The involvement of ARF6 in rapid membrane recycling during *Drosophila* spermatocyte cytokinesis

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Tag der Verteidigung:
All streams run to the sea,
but the sea is not full;
to the place where the streams flow,
there they flow again.
All things are full of weariness;
a man cannot utter it;
the eye is not satisfied with seeing,
nor the ear filled with hearing.
What has been is what will be,
and what has been done is what will be done,
and there is nothing new under the sun.
Is there a thing of which it is said,
“See, this is new”?
It has been already
in the ages before us.
_Ecclesiastes 1, verses 7-10_

When I applied my heart to know wisdom, and to see the business that is done on earth, how neither day nor night do one's eyes see sleep, then I saw all the work of God, that man cannot find out the work that is done under the sun. However much man may toil in seeking, he will not find it out. Even though a wise man claims to know, he cannot find it out.
_Ecclesiastes 8, verses 16-17_
# Molecular mechanisms of ARF6 action during cytokinesis

## ARF6 and cytokinesis

### Membranes and Cytokinesis

- **Initiation of Furrowing**
- **RhoA coordinated actomyosin ring formation**
- **Abscission**

### Cell biological functions of ARF6

- **ARF6 and endocytosis**
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- **ARF6 and secretion**
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- **ARF6 and cell migration**
- **Disassembly of cell-cell junctions**
- **Lamellipodium formation and migration**
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- **Communication between the central spindle and contractile ring**
- **RhoA coordinated actomyosin ring formation**
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My father Peter Foster had the idea of using the least sum of squares to calculate the probability of cytokinesis failure from data on the frequency of multinucleated cells. He performed the mathematical derivations leading to the cubic equation in the cell division model, and wrote a script to solve this equation numerically. He also wrote the script used to calculate the surface area of cells.

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Summary

Cytokinesis involves constriction of the cell at the equator. Without decreasing in volume, a spherical cell requires a net increase in the surface area during this constriction. The constriction is driven by formation of an actomyosin contractile ring, and the surface increase by addition of membrane during the formation of the cleavage furrow. Both events depend on the central spindle microtubules at the midzone of the spindle and, in particular, on the centralspindlin protein complex. The communication between the central spindle microtubules and the actomyosin ring involves binding of a GAP and a GEF for RhoA to the centralspindlin kinesin Pavarotti/MKLP1. However, it is still unclear which molecular machinery connects the mitotic spindle to membrane trafficking during cleavage furrow ingression.

ARF6 is a member of the ARF family of small GTPases, and previous studies suggest that it is an important regulator of membrane trafficking through the endocytic pathway, and cortical Actin remodelling. I generated an arf6 null mutant in Drosophila. arf6 null mutants survive to adulthood without obvious morphological defects, indicating that ARF6 is not required for Drosophila somatic development. However, ARF6 is required for cytokinesis in Drosophila spermatocytes. The centralspindlin kinesin Pavarotti, identified as an ARF6 interactor in a Yeast-2-Hybrid assay, binds ARF6 in GST pulldowns, and interacts genetically with the arf6 mutant. ARF6 localizes to the plasma membrane and a population of early and recycling endosomes. During cytokinesis, ARF6 is enriched on recycling endosomes at the central spindle. arf6 mutants form a cleavage furrow during cytokinesis, which later regresses. Cytokinesis in arf6 mutant spermatocytes lacks the rapid plasma membrane expansion observed during normal divisions.

The results of this study suggest that ARF6 might promote rapid recycling of endosomal membrane stores at the central spindle to the plasma membrane during cytokinesis. ARF6 might be recruited to the central spindle via its interaction with Pavarotti, and act as part of the molecular link between the central spindle cytoskeleton and the rapid plasma membrane addition necessary for cytokinesis.
Introduction

ARF GTPases

ARF family

The ADP Ribosylation Factors (ARFs) are small GTPases of the Ras superfamily. GTPases are proteins that catalyze the hydrolysis of Guanosine triphosphate (GTP) to Guanosine diphosphate (GDP). ARF proteins switch conformation, depending on whether they are bound to GDP or GTP. Isolated ARFs lack spontaneous GTPase activity in-vitro (Weiss et al., 1989). The hydrolysis of GTP is facilitated by GTPase activating proteins (GAPs), and the release of GDP by Guanine Nucleotide Exchange Factors (GEFs). ARFs interact with membranes via an N-terminally attached myristoyl group (Haun et al., 1993). The ARF family is divided into class I, class II and class III ARFs. ARFs have been functionally characterized in most detail in mammalian cells. ARFs were first identified (and named) as cofactors in the cholera toxin mediated ADP-ribosylation of the α subunit of heterotrimeric G proteins (Kahn and Gilman, 1984). In non-infected cells, ARFs perform functions related to regulating membrane traffic and the Actin cytoskeleton. GTP bound ARFs exert these cellular functions by binding and stimulating downstream effectors.

Humans have three class I ARFs (ARF1, 2 and 3), which regulate the generation of COPI coats in the secretory pathway (Bremser et al., 1999; Spang et al., 1998). The class III ARF, ARF6, is localized to the plasma membrane and endosomes, where it is proposed to regulate endocytic trafficking and Actin cytoskeletal remodelling. The functions of class II ARFs are not yet well understood.

The ARF family also includes ARF related protein, the Arflke (Arl) families, and the less closely related Secretion-associated and Ras related (SAR) family. ARF homologues are found in animals, plants, fungi and protists, as are SAR and Arl2 proteins (Li et al., 2004). The Arls perform a wider variety of seemingly unrelated functions, and SAR proteins regulate the formation of COPII vesicles (Barlowe et al., 1994; Burd et al., 2004).

Molecular switches

The crystal structure of ARFs in GTP and GDP bound configurations have elucidated the mechanism of conformational change (Amor et al., 1994; Menetrey et
The GTP/GDP structural cycle of proteins of the Ras superfamily involves movement of two regions: switch I and switch II. These highly conserved regions are illustrated in Figure 1. In ARFs the GDP/GTP structural cycle is additionally coupled to the interaction of the protein with membranes. The second Glycine residue at the N terminal of ARFs is myristoylated, which may tether ARFs to membranes (Haun et al., 1993; Kahn et al., 1988) or allow the interaction of the N-terminal with membrane bound exchange factors (Franco et al., 1993). When ARF is not membrane associated, a N-terminal helix with a myristoyl group blocks the change from GDP to GTP bound conformations, but membrane interaction allows this change (Randazzo et al., 1995). Also, GTP binding strengthens the interaction of ARFs with membranes (Franco et al., 1995) Unzipping and rezipping of two β strands and the reregistering of an interswitch region (the amino acids between switch I and switch II, blue box in Figure 1) allows the rearrangement of the switch I and II regions (Pasqualato et al., 2001). The SAR proteins and some Arls do not share the myristoyl mechanism of membrane interaction, but they share conserved residues in strand β3, the interswitch region, and most of switch II with the ARFs (Pasqualato et al., 2002). In the GTP bound conformation, ARF1 and ARF6 have very similar structures, but they differ more in the GDP bound form (Pasqualato et al., 2001). Crystal studies have instructed and been complemented by genetic studies involving point mutations in the protein sequence and the exchange of N (amino) and C (carboxy) terminals between different ARFs. The most commonly used point mutations are illustrated in Figure 1.
Figure 1: Alignment of Drosophila ARF protein sequences

Red boxes: “Dominant Negative” and “Dominant Active” mutations

T27 Mutation of the threonine (T) at position 27 to asparagine (N) results in a lowered affinity for GTP. ARF6T27N was initially believed to be locked into the GDP bound configuration, and has been used in many subsequent studies as the classic “dominant negative” mutant (D’Souza-Schorey et al., 1995).

T44 Mutation of the threonine (T) at position 44 to asparagine (N) results in lowered affinity for GTP, but the GDP affinity remains high (Macia et al., 2004). This T44N mutation was engineered because the T27N mutant was shown to have lowered affinity for both GTP and GDP.

Q67 Mutation of glutamine (Q) at position 67 to leucine (L) results in GTPase defective ARF proteins, locked into the GTP bound form (D’Souza-Schorey et al., 1995). Q67L is the classic “dominant active” mutant used in subsequent studies.

N48 Mutation of asparagine (N) at position 48 to arginine (R) or isoleucine (I), is an “effector domain mutation”, which prevents ARF6GTP from activating PLD (Jovanovic et al., 2006; Vitale et al., 2002b).

Brown box: Glutamine (Q) at position 37 and serine (S) at position 38 in ARF6 can be substituted by glutamic acid (E) at 37 and isoleucine (I) at 38. Glutamic acid and isoleucine are found in the equivalent position in ARF1. These residues were discovered by ARF1/ARF6 domain swaps to be in the “effector domain”: important for Actin remodelling (protrusion) functions of ARF6, but not membrane trafficking (Al-Awar et al., 2000).

