0 (Adobe Systems).

**MATERIALS AND METHODS**

*Drosophila* stocks

Imprecise excision of EP(2)2612 generated the deletions arf60, arf65 and arf66. Thirty nucleotides flanking sequence on each side of the deletions and the excision scar (italics) are shown, with asterisks indicating the boundaries of arf6 sequence: arf60: TATACTATGTAATGGTGATGCCCCCGGTCTTGTTTCTTTTCTCAGTCTAGTCTACACAGTGCGGCCCACTATACTGATCATCGTACAAATGTTAAAAATG; arf65: TATACTATGTAATGGTGATGCCCCCGGTCTTGTTTCTTTTCTCAGTCTACACAGTGCGGCCCACTATACTGATCATCGTACAAATGTTAAAAATG; arf66: TATACTATGTAATGGTGATGCCCCCGGTCTTGTTTCTTTTCTCAGTCTACACAGTGCGGCCCACTATACTGATCATCGTACAAATGTTAAAAATG.

**Live imaging**

Spermatocyte imaging was carried out as described (Rebollo and Gonzalez, 2004). Cell perimeter and diameter were measured in ImageJ (http://rsb.info.nih.gov/ij/). Furrow progression, perimeter and surface area rates are linear regression line slopes. Four to six confocal sections were maximally projected, except Sqh-GFP.

**Germ line clones of arf6 mutants**

Germline clones were generated using the flip/FRT systems described (Chou and Perrimon, 1992). Females (genotype y w hsflp; FRTG13Ovop2/FRTG13arf6) raised at 25°C were heat shocked for 2 hours at 38°C as third instar larvae to activate the flipase, generating germ line clones (genotype y w hsflp; FRTG13arf6/FRTG13arf6). y w hsflp; FRTG13Ovop2/FRTG13arf6 females were crossed for 3 days to wild-type males in vials supplemented with fresh yeast before collecting eggs. The same procedure was used for arf65.

**Yeast two-hybrid analysis**

PCR from clone LD22876 generated a Glu67-Leu substitution of the ARF6 ORF. Norl and SpeI overhang sites were generated by PCR and the resulting fragment cloned into pB27 bait plasmid derived from pBTM116 (Vojtek and Hollenberg, 1995). A random-primed cDNA library from 0-24 hour *Drosophila* embryo poly(A+) RNA was constructed into the pP6 plasmid derived from pGADGH (Bartel et al., 1993). The two-hybrid system was used to detect protein-protein interactions (Bartel, 1993). The library was transformed into the Y187 yeast strain. Around 10 million independent yeast colonies were collected, pooled and stored at –80°C, and over 50 million interactions tested using a previously described mating protocol (Fromont-Racine et al., 1997). Prey fragments of positive clones were PCR amplified and sequenced at 5’ and 3’ junctions. Corresponding genes were identified in the GenBank database (NCBI) using an automated procedure (Formstecher et al., 2005).

**Pav-binding assay**

DNA encoding Pav655-865 was cloned into pGEX4T1 at the GST C-terminal His6 tag was generated by PCR and the resulting fragment cloned into TOPO-XL. The 3.8 kb fragment from TOPO-XL was cloned into the pUAST vector. I and Xba overhang sites were generated by PCR and the resulting fragment excised from pUAST was cloned between the polyubiquitin vector and XbaI fragments from pUAST were cloned between polyubiquitin vector I and NotI sites of pSRalpha. A rescue construct, a 3.8 kb PCR product containing Pav655-865 was cloned into pGEX4T1 at the GST C-terminus. The XhoI-XbaI fragment from pGEX4T1 was inserted into the pUAST vector. In all cases, GST-Rab containing Notl-XbaI fragments from pUAST were cloned between polyubiquitin vector Notl and XbaI sites.