GEFs, GAPs and Effectors

GEFs

In-vitro, ARF GEFs have can activate multiple ARFs, but colocalise with distinct ARFs in-vivo, suggesting specificity. ARF GEFs contain a Sec7 domain, which is sufficient for the GEF catalytic activity in vitro (Chardin et al., 1996). ARF
GEFs can be divided into low and high molecular weight classes. ARF6 GEFs are mainly low molecular weight, insensitive to the fungal toxin Brefeldin A (BFA), and contain a Pleckstrin Homology (PH) domain that recruits the protein to membranes containing Phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2) or Phosphatidylinositol (3,4,5) triphosphate (PtdIns(3,4,5)P3), (reviewed in Jackson and Casanova, 2000). ARF1 GEFs are typically high molecular weight and BFA sensitive. Identified ARF6 GEFs include ARF nucleotide-binding site opener (ARNO) and exchange factor for ARF6 (EFA6) (Franco et al., 1999; Frank et al., 1998).

GAPs

ARF GAPs contain a conserved zinc-finger motif catalytic domain. They tend to be large, multidomain proteins that may have diverse cellular functions in addition to their ARF GAP activity (Randazzo and Hirsch, 2004). At least for ARF1, interaction with different GAPs may involve different parts of the ARF protein: ASAP (ARF-GAP containing an SH3 domain (Src homology domain 3), ankyrin repeats, and a PH (Pleckstrin Homology) domain) binds Switch 1 and ARF GAP1 binds Switch 2 and helix α-3 (Goldberg, 1999; Jacques et al., 2002).

GAPs with activity on ARF6GTP include ACAP1 (Arf GAP with coiled coil, ANK repeat and PH domains) and GIT (G-protein-coupled receptor kinase-interacting target) 1 and 2. Residues Q37 and S38 in the Switch I region of ARF6 are necessary for the specific action of AKAP1 on ARF6, as substituting these residues with the equivalent residues in ARF1 (E1) blocks the response of ARF6 to AKAP1 (Klein et al., 2006).

Effectors

As in the case of GEFs and GAPs, *in-vivo* studies of ARF activity on effectors reveal a higher specificity than *in-vitro* studies. This is probably due to the specific subcellular localisations of ARFs and their effectors, which ensure that inappropriate interactors do not meet *in-vivo*. Effectors interact specifically with GTP bound ARFs. ARF6 effectors include Phospholipase D 1 (PLD1) and Phosphatidylinositol (4) Phosphate 5 Kinase α (PI(4)P5Kα).

PLD1 catalyses the hydrolysis of phosphatidylcholine (PC) to phosphatidic acid (PA) and free choline. ARF and Rho GTPases and PtdIns(4,5)P2 stimulate the
low basal activity of PLD1. PA can stimulate PI4P5 Kinase type 1 (Jenkins and Frohman, 2005). PA is a fusogenic lipid: the small head group and two fatty acyl chains give PA a cone shape that may lower the activation energy for negative membrane curvature (Kozlovsky et al., 2002). Additionally, PA may be further converted to diacylglycerol (DAG), also a fusogenic lipid and second messenger in signalling pathways.

ARF6 was identified as a GTPγ-S dependent PI(4)P5Ka activator (Honda et al., 1999). Although class I and II ARFs have PI(4)P5Kα stimulation potential in-vitro, only ARF6 colocalises with PI(4)P5Kα in membrane ruffles. PI(4)P5Ka phosphorylates PdtIns4P to produce PdtIns4,5P2.

The stimulation of PI(4)P5Kα and PLD1 by ARF6 are intricately intertwined by the PA dependence of PI(4)P5Kα and the PdtIns4,5P2 stimulation of PLD1. It has been hypothesized that ARF6 GTP can trigger large increases in PdtIns4,5P2 on the plasma membrane and endocytic membranes where it is localized (D'Souza-Schorey and Chavrier, 2006). In addition to membrane ruffling, PdtIns4,5P2 is involved in the regulation of diverse Actin cytoskeletal dynamics and membrane trafficking (reviewed in Toker, 1998). PdtIns4,5P2, PA and their derivatives are likely to mediate many of the membrane trafficking and Actin cytoskeletal regulating functions of ARF6.

**ARF6 subcellular localisation**

The distinct subcellular localisations of ARFs provide useful information about their different functions. ARF1 is localized to the Golgi apparatus (Stearns et al., 1990). In contrast, ARF6 is localized to the plasma membrane and endosomes (D'Souza-Schorey et al., 1995; D'Souza-Schorey et al., 1998; Song et al., 1998).

The distribution of ARF6 depends on cell type, GTP/GDP binding and level of expression, which has led to some controversy over ARF6 localisation. Initially, the GTPase defective mutant ARF6Q67L, and the GTP binding defective mutant, ARF6T27N, were used as models for ARFGTP and GDP localisation. D'Souza-Schorey et al monitored the localisation of transiently overexpressed ARF6, ARF6T27N and ARF6Q67L in Chinese hamster ovary (CHO) cells using an ARF6 specific antibody. In their study, wild type (WT) ARF6 was localized to the plasma membrane and some intracellular structures, whereas ARF6T27N was exclusively on
intracellular structures and ARF6Q67L exclusively at the plasma membrane (D'Souza-Schorey et al., 1995). In the same series of experiments, overexpression of ARF6 or ARF6Q67L blocked the receptor-mediated uptake of Transferrin into the cells, whereas ARF6T27N did not block uptake, instead leading to an accumulation of intracellular Transferrin and a block in recycling. This prompted the hypothesis that ARF6 is a regulator of receptor-mediated endocytosis, with the site of action possibly being endosomes. This result was contradicted by the results of a study by Cavenagh et al, using free flow electrophoresis, a fractionation approach to separate intracellular membranes on the basis of net surface charge. In this study, endosomes were identified on the basis of Horseradish Peroxidase (HRP) content after 10 minutes of uptake, and the results suggested that endogenous ARF6 protein in CHO (Chinese Hamster Ovary) cells is exclusively localized to the plasma membrane (Cavenagh et al., 1996). The different results were attributed to overexpression in the D'Souza-Schorey study. D'Souza-Schorey et al replied to this finding with a cryoimmunogold electron microscopy study using low levels of protein overexpression. This approach showed that ARF6 was localized to intracellular vesicles close to the trans Golgi network (TGN), which contained Transferrin receptor and Cellubrevin, in addition to the plasma membrane and cytosol (D'Souza-Schorey et al., 1998). These vesicles lacked the early endosomal marker HRP, the late endosomal marker mannose-6-phosphate receptor and the lysosomal associated membrane protein 3 (LAMP-3). Based on this cargo and their pericentriolar localisation and tubular-vesicular morphology, these endosomes were judged to be a recycling compartment. D'Souza Schorey et al also confirmed the earlier observations that ARF6Q67L is localized to discrete plasma membrane sites, and ARF6T27N to intracellular vesicles. Since then, using subcellular fractionation and antibodies against endogenous ARF6, ARF6 has also been found on secretory granules of neuroendocrine chromaffin cells (Galas et al., 1997) and on intracellular membranes of adipocytes by subcellular fractionation (Yang et al., 1998).

ARF6T27N, which was used in many early studies, may not have been a good model for ARF6 GDP, as it does not bind tightly to GDP, and on losing GDP it forms aggregates. Binding to the GEF EFA6 can prevent ARF6T27N from aggregating, explaining why it can sometimes still act as a dominant negative. A second mutant, ARF6T44N, has lowered GTP affinity but binds tightly to GDP. The
localisation of the ARF6T44N mutant suggests that ARF6 GDP is localized on the plasma membrane (Macia et al., 2004).

ARF6 localisation shows dynamic behaviour during the life of a cell, suggesting that the localisation and activation of ARF6 is controlled by both intracellular and extracellular stimuli. During cytokinesis, ARF6 is enriched at the cleavage furrow and central spindle region, and the levels of ARF6 GTP are raised during this stage of the cell cycle (Schweitzer and D'Souza-Schorey, 2002). In chromaffin cells, ARF6 is recruited from secretory granules to the cell surface in a calcium triggered exocytosis event triggered by treatment of cells with high levels of potassium (Vitale et al., 2002a; Vitale et al., 2002b). Recently, a fluorescence resonance energy transfer (FRET) based biosensor exploiting the interaction of ARF1 GTP and ARF6 GTP with the GGA (Golgi-localizing, gamma-adaptin ear homology domain, ARF-binding protein) and TOM (GAT) ARF binding domain of GGA1 has been developed. This allowed the visualization of the activation of ARF6-CFP (Cyan fluorescent protein) at the advancing tips of pseudopods during phagocytosis, and at plasma membrane ruffles, and for the first time made it possible to simultaneously contrast the localisation of “total ARF” with that of ARFGTP (Beemiller et al., 2006).

Cell biological functions of ARF6

Biochemical and localisation studies have already taught us much about ARF6 function. The tools developed in such studies have been invaluable for teaching us how ARF6 acts with its GEFs, GAPs, and Effectors at cell biological and developmental levels. Here I discuss, firstly at a cell biological level, four major roles of ARF6: regulation of endocytosis, recycling, regulated secretion and Actin cytoskeletal remodelling. I then go on to illustrate how the knowledge of biochemical and cell biological ARF6 functions has facilitated investigations of ARF6 function at developmental level with three examples: cell migration, myoblast fusion, and neural development. Finally, I move on to cytokinesis, an event in which ARF6 has recently been implicated, and which is the main topic of investigation in this thesis.

ARF6 and endocytosis

ARF6 has been implicated in the control of endocytosis via the clathrin-dependent pathway (D'Souza-Schorey et al., 1995). ARF6 may act in a manner analogous to ARF1 on the Golgi, by recruiting the adaptor protein AP-2 and clathrin
to the plasma membrane (Paleotti et al., 2005). However, the interaction of ARF6 with AP-2 may not be direct, but via the effector PI(4)P5K, causing the production of PtdIns4,5P2, which has been implicated in clathrin coat recruitment to synaptic membranes (Krauss et al., 2003). Alternatively, an ARF GAP might recruit clathrin coat components (Tanabe et al., 2005). In polarized Madin-Darby canine kidney (MDCK) cells, ARF6 and its GEF ARNO control clathrin mediated endocytosis of IgA from the apical side, possibly by recruiting Actin to clathrin coated pits (Altschuler et al., 1999; Hyman et al., 2006; Shmuel et al., 2006).

In addition to the clathrin-dependent pathway, ARF6 has also been implicated in the control of endocytosis via the clathrin independent pathway (Naslavsky et al., 2004; Radhakrishna and Donaldson, 1997). Cargo identified in ARF6 endosomes includes Major Histocompatibility Complex class I proteins, the Interleukin 2 receptor α subunit, E-Cadherin, Transferrin and its receptor and the Integrin β1 subunit (D'Souza-Schorey et al., 1995; D'Souza-Schorey et al., 1998; Naslavsky et al., 2004; Palacios et al., 2001; Powelka et al., 2004).

Further down the endocytic pathway, ARF6 GTP also negatively regulates the convergence of cargo endocytosed independently of clathrin, with cargo endocytosed using a clathrin-dependent pathway (Naslavsky et al., 2003).

ARF6 and recycling

ARF6 has been implicated in the endocytic recycling of the Transferrin receptor, Integrin β1 subunits and E-Cadherin (D'Souza-Schorey et al., 1998; Franco et al., 1999; Palacios et al., 2001; Powelka et al., 2004). It shows partial colocalisation with the recycling endosome markers Rab11, Transferrin receptor and Cellubrevin (D'Souza-Schorey et al., 1998). As discussed above, overexpression of mutant forms of ARF6 can lead to a recycling block. It has been speculated that ARF6 might regulate the bulk exit of membrane from recycling endosomes (D'Souza-Schorey and Chavrier, 2006). This hypothesis was prompted by the observation that in sorting endosomes in CHO cells, the bulk of the membranes and the proteins inserted in them are destined for the recycling pathway, and the bulk of the volume is destined for the lysosomal pathway (Mayor et al., 1993). Distinct populations of post endosomal vesicles bearing different proteins are produced from sorting endosomes. Formation of these vesicles is regulated by BFA insensitive cytosolic factors (Lim et al., 2001).
Use of effector domain mutants unable to bind PLD (discussed above) suggest that ARF6 uses PLD as an effector for recycling (Jovanovic et al., 2006). ARF6 may also regulate endocytic recycling via PtdIns4,5P2, since overexpression of ARF6Q67L or PI5P5K leads to the accumulation of PtdIns4,5P2-rich, Actin-coated endocytic vesicles containing cargo that is normally recycled to the cell surface (Brown et al., 2001). ARF6 also uses Sec10 as an effector for delivery of membrane from recycling endosomes to the plasma membrane (Prigent et al., 2003). Sec10 is a subunit of the exocyst (Sec6/8) complex, which targets membrane secretion to specific plasma membrane domains in yeast and human cells (Finger et al., 1998; Grindstaff et al., 1998; Hazuka et al., 1999).

**ARF6 and secretion**

ARF6 function in secretion has been investigated both in adipocytes and neuroendocrine cells. In adipocytes, in response to Insulin, ARF6 regulates secretion of the serine protease Adipsin, but not the transport of glucose transporters Glut1 and Glut4 to the cell surface (Yang and Mueckler, 1999). ARF6 is required for exocytosis of secretory granules in neuroendocrine chromaffin cells in response to Ca2+ (calcium ions) (Caumont et al., 1998; Galas et al., 1997; Vitale et al., 2002a). In neuroendocrine PC12 cells, ARF6 GDP, but not other ARFs, is localized to secretory vesicles and is recruited to the plasma membrane after the elevation of intracellular Ca2+ following stimulation of the cell with an elevated K+ (potassion ion) solution (Vitale et al., 2002b). The GEF ARNO allows the ARF6 recruited to the plasma membrane to release GDP and bind GTP. The majority of reports implicating ARF6 in secretion point to PLD as the effector, although ARF6 stimulated PI4,5P2 production has also been implicated in regulated exocytosis (Aikawa and Martin, 2003).

**ARF6 and Actin remodelling**

Control of membrane trafficking and Actin remodelling are closely interlinked functions of ARF6. ARF6 uses a similar set of GEFs, GAPs and effectors for controlling these processes, although not an identical set, since these functions can be uncoupled with an effector domain mutant (Al-Awar et al., 2000). ARF6 has been implicated in the formation of Actin-dependent structures such as membrane ruffles.
and protrusions, and a simultaneous loss of stress fibres. Activation of ARF by stimulation with aluminium fluoride, epidermal growth factor (EGF), or the overexpression of the ARF6Q67L mutant, stimulates the formation of these structures (Honda et al., 1999; Radhakrishna et al., 1999; Radhakrishna et al., 1996). ARF6 controlled membrane ruffling is mediated by the effector PI(4)P5Kinase, but since PLD2 is also translocated to ruffles it seems likely that PLD may also be used, at least to provide the PA necessary to stimulate PI(4)P5K (Honda et al., 1999). ARF6 localizes to areas of the cortex where Actin remodelling takes place, such as around the phagocytic cup, and at membrane ruffles (Beemiller et al., 2006; Song et al., 1998). Overexpression of the ARF6 GAP Centaurin-α1 inhibits EGF stimulated cortical Actin formation (Venkateswarlu et al., 2004). ARF6 can stimulate pinocytosis of fluid phase markers into moving particles with Actin tails, (Schafer et al., 2000). In physiological situations, the control of Actin cytoskeleton by ARF6 probably involves crosstalk with the Rho and Rac GTPases (Boshans et al., 2000).

**Developmental functions of ARF6**

The developmental function of ARF6 has been tested with the overexpression of GTP and GDP bound ARF mutants in cell culture, and by using an ARF6 GEF mutant in *Drosophila*. These studies have revealed roles for ARF6 in cell migration, myoblast fusion and neuronal development, which are discussed below. ARF6 function has been tested by reduction of protein levels in mouse (see below). Also, in a *C. elegans* study, RNAi (RNA interference) was used to knockdown *arf1*, *arf3* and *arf6*. ARF1 and ARF3 were required for embryonic development, but *arf6* knockdown had no effect (Li et al., 2004).

**ARF6 and cell migration**

A major mechanism of epithelial cell migration is via transition to a mesenchymal state, during which cell-cell junctions are disassembled. Epithelial polarity is reorganized into a polarization of cells in the direction of movement. Such a mechanism is used both in development, for example in *Drosophila* mesoderm formation, and in the metastasis of tumour cells (Huber et al., 2005; Smallhorn et al., 2004). ARF6 has been implicated in both of these processes as discussed below.
Disassembly of cell-cell junctions

Epithelial cells are joined apically by adherens junctions. Mammalian epithelia are additionally linked by tight junctions, which are apical to the adherens junctions. The transmembrane adherens junction component E-Cadherin mediates cell adhesion by forming extracellular Ca²⁺ dependent homophilic and heterophilic interactions, (reviewed in Leckband and Prakasam, 2006). E-Cadherin is constitutively endocytosed and recycled to the basolateral membrane in MDCK cells (Le et al., 1999).

To allow cell migration, junctions linking epithelial cells are disassembled. ARF6 impacts on cell migration by regulating the trafficking of E-Cadherin. ARFQ67L overexpression leads to adherens junction disassembly, and stimulates cell migration, by promoting the endocytosis of E-Cadherin, which is then targeted to a perinuclear endocytic compartment, where it colocalises with ARF6 and Transferrin Receptor.

Epithelial cell scattering both in cultured MDCK cells and in mouse liver development is driven by Hepatocyte Growth Factor (HGF) signal received by the tyrosine kinase receptor c-Met (Schmidt et al., 1995). Measurement of ARF6-GTP and Rac-GTP levels following HGF treatment of MDCK cells revealed an increase in ARF6-GTP levels, and for Rac-GTP levels, a transient decrease during junctional disassembly followed by an increase during cell scattering (Palacios and D'Souza-Schorey, 2003). Overexpression of ARF6T27N blocked Hepatocyte Growth Factor and Ca²⁺-induced E-Cadherin endocytosis, and inhibited cell migration (Palacios et al., 2001). ARF6Q67L expression induces dynamin dependent endocytosis of E-Cadherin by recruiting nuclear diphosphate kinase (Nm23-H1) to adherens junctions, which provides a GTP source for dynamin (Palacios et al., 2002). The transient Rac-GTP downregulation during cell scattering is dependent in ARF6 activation, since ARF6T27N overexpression blocked the transient Rac-GTP downregulation. Also, the extent of Rac-GTP downregulation was reduced by overexpression of a dominant negative version of the ARF6 effector Nm23-H1 (Palacios and D'Souza-Schorey, 2003).