**Antibodies and microscopy**

A rabbit polyclonal antibody generated (Eurogentec) against amino acids 99-112 of *Drosophila* ARF6 (ARTELHRIINDREM) binds ARF6 at 20 kDa in western blots. Rabbit anti actin (Sigma A2066) was used 1:400. Anti-ARF6 was used 1:50 for western blotting, but was unsuitable for immunofluorescence. Embryo immunofluorescence staining was performed using standard techniques. Mouse antibody BP102 (Hybribbon) was used 1:30, and Rabbit anti MHC (Kiehart and Feghali, 1986) at 1:500. arf6 embryos zygotically rescued by CyO, hh-lacZ were identified using rabbit anti β-galactosidase (Cappel) 1:500. Testes dissected in PBS were fixed for 20 minutes in PBS containing 4% paraformaldehyde, and a further 20 minutes after the addition of 0.2% Triton X-100. After washing with PBS, subsequent staining and washing steps were performed in PBS containing 0.1% Triton X-100. A 2-hour block with 0.5% BSA was followed by overnight incubation at 4°C with 0.5% BSA and primary antibodies: rat anti-HA (clone 3F10, Roche), 1:500, rabbit anti-Parv-1:250 (Adams et al., 1998). After three 20-minute washes, primary antibodies were detected using Alexa Fluor 546-conjugated anti-rabbit (Molecular Probes) and Cy5-conjugated anti-rabbit (Jackson ImmunoResearch Laboratories) antibodies at 1:500, with 2% normal goat serum for 2 hours at room temperature. Confocal images were acquired using Zeiss LSM 510 and LeicaDMIRE2, TCS SP2 SP2 microscopes, with 63X (NA 1.4) and 100X (NA 1.4) objective lenses. Colocalization (± s.e.m.) was quantified in unprocessed images in the Zeiss LSM image browser by manually counting punctae. In dividing cells, punctae within 3 µm of Pav staining were classified as ‘central spindle’ localized, other punctae as ‘non central spindle’. Images were processed for contrast/brightness, levels and ‘dust and scratches’ with Adobe Photoshop 7.0 (Adobe Systems).
by western blotting with rabbit anti-HA antibody at 1:500 (Roche) and affinity-purified polyclonal rabbit anti-GST at 1:10,000 (Protein Expression and Purification Facility, MPI-CBG, Dresden).

Perimeter and surface area measurement
To test the suitability of perimeter as a measure of surface area, six control cells were cultured with 8 μM FM4-64 (Molecular Probes) in Schneider’s medium with 10% fetal calf serum. In these conditions spermatocytes are almost rotationally symmetrical. The cell outline of the mid confocal plane of non-tilted cells was used to calculate surface area capitalizing on this rotational symmetry, approximating the cell by a series of around 20 cone segments. Software used to calculate the surface area available upon request (pdfoster@ntlworld.com). For each cell, perimeter was roughly linearly related to surface area:

\[ P = mS + c, \]

where \( P \) is perimeter; \( S \), surface area; \( m \), slope of the linear regression line; and \( c \), intercept on the perimeter axis. The value of \( m \) was similar for different cells, whereas \( c \) was more variable (see Fig. S4C, Table S2 in the supplementary material). Therefore, without making a complete set of measurements on a cell, it is difficult to infer the surface area from the perimeter. However, the rate of perimeter change indicates of the rate of surface area change well.

Let \( P_1 \) be the perimeter, and \( S_1 \) the surface area at time \( t_1 \), and \( P_2 \) be the perimeter and \( S_2 \) the surface area at time \( t_2 \). The perimeter change between \( t_1 \) and \( t_2 \) is:

\[ P_2 - P_1 = (mS_2 + c) - (mS_1 + c) = m(S_2 - S_1). \]

Therefore, the rate of perimeter change for time period \( t_1 \) to \( t_2 \) is:

\[ \frac{P_2 - P_1}{t_2 - t_1} = m(S_2 - S_1)/(t_2 - t_1). \]

Therefore, rate of perimeter change can be used to indicate surface area rate change, since the cell-specific \( c \) term is eliminated: only the slope of the relationship between surface area and perimeter is relevant.

Cell volume was calculated as the product of the cell area in each slice and the slice separation, from stacks of optical slices taken at 0.5 μm intervals through spermatocytes expressing DE-cad-GFP.

**RESULTS**

*Drosophila arf6* is a non-essential gene required during spermatogenesis
To study ARF6-dependent endocytic trafficking, we generated deletions in the *arf6* gene by imprecise excision of the EP(2)2612 transposable element in intron 1 (Fig. 1A). A null mutation, *arf6*1, corresponding to a 1709-nucleotide deletion in the transcribed region, entirely removed the ORF (Fig. 1A). Consistently, an antibody against *Drosophila* ARF6 detected no ARF6 protein in western blots from homozygous *arf6*1 flies (see Fig. S1A in the supplementary material).

ARF6 is not essential for fly viability. Homozygous *arf6*1 progeny from homozygous mutant mothers (maternal/zygotic mutants) were viable until adulthood, presenting no overt external morphological phenotype. Recent reports show that expression of a GDP-bound dominant negative ARF6 protein (ARF6TN) impairs myoblast fusion and axon path finding during embryogenesis (Chen et al., 2003; Onel et al., 2004). Both developmental events occurred normally in *arf6* null mutant embryos (see Fig. S1B-E in the supplementary material), indicating that the ARF6TN protein causes secondary defects beyond the suppression of ARF6 function.
Female arf61 flies showed reduced fertility because of a partially penetrant requirement for ARF6 during chorion formation in the germ-line (see Fig. S2 and Table S1 in the supplementary material). Male arf61 flies were completely sterile. Mutant spermatids showed a ‘four-wheel-drive’ phenotype, indicating a cytokinesis defect during spermatocyte meiosis (Fuller, 1993) (Fig. 1B-E): over 90% of spermatids contained more than one nucleus, and 41% had four nuclei per mitochondrial Nebenkern derivative (Fig. 1E), corresponding to 79% failure in cytokinesis during the two meiotic divisions (see Fig. S3 in the supplementary material). 1.9% of cells showed 8:1 nuclei-Nebenkern ratios, suggesting that cytokinesis of gonial cell mitosis prior to meiosis is also occasionally affected, as previously suggested for other cytokinesis mutants (Brill et al., 2000; Giansanti et al., 2004).