An exciting extension to the cell culture studies on ARF6 and cell migration in response to HGF has recently been provided by a mouse arf6 knockout (Suzuki et al., 2006). arf6 knockout mice show embryonic lethality with a major reduction in
liver size, with an increase in apoptosis of both hepatocytes and erythroid cells. The primary defect however appears to be a failure of hepatocytes to respond to HGF. The mouse knockout provides two functional conformations of the involvement of ARF6 in the HGF response, which was previously suggested by cell culture investigations. Firstly, hepatic cord formation is defective in the embryonic liver, which resembles the HGF knockout phenotype, and secondly, foetal hepatocytes from knockout mice have a reduced capacity to form hepatic cord like structures in response to HGF (Schmidt et al., 1995; Suzuki et al., 2006).

In addition to the membrane trafficking-mediated effects of ARF6 on cell migration, ARF6-regulated Actin dynamics may also play a role. ARF6 Q67L:Q37E:S38I, is a constitutively active effector domain mutant of ARF6, able to drive trafficking functions, including the shift of E-Cadherin to the perinuclear compartment, but unable to generate Actin-based protrusions and ruffles. However, overexpression of ARF6 Q67L:Q37E:S38I is not sufficient to stimulate MDCK cell migration (Palacios et al., 2001). In the presence of Ca$^{2+}$, MDCK cells form first adherens junctions, and then tight junctions. The ARF6 GEF, EFA6, promotes the formation of tight junctions, and delays their disassembly after Ca$^{2+}$ removal using a mechanism dependent on its ARF6 GEF activity and involving the stabilization of apical Actin (Luton et al., 2004).

Lamellipodium formation and migration

Once the junctions linking epithelial cells have been disassembled, cells repolarize in the direction of migration, and may form a lamellipodium at the leading edge. The ARF6 GEF ARNO stimulates MDCK cell migration, leading to the formation of lamellipodia in a manner dependent on its ARF GEF activity, Rac activation and PLD activation (Santy and Casanova, 2001). In migrating cells, ARF6 and Rac work in tandem. During cell migration, it is necessary to restrict Rac activity to the leading edge of lamellipodia, so that movement occurs in one direction. Failure to restrict Rac activity to the leading edge would result instead in cell spreading and flattening. At the leading edge, ARNO induces ARF6 to activate Rac, using a bipartite Rac GEF, the Dock180/Elmo complex (Santy et al., 2005).

Phosphorylated α-4 integrin is localized exclusively at the leading edge of the lamellipodium, where ARF6 and Rac are active. At the sides and rear of the cell,
α-4 integrin is not phosphorylated, which allows it to bind the cytoplasmic adaptor protein Paxillin (Goldfinger et al., 2003). Paxillin binds the ARF6 GAP GIT1 (Turner et al., 1999; Zhao et al., 2000). The ARF6 GAP domain of GIT1 was necessary for the effects of GIT1 and cell spreading and migration, leading to the conclusion that downregulation of ARF6 at the sides and rear of the cell is responsible for Rac downregulation (Nishiya et al., 2005).

There is still much to discover about the function of ARF6 in cell migration in developmental systems rather than in cell culture. In addition to the cell biological functions of ARF6, an understanding of the role of ARF6 in development requires knowledge of its expression pattern. *In-situ* hybridization suggests that arf6 is expressed in a ubiquitous pattern in *Drosophila* embryos, but Northern blotting shows that arf6 mRNA is enriched in the head of adult flies, probably indicating a higher levels of expression in neurons (Lee et al., 1994; Tomancak et al., 2002). Reporter construct expression in *C. elegans* revealed expression of ARF6 in various tissues including muscle, intestine and ventral nerve cord (Li et al., 2004). In mammals, antibody staining in rat kidney and Northern Blotting on extracts from human tissues showed that ARF6 is expressed at least in brain, ling, liver, kidney and heart tissue, (El-Annan et al., 2004; Lebeda et al., 2003; Tsuchiya et al., 1991). Tissue or cell type specific GAPs and GEFs could regulate ARF6 differentially in different tissues during development and adult life. Indeed, in *Drosophila*, the ARF6 GEF Loner/Schizo is expressed in a more limited expression pattern than ARF6 itself, and may regulate ARF6 function in myoblasts and midline glial cells (Chen et al., 2003; Onel et al., 2004). Loner/Schizo function is discussed in more detail below.

**Myoblast fusion**

A role for ARF6 has been suggested in myoblast fusion, a process essential for the development of multinucleated muscle cells from mononucleated myoblasts. Myoblast fusion requires recognition and adhesion of the muscle founder cell and myoblast, alignment of these cells, and finally cell fusion (Doberstein et al., 1997; Wakelam, 1985). The ARF GEF Loner/Schizo was identified in a screen for mutants affecting somatic musculature in *Drosophila* (Chen et al., 2003). Chen et al showed that the GEF activity of Loner is required for the fusion, but not the specification, recognition or adhesion of myoblasts to founders, since versions of Loner lacking the
GEF domain or with a point mutation in the GEF domain were unable to rescue the loner mutant phenotype. Loner was necessary for the localisation of Rac to fusion sites between founder cells and myoblasts. Drosophila Rac genes Rac1 and Rac2 are redundantly essential for myoblast fusion in Drosophila (Hakeda-Suzuki et al., 2002). Loner localizes to discrete foci in muscle founder cells. Dumbfounded/Kin of Irregular-Chiasm-C (Duf), an immunoglobulin domain-containing homophilic adhesion molecule (Ruiz-Gomez et al., 2000), or Roughest (Rst), a Duf-related protein (Strunkelnberg et al., 2001), could recruit Loner to sites of contact between neighbouring S2 cells, and in duf rst double mutants, less Loner was localized to foci. Chen et al used in-vitro GEF assays to show that Loner had efficient GEF activity specifically on ARF6 and not on ARF1. Consistently, ARF6T27N overexpression in founder cells also led to a myoblast fusion defect.

In a parallel pathway to Loner localisation, Antisocial/Rolling Pebbles, a founder cell specific adaptor protein, is recruited to sites of fusion by Duf and Rst, linking adhesion receptors at the membrane with cytoskeletal components implicated in myoblast fusion, such as Myoblast City (Chen and Olson, 2001; Erickson et al., 1997; Menon and Chia, 2001). Intriguingly, the vertebrate homologue of Myoblast City is DOCK180, a protein implicated in the crosstalk between ARF6 and Rac (Santy et al., 2005).

Neural development

The roles of ARF6 in neural development have been studied both in cell culture and in Drosophila. Cell culture studies have revealed a role for ARF6 in branching and growth of processes on neural cells, whereas in Drosophila ARF6 has been implicated in axon guidance.

ARF6 and neurite elongation and branching

ARF6 has been implicated in neurite elongation and branching. During the development of a neuron, multiple processes called neurites extend from the cell body. Later, the cell becomes polarized, with one long process, the axon, and multiple short dendrites (Arimura and Kaibuchi, 2005). In cultured mouse hippocampal neurons, the ARF6 GEF ARNO is localized to sites of active process extension.
ARF6 GTP may have a negative regulatory role in dendritic branching. Overexpression of dominant negative ARF6T27N or the ARNO-E156K (ARF GEF defective) mutant enhanced dendritic branching, whereas ARF6Q67L caused a decrease in branching, and blocked the ARNO-E156K induced branching. ARF6, regulated by the GEF ARNO, may act together with Rac 1 in the control of dendritic branching, as expression of dominant negative Rac1 also resulted in enhanced dendritic branching, and Rac1 overexpression blocked ARNO-E156K induced branching (Hernandez-Deviez et al., 2002).

ARF6 may not act in the same way in the control of elongation as opposed to branching of neuronal processes, although crosstalk with Rac is also involved. An investigation in chick neurons revealed that found that ARF6 GTP positively regulates outgrowth, since overexpression of ARF6T27N inhibited rather than stimulated neurite outgrowth (Albertinazzi et al., 2003). In these neurons, the GIT family ARF GAP, p95APP1, forms a complex with the ARF GEF PIX and the focal adhesion protein Paxillin. PIX can bind the Rac effector PAK allowing cross talk between ARF and Rac mediated activities (Manser et al., 1998; Turner et al., 1999). Overexpression of p95APP1 mutants lacking GAP activity, but still able to bind PIX, led to the accumulation of this p95 complex at Rab11 recycling endosomes, and an inhibition of neurite outgrowth (Albertinazzi et al., 2003). This suggests that p95APP1-stimulated GTP hydrolysis on ARF6 is necessary for recycling and neurite outgrowth. Overexpression of p95APP1 mutants lacking GAP activity could possibly have a dominant negative effect and increase the amount of ARF6GTP, which, unlike the ARF6T27N overexpression experiment, would be consistent with the results of Deviez et al in suggesting a negative regulatory role for ARF in outgrowth. These contrasting results serve to demonstrate that the use of ARF6Q67L and ARF6T27N mutants can produce confusing results, since normal function of ARF6 probably includes cycles of GTP binding and hydrolysis.