ARF6 is required for cleavage furrow progression

Videomicroscopy of control and mutant cells during meiosis I was performed to characterize the cytokinesis defect. arf6 mutant spermatocytes expressing Histone2A-GFP and γ-Tubulin GFP showed that chromosome segregation and centrosome behavior occurs normally (not shown). α-Tubulin GFP fusion (GFP-α-tubulin) indicated that spindle initially forms normally in arf63 mutant spermatocytes (see Movies 1, 2 in the supplementary material). In arf6 mutant males, a cleavage furrow was established, but later regressed. We therefore analyzed furrow progression in wild-type and arf6 mutant spermatocytes.

In control cells, cell shape and diameter were constant until anaphase onset (see Fig. 4B and Movies 1, 3, 4, 7 and 9 in the supplementary material). After anaphase onset, cells elongated, decreasing in equatorial diameter at 0.4 μm/minute (Fig. 3B, Fig. 4C). During anaphase B, which starts 2 minutes after anaphase onset (min AA), Pav accumulated at the central spindle (Minestrini et al., 2003) (Fig. 2A, Fig. 3A, Fig. 4C; see Movie 3 in the supplementary material). The centralspindlin complex subsequently signals to the cortex, and the actomyosin contractile ring forms. Myosin regulatory light chain (Sqh-GFP) accumulated at the future cleavage furrow 1 minute after the onset of Pav accumulation at the central spindle (Fig. 2D, Fig. 3A; see Movie 4 in the supplementary material) (Adams et al., 1998; Royou et al., 2004; Somers and Saint, 2003). Shortly after Pav and Sqh accumulation, equator contraction accelerated to

Fig. 2. ARF6 is not required for central spindle or contractile ring formation. (A-E) Time-lapse images of Pav-GFP (A-C) and Sqh-GFP (D,E) during cytokinesis in control (A,D), arf6 (B,E) and chic13E mutant spermatocytes (C). Times are minutes:seconds after anaphase onset (min AA). Scale bars: 5 μm. (A) Control, Pav-GFP accumulates at the central spindle during anaphase B (arrowhead, 03:29). Central spindle microtubules labelled with Pav-GFP bundle and compact into a dense midbody (arrowheads, 09:14-59:54). (B) arf61, anaphase B Pav-GFP central spindle accumulation occurs (arrowhead, 06:54). Pav-GFP-labelled microtubules bundle (arrowhead, 15:20), a cleavage furrow initiates, but central spindle Pav-GFP signal declines (arrowhead, 24:21). After furrow regression, only a tiny amount of Pav-GFP remains at the cortex (arrowhead 50:21). (C) chic13E, anaphase B Pav-GFP central spindle accumulation occurs (arrowhead, 03:00, 06:54), but no furrow is initiated. 17:30 min AA: very little Pav-GFP remains at the central spindle. (D) Control, Sqh-GFP transfers to the cortex (arrowhead, 04:55), accumulates at the future cleavage furrow site (arrowheads, 07:05, 08:11) which then invaginates (arrowhead, 22:14). (E) arf63, Sqh-GFP transfers to the cortex (arrowhead, 04:58) concentrating at the future cleavage furrow site (arrowheads 07:01, 14:09). Sqh-GFP remains at the cortex during and after regression (arrowhead, 25:22). Genotypes: w; arf61/CyO; Pav-GFP/TM6B (A), w; arf61 arf63; Pav-GFP/TM68 (B), chic13E chic13E; Pav-GFP/TM68 (C), y w sqhX2; +; P (w+ sqh-gfp) (D), y w sqhX2; arf61arf63; P (w+ sqh-gfp) (E).
anaphase onset.

With control cells: *late regressors (blue) membrane was also added with normal kinetics (see below; Fig. 4B; the supplementary material). During furrow progression, plasma membrane indentation, the ‘cleavage furrow' appeared, when it is around 15 μm wide, the furrow regresses. After cleavage furrow collapse, Pav dissociates from the central spindle, although Sqh remains at the cortex during regression (Fig. 2E; see Movie 6 in the supplementary material).

These observations indicate that ARF6 is not necessary for targeting Pav to the central spindle, or for actomyosin ring formation. The mutant phenotypes reveal two critical phases for ARF6 function during cytokinesis: (1) an early role during cleavage furrow progression after furrow initiation, and (2) a later role in ring canal establishment at the end of cytokinesis. Since ring canal stabilization may be a specialized event restricted to germ cells, and may be due to the higher frequency of the early regressors, we decided to concentrate on the early regressors and the early role of ARF6 in furrow progression.

**Fig. 3. ARF6 is required for rapid cleavage furrow invagination.** (A) Representative results for control, arf6 mutant ‘early regressor', ‘late regressor' and chic13E illustrate furrow progression, timing of Pav accumulation at central spindle (arrowheads) and indented cleavage furrow appearance (arrows). Diameter is measured at furrow tip or future furrow site. (B) Furrow ingression rates in control (black), arf6 late regressors (blue) chic13E (green) and arf6 early regressors (red). Furrow ingression is significantly impaired in arf6 mutants compared with control cells: *P<0.01, Student's t-test. min AA, minutes after anaphase onset.

In ‘early regressors', anaphase B-cell elongation occurred. The equator contracted at 0.3 μm/minute (Fig. 3, Fig. 4C). Five minutes after Pav accumulation, a plasma membrane indentation, the ‘cleavage furrow', appeared (Fig. 2A, Fig. 3A, Fig. 4C). The furrow progressed at 1 μm/minute, finally decelerating to stop around 35 minutes after anaphase onset at width 3-5 μm (Fig. 2A, Fig. 3A, Fig. 4C; see Fig. S4A in the supplementary material). This narrow opening between the two daughter cells differentiates into the ring canal (Hime et al., 1996).

In time-lapse movies, cytokinesis failed in 89% of arf61 cells, consistent with the frequency of multinucleated spermatids (see Fig. S3B in the supplementary material). Cytokinesis failed early during furrow progression in 55% of the failing cells. The remaining 45% of failing cells were ‘late regressors', in which cytokinesis proceeds with normal kinetics of furrow formation and progression until the furrow is less than 10 μm wide. The furrow stays at this diameter for a variable time period before collapsing (Fig. 3A; see Fig. S4A in the supplementary material). During furrow progression, plasma membrane was also added with normal kinetics (see below; Fig. 4B; see Fig. S4B in the supplementary material).

In ‘early regressors', anaphase B-cell elongation occurred. The equator contracted at 0.3 μm/minute, only slightly slower than in the wild type (Fig. 3B, Fig. 4C). Pav and Sqh central spindle and contractile ring targeting occurred only slightly later than in the wild type (Fig. 2B,E, Fig. 4C; see Movies 5 and 6 in the supplementary material). However, fast equator contraction did not occur, only accelerating to 0.5 μm/minute, and indentation occurred 10 minutes, instead of 5 minutes after central spindle Pav accumulation (Fig. 2B, Fig. 3A, Fig. 4C). Shortly after the cleavage furrow indentation appeared, when it is around 15 μm

**ARF6 is required for rapid plasma membrane addition during cytokinetic cleavage furrow progression**

Spermatocytes divide, producing two daughter cells with half the volume of the mother (volume change 0.8±1.4%. n=5 cells). For spherical cells, this implies that the membrane surface increases by 26% during cytokinesis. We therefore investigated whether the arf6 phenotype is caused by a defect in membrane addition to the cell surface. The absence of surface increase could lead to an increase in membrane tension, which would counteract the forces generated by the contractile ring. This hypothesis was prompted by the established role of ARF6 during endocytic membrane recycling (D’Souza-Schorey et al., 1998; Prigent et al., 2003; Radhakrishna and Donaldson, 1997) which might be essential for rapid membrane addition from an endosomal, ARF6-dependent membrane store.

The kinetics of plasma membrane increase during meiosis I was studied by measuring cell perimeter of spermatocytes in confocal images, as well as the total surface area calculated for 3D-reconstructed cells (Fig. 4; see Fig. S4 in the supplementary material). Experiments on the relationship between perimeter and surface area changes indicated that perimeter increase correlates well with surface area increase (see Fig. S4C in the supplementary material). Plasma membrane growth during meiosis I was negligible prior to anaphase. Membrane increase started during anaphase B cell elongation at 0.6 μm/minute. This ‘perimeter rate' corresponds to a membrane addition of around 8 μm²/minute (see models in the supplementary material). The perimeter rate increased greatly (2.5-fold, corresponding to around 22 μm²/minute) directly after the onset of Pav accumulation, peaking around 15 min AA at the time of maximum furrow ingression rate and the appearance of membrane indentation (Fig. 4B,C). Subsequently, the rate decreased until the completion of cytokinesis. In arf6 mutants, slow membrane addition characteristic of early cytokinesis was maintained after furrow membrane indentation, and the rapid membrane addition phase never occurred (Fig. 4B,C; see Fig. S4B in the supplementary material). These data suggest that ARF6 is involved in rapid membrane addition to the plasma membrane, which is necessary during the rapid contraction of the actin ring during cytokinesis.