ARF6 can also affect neural morphology independently of Rac. In the axons of cultured rat hippocampal neurons, the effects of ARF6 are mediated not by Rac1 but by PI(4)P5Kα, since in this context Rac1 overexpression did not suppress the ARNO-E156K induced branching, but PI(4)P5Kα expression massively reduced the growth and branching induced by ARNO-E156K (Hernandez-Deviez et al., 2004).
ARF6 and axon guidance

In *Drosophila*, ARF6 has been implicated in axon guidance, but the site of action is thought not to be the growing axon itself but glial cells signalling to it. The ARF6 GEF Loner/Schizo regulates the midline crossing of commissural axons, (Onel et al., 2004) by impairing signaling of Slit, a protein secreted from midline glial cells which signals to the Roundabout family ligands expressed in neurons of the central nervous system (CNS) (Battye et al., 1999; Kidd et al., 1999). *schizo* was identified in a screen for mutants affecting CNS development, as a mutant with a defect in commissure formation: in *schizo* mutants, fewer axons cross the midline of the central nervous system than in wild type (Hummel et al., 1999). Schizo expression in the midline glial cells is sufficient to rescue the *schizo* mutant phenotype. Overexpression of dominant negative Shibire (Dynamin), or ARF6T27N, also resulted in a reduction in axons crossing the midline, leading to the conclusion that Schizo might regulate Slit by modulating membrane dynamics in midline glial cells (Onel et al., 2004). In this study, the link between ARF6 and Rac activity was not investigated, although mutations in *Drosophila* Rac homologues mtl, rac1 and rac2 lead to the misrouting of longitudinal axons across the midline (Hakeda-Suzuki et al., 2002).

Cytokinesis

Cytokinesis is the division of a cell into two after the separation of the chromosomes during anaphase. Cytokinesis must be coordinated with the separation of the chromosomes in space and time, to ensure the correct partition of the cell into two sections of controlled sizes without chromosome damage. ARF6 has been implicated in cytokinesis in mammalian cells, by its localisation to the cleavage furrow and functional requirement for cytokinesis completion (Schweitzer and D'Souza-Schorey, 2002; Schweitzer and D'Souza-Schorey, 2005). In mammalian cells, ARF6 helps to complete cytokinesis by recruiting a Rab11 binding protein and Rab11 recycling endosomes to the mitotic midbody (Fielding et al., 2005; Wilson et al., 2005).

In this section I introduce first the requirements for cytokinesis in terms of the cell cycle defined timeframe in which it must take place. I then describe the stages
of cytokinesis. Third, I discuss the general role of membrane trafficking components during cleavage furrow invagination and abscission. Finally, I summarize the investigations on the specific role of ARF6 in cytokinesis in mammalian cells in culture.

The time window for cytokinesis

Cell cycle regulators must ensure that cytokinesis can only start after chromosomes are fully segregated, and may also put an upper limit on the time allowed for cytokinesis completion.

Cyclin dependent protein kinase (CDK) is a major player in the regulation of cell division. CDK is active only when bound to a Cyclin. The specific Cyclin bound to CDK plays a role in CDK target selection, so Cyclin degradation controls CDK activity and targets (Miller and Cross, 2001). Cyclin degradation requires a destruction box in the N-terminal. In Drosophila, A and B type Cyclins control distinct stages of mitosis. Cyclin A is required for entry into mitosis, and Cyclin A degradation helps to control the metaphase anaphase transition (Lehner and O'Farrell, 1990; Sigrist et al., 1995). Cyclin B is degraded at the metaphase to anaphase transition, and overexpression of Δ-Cyclin B, which lacks the N terminal destruction box and is thus non-degradable, leads to anaphase arrest after sister chromatid separation, and blocks anaphase B spindle elongation and cleavage furrow invagination (Parry and O'Farrell, 2001; Sigrist et al., 1995; Whitfield et al., 1990). Cyclin B degradation allows cytokinesis onset: a chemical inhibitor specific for CDK1 induces cytokinesis onset before chromosome separation (Niiya et al., 2005)

Cyclin B3 is normally degraded during anaphase, and Δ-Cyclin B3 overexpression blocks chromosome decondensation, compact midbody formation and nuclear envelope formation. Δ-Cyclin B3 overexpression slows but does not block the cytokinesis cleavage furrow invagination (Echard and O'Farrell, 2003; Parry and O'Farrell, 2001; Sigrist et al., 1995). Single cyclin B and cyclin B3 mutants do not prevent mitotic progression in the somatic tissues, although cyclin B mutants show some aberrant divisions, and both proteins are required for germline cell divisions. Double cyclin B/cyclinB3 mutants do block cell cycle progression, indicating that they act degenerately during mitosis (Jacobs et al., 1998; Knoblich and Lehner, 1993). After the degradation of a Cyclin, it may take some time before the relevant
phosphatase dephosphorylates the phosphorylated Cyclin/CDK1 targets. After Cyclin B3 degradation, reformation of the nuclear envelope and subsequent nuclear input of many proteins necessary for cytokinesis may close the time window available for cytokinesis, since these factors are then no longer available to perform their cytokinesis functions.

**Stages of cytokinesis**

During cytokinesis, the cell shape changes dramatically. The dramatic shape changes of the cell during cytokinesis are driven by the activity of cytoskeletal components and their regulators: the microtubules of the spindle and the actomyosin contractile ring, which serves to pinch animal cells into two, (reviewed in Glotzer, 2005). Cytokinesis can be divided into four sections, illustrated in Figure 2: cleavage site selection, cleavage furrow initiation, cleavage furrow invagination, and finally abscission. Since the central spindle plays a key role in coordinating the events of cytokinesis, the formation of the central spindle and the communication between the central spindle, RhoA and the forming actomyosin ring are also described in this section.
Figure 2: Stages of cytokinesis in an animal cell

(A) Selecting the cleavage furrow site. Astral and/or spindle microtubules send a signal to the cortex.

(B) Initiation of furrowing. Kinetocore-attached microtubules have separated the chromosomes. Chromosomal passenger proteins and the centralspindlin complex concentrate on non-kinetocore spindle midzone microtubules (and equatorial microtubules in Drosophila). Active, GTP-bound RhoA concentrates in the midzone of the cell. Phosphorylated rMLC accumulates at the cleavage furrow cortex and binds myosin heavy chain, where Actin filaments are organised into a contractile ring.

(C) Cleavage furrow ingression. Actin binding proteins such as formins and profilin organise the actomyosin contractile ring. Equatorial astral microtubules and non-kinetocore spindle midzone microtubules bundle and compact together to form a midbody. Vesicles derived from the secretory and endocytic recycling pathways associate with spindle microtubules and deliver membrane to the plasma membrane, using SNAREs (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors) and the exocyst complex. In some cell types, the plasma membrane at the cleavage furrow has a distinct composition. Septins at the furrow cortex can act as a diffusion barrier to maintain this composition.

(D) Abscission or progression to a stable ring canal. The chromosomes decondense, and the nuclear envelope reforms. Actin microfilament severing proteins such as Cofilin help to disassemble the actomyosin contractile ring. The compact midbody is enriched in membrane vesicles.

Furrow site selection

The furrow forms between the spindle poles at the equatorial cortex overlying the former metaphase plate. Microtubules are required to position the furrow, but the mechanism of cleavage site selection remains a contentious issue (reviewed in Burgess and Chang, 2005). Furrow site selection can occur correctly without chromosomes (Bucciarelli et al., 2003; Zhang and Nicklas, 1996) or centrosomes (Basto et al., 2006; Hinchcliffe et al., 2001; Matthies et al., 1996). There are four prevailing models: i) that the equatorial astral microtubules send a positive signal to the cortex at the division plane (equatorial stimulation model), ii) outer astral microtubules send a negative signal to the poles (polar relaxation model), iii) a signal from kinetocores travels via a subset of astral microtubules to the division plane (chromosomal passenger protein model) or iv) that a signal emanates from the antiparallel, non-kinetocore microtubules of the spindle midzone (spindle midzone model, reviewed in Burgess and Chang, 2005). Possibly these methods overlap and act redundantly, with each mechanism having a different relative importance in different cell types.
Initiation of Furrowing

Once the site of furrowing is selected, the physical process of initiating the furrow takes place, which is driven by the actomyosin contractile ring. The construction of the contractile ring is dependent in many cell types on the microtubules of the central spindle. Below I discuss the formation of the central spindle and the communication between central spindle and actomyosin ring, which is essential for cytokinesis. The exact function of the central spindle in cytokinesis seems to vary between model systems, but at least in *Drosophila*, central spindle components are necessary for the initiation of furrowing. One of the earliest events to be detected in cytokinesis before a visible initiation of furrowing is the accumulation of active (GTP bound) RhoA at the cortex beneath the cleavage furrow (Bement et al., 2005; Yoshizaki et al., 2003). As discussed below, active RhoA plays a key role in activating Actin-binding proteins such as myosin to form an actomyosin contractile ring.