**Membrane addition to the plasma membrane is uncoupled from actomyosin ring contraction**

The arf6 mutant phenotype reveals a link between cleavage furrow progression and rapid membrane surface increase. To find out whether cleavage furrow progression defects lead to a defect in surface increase, or vice versa, we studied furrow progression and membrane addition rates in profilin chicadée (chic) mutants (Cooley et al., 1992; Giansanti et al., 1998). In chic13E mutants, the...
central spindle initially formed normally and Pav was targeted properly (Fig. 2C), but actomyosin contractile ring formation fails (Giansanti et al., 1998). As a consequence, furrow ingression kinetics are even more affected than in arf6 mutants (Fig. 3). However, in these chic13E cells, membrane addition initially occurred with kinetics similar to control cells, until the premature disassembly of the central spindle and Pav-GFP disappearance from the central spindle area (Fig. 2C, Fig. 4A,B; see Fig. S4B in the supplementary material).

These results imply that the actomyosin ring contraction and cleavage furrow progression are not essential for the rapid membrane-addition phase. Lack of rapid membrane addition is a specific feature of the arf6 mutants, not a trivial consequence of furrow ingression failure. The data leave open a possible role for the Pav central spindle during the process.

**ARF6 endosomes are associated with the Pav central spindle during cytokinesis**

We then studied the subcellular localization of ARF6, its possible association with intracellular endosomal membranes, and the Pav central spindle. We used GFP-Rab4 as a marker for early endosomes along the fast recycling route to the plasma membrane, and GFP-Rab11 to label recycling endosomes along the kinetically slower recycling route (Sheff et al., 1999; van der Sluijs et al., 1992). Functional HA-tagged ARF6 was expressed from the polyubiquitin promoter. This expression rescued the arf6 mutant cytokinesis and chiorion phenotypes (Fig. 1E; see Fig. S2D in the supplementary material). ARF6-HA was present in the cytosol and enriched at endosomes and the plasma membrane, as previously reported in mammalian cells (Fig. 5; see Fig. S5 in the supplementary material) (D’Souza-Schorey and Chavrier, 2006).

The results of the localization analysis are summarized in Table 1. Early during meiosis I cytokinesis, ARF6-positive endosomes (66% of punctae) associated with Pav-positive central spindle microtubules including the cortical microtubule population where the cleavage furrow forms (Table 1, Fig. 5A-B,D, arrowheads; see Fig. S5D in the supplementary material). This contrasts with 52% of Rab4 endosomes and 40% of Rab5 endosomes associated with the central spindle during meiosis I (Table 1). The ARF6 central spindle endosomal population corresponds mainly (87%) to Rab4-labelled endosomes.

Rab11 recycling endosomes are also recruited to the central spindle and decorated with ARF6 (Table 1, Fig. 5B). Unlike ARF6/Rab4 endosomes, Rab11/ARF6 endosomes are only enriched at the central spindle late in cytokinesis when the cleavage furrow is almost fully ingressed [4.9±0.4 μm across (mean ± s.e.m.), n=11 cells]. This suggests that both Rab4 and Rab11 recycling endosomes participate in plasma membrane addition during cytokinesis. However, the late appearance of Rab11 endosomes at the central spindle in control cells, at a time when the furrow had already collapsed in arf6 early regressors, instead suggests a role for Rab11 ring canal stabilization, which might be critically affected in the arf6 ‘late regressors’.

In summary, recycling endosomes at the central spindle contain ARF6. Is ARF6 specifically enriched in the central spindle population of Rab4 and Rab11 recycling endosomes? Most central spindle Rab4 endosomes (73%) were decorated by ARF6 whereas only 22% of the Rab4 endosomes not localized to the central spindle contained ARF6 (Table 1, Fig. 5C). Similarly, 85% of Rab11 endosomes at late central spindles contained ARF6, versus 10% elsewhere in the cell (Table 1, Fig. 5C). These data indicate that ARF6 targeting to recycling endosomes is specifically biased towards the central spindle endosomal population.

Since central spindle recycling endosomes are enriched in ARF6, we asked whether ARF6 itself targets recycling endosomes to the central spindle. We therefore observed Rab4 and Rab11 endosome distribution in time-lapse movies. In arf6’ mutants, rab4 endosomes were targeted to the central spindle as in wild-type controls (Fig. 6A,B; see Movies 7 and 8 in the supplementary material). Therefore, ARF6 does not target endosomes to the spindle, but instead functions downstream of endosomal targeting. ARF6 does not seem
ARF6 and cytokinesis

Fig. 5. ARF6 endosomes at the Pav central spindle during cytokinesis. (A,B) Fixed primary spermatocytes. (A) ARF6HA (red) and Pav (blue) immunostaining and Rab-GFP (green; Rab4 (A) and Rab11 (B)). ARF6HA colocalizes with GFP-Rab4 (A) and GFP-Rab11 (B) at the central spindle (arrowheads). (C) Colocalization frequency of GFP-Rab4 (n=17 cells) and GFP-Rab11 (n=13 cells) with ARF6HA at the central spindle during cytokinesis (grey) or elsewhere in the cell (blue). Error bars represent s.e.m. (D) Primary spermatocyte initiating furrow. ARF6HA (red) is already partially localized to the central spindle (arrowheads), labelled with Pav-GFP (green). DAPI labels chromosomes (blue). Scale bars: 5 μm.

In summary: (1) Rab4 and Rab11 recycling endosomes are targeted to the central spindle; (2) ARF6 targeting is biased towards the central spindle population of recycling endosomes; but (3) ARF6 does not target recycling endosomes to the central spindle. Instead, another machinery must target ARF6 to central spindle endosomes. ARF6 could then endow the endosomal membrane stores with rapid recycling kinetics necessary for rapid membrane addition during fast cleavage furrow progression.

ARF6 binds the centralspindlin component Pavarotti

What targets ARF6 to central spindle endosomes? Using a Drosophila embryo cDNA library, Pav was identified in a two-hybrid screen for proteins interacting with Drosophila ARF6Q67L mutant (Fig. 7A). Five clones corresponding to the Pav ORF define the ARF6-binding domain: a region adjacent to the coiled-coil domain of Pav (amino acids 727-844; Fig. 7B). Binding assays confirmed this interaction (Fig. 7C). These results suggest that Pav might contribute to ARF6 recruitment to central spindle endosomes.

DISCUSSION

Dividing a sphere into two spheres that sum up to the same volume requires a net surface area increase of 26%. During meiosis I cytokinesis in Drosophila spermatocytes, 500 μm² of plasma membrane are added in around 20 minutes, posing a logistical problem of adding plasma membrane at an average rate of over 0.4 μm²/second. In this report, we have shown that ARF6 helps to solve this problem by mediating the rapid mobilization of endocytic membrane stores in recycling endosomes at the central spindle. This is supported by two lines of evidence: (1) In arf6 null mutants, cytokinesis failed in 80% meiotic divisions (Fig. 1; see Fig. S3 in the supplementary material) owing to a defect in rapid plasma membrane addition (Fig. 4; see Fig. S4 in the supplementary material) ultimately causing furrow regression (Fig. 3; see Fig. S4 in the supplementary material); and (2) Rab4 and Rab11 endosomes at the central spindle are enriched in ARF6 (Fig. 5). Without ARF6, recycling endosomes are still targeted to the central spindle, but membrane insertion is slow.

These data suggest that ARF6 modifies the membrane dynamics of recycling endosomes to achieve a high recycling rate. The results pose the following questions: why are recycling endosomes targeted to the central spindle? How does ARF6 modify the membrane dynamics? Why are there arf6 early and late regressors? Why is ARF6 only required for male meiosis? We discuss these four issues below.

Plasma membrane addition during cytokinesis: secretory versus endocytic trafficking

Four solutions exist to the demand for rapid surface area increase during cytokinesis: (1) decreasing cell volume; (2) stretching existing membrane; (3) resolving membrane microvilli; and (4)

Table 1. Colocalization statistics of ARF6 with Rabs at endosomes

<table>
<thead>
<tr>
<th></th>
<th>ARF6*</th>
<th>Rab4*</th>
<th>Rab5*</th>
<th>Rab11†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Punctae at central spindle</td>
<td>66±4 (n=41)</td>
<td>52±6 (n=17)</td>
<td>40±5 (n=10)</td>
<td>42±8 (n=13)</td>
</tr>
<tr>
<td>Colocalising with ARF6 (central spindle)</td>
<td>73±7 (n=17)</td>
<td>ND</td>
<td>ND</td>
<td>84±6 (n=13)</td>
</tr>
<tr>
<td>Colocalising with ARF6 (not central spindle)</td>
<td>22±5 (n=17)</td>
<td>ND</td>
<td>ND</td>
<td>10±4 (n=13)</td>
</tr>
<tr>
<td>Colocalising with Rab4 (central spindle)</td>
<td>87±5 (n=17)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colocalising with Rab4 (not central spindle)</td>
<td>56±11 (n=17)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colocalising with Rab11 (central spindle)</td>
<td>37±11 (n=12)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Colocalising with Rab11 (not central spindle)</td>
<td>43±8 (n=12)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Data are presented as % of punctae ± s.e.m.; n, number of cells quantified; ND, not determined.
* Cells in all stages of cytokinesis after initiation of cleavage furrow invagination.
† Only cells in late cytokinesis (maximum diameter at cleavage furrow 13.5 μm).
delivering membrane to the surface. There are no reports of cell volume decrease during cytokinesis. Under tension, the surface of biological membranes stretches by only 2-3% before lysis, (Needham and Hochmuth, 1989). In P815Y mastoma cells, unfolding of microvilli accumulated during interphase is sufficient to account for the surface area increase during cytokinesis (Knutton et al., 1975). By contrast, microvilli in some ascidian eggs show the converse behavior, increasing in number during cleavage furrow progression and disappearing during interphase, suggesting that this mechanism is not conserved (Satoh and Deno, 1984). Cells that do not produce sufficient extra surface area between G1 and cytokinesis, must increase membrane surface during cytokinesis by delivering membrane to the surface.