Formation of the central spindle

The central spindle consists of antiparallel, non-kinetocore microtubules. Conserved central spindle components have been given different names in different model systems, and are referred to here in the following order: *Drosophila/human/C.elegans* homologue. At anaphase onset, inactivation of CDK1 allows the dephosphorylation of the microtubule bundling protein Fascetto/PRC1/SPD-1, allowing it to bind microtubules and organize the central spindle (Mollinari et al., 2002; Verbrugghe and White, 2004; Verni et al., 2004). The kinesin like protein, KLP3A/KIF4/KLP-19 helps to organise the central spindle by restricting Fascetto/PRC1/SPD-1 localisation to a narrow region of the central spindle (Kurasawa et al., 2004; Williams et al., 1995). Chromosomal passenger proteins of the AuroraB complex (AuroraB/AuroraB/AIR-2, Incenp/Incenp/ICP-1, Deterin/Survivin/BIR-1 and Borealin/Borealin/CSC-1) transfer from chromosomes to the central spindle, where they are required to localize the central spindlin complex to a narrow region (Schumacher et al., 1998; Severson et al., 2000). The centralspindlin complex, which consists of the Kinesin Pavarotti/MKLP1 (mitotic kinesin-like protein 1) /ZEN-4, RacGAP50C/MgcRacGAP/CYK-4, and a non stochiometric
component, Pebble/ECT2/LET-21 (a Rho GEF), is also inhibited from binding microtubules prior to anaphase onset by CDK1 phosphorylation of Pavarotti (Mishima et al., 2002; Mishima et al., 2004; Somers and Saint, 2003). After anaphase onset, Pavarotti/MKLP1/ZEN-4 is released from this inhibition by CDC14-mediated dephosphorylation, allowing the centralspindlin complex to bind and bundle microtubules (Mishima et al., 2004; Nislow et al., 1992). Pavarotti/MKLP1/ZEN-4 additionally binds Polo/Plk/Plc, a Kinase, with which it is codependent for localisation to the central spindle during cytokinesis (Adams et al., 1998; Carmena et al., 1998). The exact role of Polo/Plk/Plc in cytokinesis has been difficult to establish because it is also necessary for the earlier events such as formation of an organised spindle (Donaldson et al., 2001; Llamazares et al., 1991; Sunkel and Glover, 1988). However, a hypomorphic polo mutant in Drosophila exhibits cytokinesis defects in the spermatocytes (Carmena et al., 1998).

Communication between the central spindle and contractile ring

The mode of communication between the microtubules of the central spindle and the forming actomyosin contractile ring has already been partially unravelled. The centralspindlin complex is essential for this communication in Drosophila. In Drosophila pavarotti mutants, the central spindle is defective and no contractile ring is formed, although there may be redundant pathways for furrow formation in other systems, since in C. elegans and H. sapiens, depletion of Pavarotti homologues does not block the initiation of furrowing, instead leading to a later regression (Adams et al., 1998; Raich et al., 1998; Verni et al., 2004). In higher eukaryotes, the centralspindlin complex probably communicates with the contractile ring by regulating the activity of the small GTPase RhoA (Somers and Saint, 2003). Pebble/ECT2/LET-21 shows GEF activity for several small GTPases in-vitro, but in-vivo probably activates Rho (Prokopenko et al., 1999; Tatsumoto et al., 1999). RacGAP50C/MgcRacGAP/CYK-4 has GAP activity on several small GTPases in vitro, including RhoA and Rac. (Jantsch-Plunger et al., 2000). It is not yet clear if the essential function of RacGAP50C during cytokinesis in-vivo is as a RhoA GAP. RacGAP50C localisation to the central spindle would position it conveniently for the regulation of RhoA activity in late cytokinesis. In Drosophila there is also genetic evidence for a role of RacGAP50C in the downregulation of Rac GTPases, necessary
for successful cytokinesis (D'Avino et al., 2004). It is not yet clear how a potential RhoA GAP and GEF could function in the same complex to regulate RhoA activity.

**RhoA coordinated actomyosin ring formation**

Active RhoA in turn is a key regulator of Actin polymerization, non muscle Myosin II and Citron Kinase (Amano et al., 1996; Matsui et al., 1996; Yamashiro et al., 2003). RhoA stimulates Actin polymerization by relieving the autoinhibition of the Actin nucleator formin (Diaphanous in *Drosophila*) (Watanabe et al., 1999). In higher eukaryotes, active RhoA also releases Myosin II from an autoinhibited state by regulating the regulatory light chain (rMlc): RhoA activates Rho Kinase, which phosphorylates rMlc (Amano et al., 1996; Matsui et al., 1996). In yeast and *Dictyostelium*, myosin is regulated by different mechanisms not discussed here. Additionally, RhoA activates the myosin phosphatase targeting subunit, preventing rMlc dephosphorylation (Kimura et al., 1996). Citron kinase, which is activated by RhoA, and Myosin Light Chain Kinase, may also contribute to rMlc phosphorylation (Shandala et al., 2004). Phosphorylated rMlc accumulates in the midzone between the separating chromosomes after their segregation, but before the onset of furrowing (Matsumura et al., 1998). Phosphorylation of rMlc is necessary for cytokinesis, since non-phosphorylatable versions cannot rescue the *spaghetti squash* (*sqh*) mutant which lacks endogenous rMlc in *Drosophila* (Jordan and Karess, 1997). rMlc concentrates at the site of cleavage furrow formation prior to invagination (Royou et al., 2004). Phosphorylation of rMlc relieves the autoinhibition of myosin heavy chain, promoting the formation of myosin filaments and myosin ATPase activity (Scholey et al., 1980).

**Furrow ingression**

Phosphorylation of rMlc triggers the formation and constriction of the contractile ring, but several other Actin-binding proteins are required to control the polymerization, capping and severing of Actin during constriction. These are Profilin, which binds Actin monomers and caps filaments, the Formins, which nucleate unbranched filaments and favour filament growth, and Cofilin/ADF, which is an Actin severing and monomer binding protein (Castrillon and Wasserman, 1994; Gunsalus et al., 1995; Romero et al., 2004). Annilin, an Actin, myosin II and Septin
binding protein, is also required for cytokinesis in some systems, but its biochemical activity is yet to be elucidated (Field and Alberts, 1995; Field et al., 2005a; Giansanti et al., 1999; Oegema et al., 2000). During contraction, some evidence supports the idea that there is feedback from the contractile ring to the central spindle. In *Drosophila* spermatocytes treated with the Actin depolymerising agent Cytochalasin B, or mutant for *chickadee* (Profilin), or *diaphanous* (formin), the actomyosin ring does not form and the central spindle is absent or less dense than normal by telophase (Giansanti et al., 1998). *twinstar* mutants, which lack Cofilin, can form a disorganized contractile ring containing too much Actin, but have normal central spindles at telophase (Gunalsal et al., 1995). This suggests that the feedback mechanism from the contractile ring to central spindle requires the presence or the constriction of the contractile ring, but not normal contractile ring morphology.

**Abscission**

Abscission is the final stage in cytokinesis in which the actomyosin contractile ring and central spindle are disassembled and the cytoplasmic connection between daughter cells is severed. The dramatic shape changes of cytokinesis can however, leave their mark on some cells in the form of a division scar or a specialized cytoplasmic bridge, called a ring canal, connecting daughter cells after cytokinesis. It is possible that fusion of vesicles between the closely apposed plasma membrane achieves abscission. The exocyst complex and vesicles with SNARES (soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptors) localize to a late “midbody ring” structure and are required for abscission (Gromley et al., 2005). Actomyosin ring disassembly is also essential for cytokinesis completion, since mutants for Cofilin, which cannot disassemble the Actin ring, fail in late cytokinesis (Gunsalus et al., 1995; Ono et al., 2003).

**Membranes and Cytokinesis**

In addition to the central spindle and actomyosin contractile ring, membrane trafficking through the secretory and endocytic recycling pathways has also been implicated in cytokinesis (Albertson et al., 2005; Glotzer, 2005). Here I discuss briefly some historical ideas about membrane trafficking during cytokinesis, why this
area may have been neglected, and what function membrane trafficking is likely to play in cytokinesis. I then go on to discuss the main mechanisms by which membranes can be changed in amount and composition during cytokinesis, namely by membrane trafficking, diffusion barriers and lipid modification. The limited information available on the control of membrane trafficking during cytokinesis is then discussed before a more detailed examination of the involvement and control of ARF6 in its role of regulating membrane traffic during cytokinesis.

A brief history of membrane traffic in cytokinesis

The idea of membrane trafficking playing an essential role in cytokinesis is not new. In plant cells, it has long been recognised that the phragmoplast, a structure of Golgi-derived vesicles associated with spindle derived microtubules and Actin filaments, brings components to the midzone to build a new cell wall between the daughter cells (reviewed in Staehelin and Hepler, 1996). In animal cells, recent RNAi screens have also repeatedly identified membrane trafficking components as necessary for cytokinesis (Echard et al., 2004; Eggert et al., 2004; Skop et al., 2004). Typically RNAi knockdown, chemical inhibition, or mutation of membrane trafficking components does not prevent the initiation of furrowing, but either cause regression of the cleavage furrow or a failure in abscission.