Two trafficking systems control membrane addition to the plasma membrane: the secretory pathway (ER-to-Golgi-to-plasma membrane) and the endocytic pathway (recycling-endosome-to-plasma membrane). Golgi-to-endosome traffic represents a mix of these two pathways (Ang et al., 2004). Both secretory and endocytic factors are implicated in cytokinesis (Echard et al., 2004; Eggert et al., 2004; Skop et al., 2004). The relative contribution of the endocytic versus the secretory pathway to cytokinesis may depend on the rate of membrane deposition that the pathway can deliver, relative to the speed of cytokinesis. Meiosis I cytokinesis in Drosophila spermatocytes is very demanding, requiring 500 μm² expansion in 20 minutes. Endocytic recycling can make large stores of membrane available rapidly without de novo synthesis (Pelissier et al., 2003). Indeed, in BSC1 cells, endocytic recycling, but not the addition of newly synthesised membrane from the Golgi, is sufficient for the increase in plasma membrane surface during cytokinesis (Boucrot and Kirchhausen, 2007).

The analysis of membrane addition kinetics in wild-type and arf6 cells reveals two components of membrane addition: a slow ARF6-independent process and, after central spindle formation, a 2.5-fold faster addition process boosted by ARF6 (Fig. 4). The accelerated addition rate coincides with the positioning of Rab4/ARF6 endosomes at the cleavage furrow early during cytokinesis. Rab11/ARF6 recycling endosomes might be involved later for stabilization of the ring canals. The slow component might correspond to secretory trafficking, or other ARF6-independent recycling routes. Indeed, in addition to ARF6, Golgi factors such as Cog5 and Syntaxin 5 have been implicated in the process of cytokinesis in Drosophila testes (Farkas et al., 2003; Xu et al., 2002). It will be interesting to see the rate effects of mutants of these factors in comparison to arf6.

Why are some arf6 mutant cells impaired in the rate of plasma membrane addition causing early regression of the cleavage furrow, whereas other cells show a normal addition rates and only a late regression phenotype? We favor the possibility that the lack of ARF6 uncovers a natural variation in the activities of other components involved in plasma membrane insertion, or in the...
amount of endocytic membrane available for recycling. Late regressors might be those cells in which these other components or amounts of available membrane are above a certain threshold level that, if not reached, would lead to early regression. In the late regressors, although membrane addition proceeds at a normal rate, the membrane inserted independently of ARF6 might lack key components that are essential for the stability of the ring canal, and thereby for completion of cytokinesis. Such a defect, which might actually occur throughout cytokinesis, would only manifest itself later by leading to late furrow regression.

Membrane recycling from the central spindle
Is central spindle targeting of recycling endosomes functionally significant? In cleaving Xenopus embryos, most membrane insertion occurs next to the furrow (Bluemink and de Laat, 1973). As in Drosophila spermatocytes, machinery for fusion of intracellular vesicles with the plasma membrane, including the exocyst complex and syntaxin, is localized to the cleavage furrow and central spindle in many cell types (Fielding et al., 2005; Gromley et al., 2005; Jantsch-Plunger and Glotzer, 1999; Low et al., 2003; VerPlank and Li, 2005). Central spindle proteins may assemble the relevant endocytic and/or secretory factors to facilitate efficient membrane addition. The central spindle might therefore function as a sensor during cytokinesis, implementing membrane trafficking at the right time and, perhaps, at the right place.

Our data showing that ARF6 binds to Pav suggest a possible molecular link between the central spindle and the trafficking machinery. If they are not at the central spindle, both Rab4 and Rab11 recycling endosomes show low levels of ARF6 colocalization during cytokinesis, whereas most of them contain ARF6 when at the spindle. Pav might ensure local enrichment of ARF6 in central spindle endosomes. Indeed, mammalian ARFs bind MKLP1, suggesting Pav-mediated ARF recruitment (Boman et al., 1999). Yeast two-hybrid and GST pull-down experiments confirmed this interaction in Drosophila, suggesting that this might be a conserved mechanism in cytokinesis (Fig. 7).