The study of membrane trafficking during animal cell cytokinesis may have been delayed by the idea of a generalised shutdown in membrane trafficking during mitosis of both secretion and endocytosis, even though membrane trafficking resumes during telophase (Berlin and Oliver, 1980; Featherstone et al., 1985; Pypaert et al., 1991; Warren et al., 1984). Endocytic recycling is also likely to slow down during division prior to telophase, since phosphorylation of the fast recycling route regulator Rab4 during mitosis by p34\(^{\text{cdc2}}\) prevents its association with early endosomes (van der Sluijs et al., 1992a). Despite multiple studies concerning the rate of membrane traffic during cell division, little is known about the mechanisms controlling it. Some studies deal with the fragmentation of the Golgi apparatus, which might help it to partition equally to daughter cells, (reviewed in Rabouille and Jokitalo, 2003). The distribution of endosomes prior to cytokinesis has also been examined, with initial studies suggesting that different compartments retain their identity throughout the division (Bergeland et al., 2001; Hobdy-Henderson et al., 2003).
Membrane trafficking is necessary for the massive increase in the surface area of the plasma membrane, which is necessary to allow the drastic cell shape changes during cytokinesis, including the constriction of the actomyosin ring (Bluemink and de Laat, 1973). In addition, membrane trafficking can be used for the local enrichment of specific components in the plasma membrane at the cleavage furrow (VerPlank and Li, 2005).

Membrane trafficking components implicated in cytokinesis

Trafficking factors implicated in cytokinesis fall into three main classes: components of the secretory pathway, endocytic/recycling factors and membrane fusion machinery. Golgi proteins required for the secretory pathway such as Cog5 and Syntaxin5 have been implicated in cytokinesis (Farkas et al., 2003; Xu et al., 2002). Blocking Golgi function with Brefeldin A, an inhibitor of high molecular weight ARF1 GEFs, prevents vesicle accumulation at the furrow apex and blocks cytokinesis (Skop et al., 2001). Endocytic recycling factors such as Rab11 and Rab11FIP3/Arfophilin have been found associated with the central spindle and furrow cortex, and are necessary for cytokinesis completion (Skop et al., 2001; Wilson et al., 2005). Machinery for heterotypic membrane fusion of vesicles with the plasma membrane such as the exocyst complex and t- and v-SNAREs is also localized at the mitotic midbody and furrow cortex, and is necessary for cytokinesis (Fielding et al., 2005; Finger et al., 1998; Gromley et al., 2005; Jantsch-Plunger and Glotzer, 1999; Low et al., 2003).

Diffusion barriers

Evidence from yeast and Xenopus suggests that different membrane compositions are maintained at the cleavage furrow and the rest of the plasma membrane by means of diffusion barriers. In cleaving Xenopus oocytes, the diffusion coefficient and mobile fraction of fluorescent lipids in the newly inserted furrow membrane and the pre-existing vegetal plasma membrane is similar. However, there is no movement of lipid between these two domains, suggesting that a diffusion barrier separates the cleavage furrow surface and the pre-existing plasma membrane (Tetteroo et al., 1984). Septins are GTPases which form filaments at the cleavage
furrow and yeast bud neck, and are required in some systems for cytokinesis (Fares et al., 1995; Neufeld and Rubin, 1994; Surka et al., 2002). In yeast, Septins form a diffusion barrier either side of the contractile ring, preventing the lateral diffusion of the exocyst complex, chitin synthase and polarizome out of the contractile ring area, although they could freely diffuse within it. Temperature sensitive septin mutants allowed diffusion of proteins out of the contractile ring area of the plasma membrane. Septin mutants are still able to make an actomyosin ring, but show defects in ring contraction and disassembly, indicating that the enrichment of certain proteins or lipids in the overlying plasma membrane might be required for normal ring contraction (Dobbelaere and Barral, 2004).

**Phosphoinositides and phospholipid composition at the cleavage furrow**

The composition and properties of membranes can be altered by membrane trafficking, but also by the modification of components already present in a membrane, or their transfer between inner and outer leaflets of the bilayer, which can also lead to the recruitment of different peripheral membrane proteins.

There is evidence that the composition of the plasma membrane changes during cytokinesis. In CHO cells, phosphatidylethanolamine (PE) is predominantly present in the inner leaflet of the plasma membrane. However, during cytokinesis, PE is enriched in the outer leaflet at the cleavage furrow (Emoto et al., 1996). Immobilization or depletion of surface PE leads to a late failure in cytokinesis (Emoto et al., 1996; Emoto and Umeda, 2000). As PE is a cone shaped lipid, this redistribution may allow different membrane curvatures and facilitate plasma membrane fusion at the end of cytokinesis.

Lipid modifying enzymes such as phosphoinosotol kinases, and their substrates and products have been implicated in cytokinesis in multiple systems. In *Drosophila*, *four wheel drive*, which encodes a phosphatidylinositol (4) Kinase (PI4K), (which produces PtdIns(4)P, a precursor of PtdIns(4,5)P2) is required for cytokinesis of spermatocytes (Brill et al., 2000). A survey of the localisation of phosphatidylinosotol phosphates using specific PH and PX domain containing probes revealed that PtdIns(4,5)P2, but not PtdIns(3)P, PtdIns(4)P PtdIns(3,4)P2 or PtdIns(3,4,5)P3, is enriched at the cleavage furrow during cytokinesis in a wide variety of mammalian cell types (Field et al., 2005b). Production of PtdIns(4,5)P2, by
PI(4)P5Kinase, is necessary for cytokinesis of mammalian cells. Overexpression of the Phospholipase C δ PH domain sequesters PtdIns(4,5)P2 and causes a cytokinesis delay or detachment of the contractile ring from the plasma membrane (Emoto et al., 2005; Field et al., 2005b). In Drosophila, PtdIns(4,5)P2 is also present at the plasma membrane of dividing spermatocytes, although it is not enriched at the cleavage furrow (Wong et al., 2005).

What role might PtdIns(4,5)P2 play in the plasma membrane of the cleavage furrow? Since several components at the furrow cortex and actomyosin contractile ring, including septin and profilin, contain PtdIns(4,5)P2-binding PH domains, PtdIns(4,5)P2 may serve to anchor the contractile ring to the plasma membrane (Lassing and Lindberg, 1985; Zhang et al., 1999). However, the role of PtdIns(4,5)P2 at the plasma membrane overlying the cleavage furrow may not be direct. Cleavage of PtdIns(4,5)P2 generates Inositol(1,4,5) trisphosphate (IP3) and DAG. IP3 is involved in the regulation of Ca2+ release from intracellular stores, (reviewed in Berridge, 1993). Ca2+ may regulate contraction of the contractile ring or stimulate vesicle fusion with the plasma membrane by triggering SNARE complex formation (Chen et al., 1999). The presence of PtdIns(4,5)P2 and its subsequent hydrolysis is necessary for the completion but not the initiation of furrow ingression in Drosophila spermatocytes (Wong et al., 2005).

Control of membrane traffic during cytokinesis

Insertion of internal membrane stores to the plasma membrane seems to be dependent on spindle microtubules, but independent of the actomyosin contractile ring. Cleaving Xenopus eggs are a useful system to study membrane insertion, because of their large size, and because newly inserted unpigmented membrane at the cleavage furrow can be distinguished from original pigmented plasma membrane. Cytochalasin B, which depolymerises Actin filaments, does not block new membrane insertion in Xenopus eggs, although cleavage furrow ingression is prevented (Bluemink and de Laat, 1973). Inactivation of Rho by treatment with C3 transferase or injection of dominant active or dominant negative forms of Rho or Cdc42 blocks the ingression of the actomyosin contractile ring but membrane insertion still occurs, as assayed both using unpigmented membrane and surface labelling experiments (Drechsel et al., 1997). On the other hand, depolymerization of microtubules by cold
or nocodazole during cleavage blocks new membrane insertion in *Xenopus* eggs, and D$_2$O (heavy water), which induces ectopic monasters of microtubules, causes ectopic membrane expansion (Danilchik et al., 2003; Danilchik et al., 1998).

Beyond the concept that microtubules regulate membrane traffic during cytokinesis, there has been little progress in the understanding of the molecular mechanisms involved. One further example is provided by the phosphatidylinositol proteins (PITPs), which bind lipid monomers of phosphatidylinositol and phosphatidylcholine, facilitating their transfer between cellular membranes. PITPs have been proposed to play a role in the formation of secretory granules, in trafficking through the Golgi apparatus and in lipid metabolism (reviewed in Cockcroft, 2001). In human and *Drosophila*, PITPs have been implicated in the ingestion of the cleavage furrow during cytokinesis (Litvak et al., 2002). Nir2, a human PITP, is Golgi-localized during interphase, but during mitosis it is phosphorylated by CDK1, allowing it to interact with Polo Kinase and thus be targeted to the cleavage furrow and central spindle (Litvak et al., 2004). In the *Drosophila* PITP mutant *giotto/vibrator*, the central spindle and actomyosin ring form normally, but the furrow ingresses more slowly than in normal cells, and then regresses (Gatt and Glover, 2006; Giansanti et al., 2006). Golgi derived vesicles accumulate in the central spindle region, suggesting a role for PITPs for facilitating fusion of such vesicles with the plasma membrane in this context (Giansanti et al., 2006).