Our data show that in the absence of ARF6, Rab4 recycling endosomes are still targeted to the spindle. Similarly, Rab11 recycling endosomes also reach the central spindle in late arf6 recessors. It therefore seems that ARF6 and Rab11 are recruited independently, with ARF6 acting downstream of Rab4/Rab11 endosome localization to mediate rapid membrane recycling. Rab11, recruited late to the central spindle, may act in cytokinesis completion as previously suggested (Fielding et al., 2005; Wilson et al., 2005). It has been proposed that ARF6 recruits Rab11 recycling endosomes to the central spindle (Fielding et al., 2005). The contrasting situation in Drosophila spermatocytes may be due to the fact that the interaction between ARF6 and human FIP3/4 is not conserved for the Drosophila FIP3/4 homologue Nuclear fallout and ARF6 (Wilson et al., 2005).

ARF6-dependent rapid recycling at the central spindle
How does ARF6 boost the recycling rate? One possibility is that ARF6 connects the recycling endosomes concentrated at the central spindle with exocyst-defined fusion sites at the plasma membrane of the cleavage furrow. The exocyst complex localizes to vesicular structures at the central spindle and cleavage furrow, which would be adjacent to the cortical central spindle ARF6 endosomes shown in this report (Fielding et al., 2005; Gromley et al., 2005). ARF6-GTP interacts with the exocyst complex subunit Sec10 (Prigent et al., 2003). ARF6 interaction with the exocyst complex may therefore mediate targeted recycling of membrane to discrete plasma membrane domains (D’Souza-Schorey and Chavrier, 2006). ARF6 might alternatively influence recycling endosome or plasma membrane phospholipid metabolism using the effector phospholipase D, a mechanism frequently implicated in regulated recycling and secretion (Brown et al., 1993; Caumont et al., 1998; Jovanovic et al., 2006; Vitale et al., 2002). Our data suggest that in the absence of ARF6, Rab4/Rab11 endosomes still contribute to a basic rate of membrane recycling, but ARF6 recruitment contributes to more efficient membrane insertion by endowing recycling vesicles with a label to perform directed exocytosis.

Life without ARF6
ARF6 is essential for meiotic cytokinesis in the testes. Occasional spermatids containing more than four nucleii in arf6 mutants are consistent with cytokinesis failure during the mitosis prior to meiosis in the spermatocytes (Fig. 1). Additionally, karyotyping of third instar homozygous arf6 larval brains revealed a low but significant frequency of tetraploidy: 4.2±2.2% (n=5 brains, 511 mitoses) in arf6L51b/arf6L51b mutants versus 0% (n=4 brains, 385 mitoses) in arf6L51b+/ heterozygotes and 0% (n=4 brains, 150 mitoses) in wild-type animals. This suggests a cytokinesis failure during mitosis in this tissue. Furthermore, there is an incompletely penetrant germ line ARF6 requirement during chorioan gonogenesis (see Fig. S2 in the supplementary material). However, most somatic mitosis and other developmental processes occur normally in individuals completely lacking ARF6.

Many other Drosophila cytokinesis mutants (e.g. fwd, gio, klp3A, fws) preferentially affect spermatocyte cytokinesis, with little or no effect on somatic cells (Brill et al., 2000; Farkas et al., 2003; Giansanti et al., 2006; Williams et al., 1995). However, it is surprising that many previously proposed ARF6-dependent processes are not affected in arf6 maternal/zygotic null mutants. For example, Loner/Schizo, which plays a role during myoblast fusion and axon path finding in Drosophila has a specific GEF activity on ARF6, but not ARF1 in vitro, and overexpression of a dominant negative GDP-bound ARF6 mutant partially phenocopies loner/schizo mutants (Chen et al., 2003; Onel et al., 2004). The lack of myoblast/neuronal phenotypes of the arf6 null mutant suggests that the real target of Loner/Schizo is another GTPase or second redundantly acting target.

In mammalian cultured cells, ARF6 mediates essential processes including cell migration, cell-cell adhesion and phagocytosis (D’Souza-Schorey and Chavrier, 2006). The mouse arf6 knockout shows a developmental phenotype consistent with impaired cell migration during hepatic cord formation (Suzuki et al., 2006). Although we have not analyzed cell adhesion, migration or phagocytosis in detail, arf6 null mutants survive to the adult stage with no overt morphological defects, which requires all these processes. Drosophila has no second arf6 gene (Lee et al., 1994). The closest homologue encoded in the genome, ARF1 (68% identical), is involved in secretory, but not endocytic trafficking (reviewed in D’Souza-Schorey and Chavrier, 2006). The mouse arf6 knockout (Suzuki et al., 2006) will tell us in the future whether these functions of ARF6 are vertebrate specific.

The authors thank Peter Foster for writing programs for the cell division model and surface area calculation, and Hybrigenics staff for the yeast two-hybrid analysis. This work was supported by HFSR, DFG, VW and EU grants to M.G.G., RTN-HPRNCT 2002-00260 COMBIO 503568 and SAF 2003-07620 grants to C.G., and a GénHommé Network Grant (02490-6088) to Hybrigenics and Institut Curie.
Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/2/4437/DC1

References