**ARF6 and cytokinesis**

Here I discuss in more detail the function of ARF6 in cytokinesis. An initial investigation on ARF6 localisation and activation during cytokinesis was followed by a confirmation that ARF6 is required for cytokinesis by Schweitzer and D’Souza Schorey. Meanwhile, work on the Arfophilin family of ARF/Rab11 interacting proteins provided insight into the molecular mechanism of ARF6 function during cytokinesis in mammalian cells.

**ARF6 localisation and activation during cytokinesis**

One of the membrane trafficking components recently implicated in cytokinesis is the class III ARF, ARF6 (Fielding et al., 2005; Schweitzer and D'Souza-Schorey, 2002; Schweitzer and D'Souza-Schorey, 2005). The first indication
that ARF6 is involved in cytokinesis was a study in which ARF6 mutants were overexpressed in HeLa and Jurkat cells (Schweitzer and D'Souza-Schorey, 2002). Endogenous ARF6 concentrates adjacent to the cleavage furrow and midbody. During cytokinesis ARF6Q67L concentrated first at the cleavage furrow, and at later stages concentrated in the midbody. In contrast, ARF6T27N was cytoplasmic, showing no enrichment at the mitotic midbody. Overexpression 80 to 100 fold over endogenous levels of ARF6Q67L, but not ARF6T27N, caused late cytokinesis defects in cells that went through mitosis. Both ARF6Q67L and ARF6T27N overexpression lowered the proportion of cells going through mitosis with respect to controls. A pulldown assay revealed that between 90-110 mins after release from a nocadazole arrest, ARF6GTP levels were raised to five times the normal level, although total levels of ARF6 protein remained constant, suggesting that ARF6 is activated during late cytokinesis. PtdIns(4,5)P2 localisation during cytokinesis was assayed using a PH-GFP (Pleckstrin Homology-Green Fluorescent Protein) fusion, and found to label the plasma membrane evenly. Overexpression of ARF6, ARF6Q67L, ARF6Q67L/Q37E/S38I or ARF6T27N did not cause obvious defects in actomyosin ring formation or PtdIns(4,5)P2 localisation. Schweitzer and D’Souza Schorey concluded from this initial study that the role of ARF6 in cytokinesis is likely to involve its membrane trafficking functions and not its functions in phospholipid metabolism or Actin remodelling activity (Schweitzer and D'Souza-Schorey, 2002).

Requirement for ARF6 during cytokinesis

ARF6 function is required for cytokinesis completion in HeLa cells (Schweitzer and D'Souza-Schorey, 2005). Reduction of ARF6 levels by 50-70% using siRNA (short interfering RNA) caused a late cytokinesis block. In the same study by Schweitzer and D’Souza-Schorey, the DNA (deoxyribonucleic acid) binding protein Ku70 was identified by a Yeast-2–hybrid screen as an ARF6 interactor. Ku70 is part of the DNA-dependent protein kinase complex, (reviewed in Tuteja and Tuteja, 2000) which is required for growth and cell proliferation in mice (Nussenzweig et al., 1996). Co-immunoprecipitation confirmed that Ku70 interacts with ARF6, and pulldowns on mitotic extracts suggested that more Ku70 interacts with ARF6 during mitosis than during interphase. However, Ku70 distribution is not affected in cells overexpressing

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ARF6 mutants, and 95% knockdown of Ku70 using siRNA had no effect on mitotic progression or mitosis, casting doubt on the in-vivo relevance of this interaction.

Molecular mechanisms of ARF6 action during cytokinesis

The Arfophilin family of ARF interacting proteins

A second line of investigation that has implicated ARF6 function in cytokinesis is the study of the Rab11/ARF interacting protein family, the Arfophilins/Arfaptins/FIPs. Arfpatin 1 and 2 were identified in a yeast-2-hybrid screen as interactors of the GTP bound form of the class I ARF, ARF3 (Kanoh et al., 1997). Arfophilin was first identified in a yeast-2-hybrid screen as an interactor of the class II ARF, ARF5 (Shin et al., 1999). The C terminal coiled-coil region of Arfophilin bound ARF5 in a GTP dependent manner in GST (Glutathione S-Transferase) pulldowns. It was later reported that in GST pulldowns, ARF6 in CHO cell lysates also binds full length or the C-terminal of Arfophilin and Arfaptin-2, although an attempt to confirm the results using yeast-2-hybrid led to inconclusive results (Shin et al., 2001). Arfaptin-2 was again identified as an interactor of ARF5 in a yeast-2-hybrid screen in 2003, and renamed Arfophilin-2, with Arfophilin being renamed Arfophilin-1 (Hickson et al., 2003). By this stage, Arfophilin 1 had two other names: Eferin, as it was identified as an EF hand containing Rab11 Interacting protein (Prekeris et al., 2001) and Rab11-FIP3 (Family of Rab11 interacting proteins) (Wallace et al., 2002). In human, Arfophilin-2 was found to be abundant in testes and to localize to a perinuclear compartment, near centrosomes and focal adhesions. Overexpression of GFP-arfophilin-2 led to an accumulation of Rab11 and Transferrin receptor in a perinuclear compartment, although it did not appear to cause quantifiable effects on Transferrin internalization or recycling (Hickson et al., 2003).

Arfophilins, ARF6 and Rab11 action during cytokinesis

Accumulating evidence implicating Rab11 in cytokinesis prompted another study on Arfophilin, which revealed that Arfophilin-1/Rab11-FIP3 concentrates at the cleavage furrow and “intracellular bridge” (late mitotic midbody) during cytokinesis (Horgan et al., 2004). In addition to Arfophilin-1, Arfophilin-2 (now also called Rab11-FIP4) was also shown to localize to the late mitotic midbody during...
cytokinesis (Wilson et al., 2005). The requirement of Rab11-FIP3 and Rab11 in mammalian cells for a late cytokinesis event, subsequent to midbody formation, was confirmed by using siRNA knockdown. A mutant version of Arfophilin1/FIP-3 unable to bind Rab11 showed that Rab11 binding is necessary for the recruitment of Arfophilin1/FIP3 to endosomes, but not for Arfophilin1/FIP3 targeting to the mitotic midbody. The signal targeting Arfophilin1/FIP3 to the midbody was not identified. Overexpression of mutant Arfophilin1/FIP3 unable to bind Rab11 blocked cytokinesis. Timelapse imaging of Arfophilin1/FIP3 GFP showed that Arfophilin1/FIP3-labelled structures transfer to centrosomes just after furrow initiation, then to the cytokinesis cleavage furrow until abscission, when they move back to centrosomes. Finally, GTP bound ARF6 was proposed as the signal targeting Arfophilin1/FIP3 and Arfophilin2/FIP4 to the midbody during cytokinesis (Fielding et al., 2005). ARF6Q67L overexpression increased the recruitment of Arfophilin1/FIP3 and Arfophilin2/FIP4 to the midbody, but had no effect on Rab11 targeting, whereas ARF6T27N blocked this recruitment, and also reduced the amount of Rab11 at the midbody. In the same study, the Exocyst complex component Exo70p was shown to interact with Arfophilin1/FIP3 and Arfophilin2/FIP4 by coimmunoprecipitation. Exo70p knockdown inhibited cytokinesis and reduced the amount of Arfophilin1/FIP3 and Rab11 recruitment to the midbody. Fielding et al proposed the model shown in Figure 3.
**Figure 3**

1. FIP proteins can bind Rab11 and ARF6. During late anaphase, Rab11 recycling endosomes with FIPs transfer to centrosomes.
2. During cytokinesis, recycling endosomes with FIPs and Rab11 are transported to the cleavage furrow using motors along microtubules.
3. ARF6GTP, which is independently targeted to the cleavage furrow, possibly by the exocyst complex, might recruit FIPs and hence Rab11, and tether them to the furrow. ARF6 might target Rab11 recycling endosomes for exocytosis via its interaction with the exocyst complex.
4. Late in cytokinesis, clusters of FIP3 positive vesicles might be tethered at the midbody ring, where their fusion might aid abscission.

**Is ARF6 function during cytokinesis conserved in other organisms?**

In a bioinformatic analysis, *Drosophila* Nuclear Fallout (Nuf) was identified as the homologue of Arfophilin-1 and Arfophilin-2 (Hickson et al., 2003). Nuclear Fallout is required for proper Actin organization during metaphase furrow formation of cortical syncytial nuclear divisions (Rothwell et al., 1998; Sullivan et al., 1993). During prophase, Nuf is associated to centrosomes. Together with Rab11, Nuf recruits membrane vesicles containing Discontinuous Actin Hexagon to the furrow tips (Riggs et al., 2003; Zhang et al., 2000). However, the mechanism via which Nuf/Arfofilin/FIP is involved in cytokinesis may be slightly different in Mammalian and *Drosophila* cells. *Drosophila* Nuclear fallout bind Rab11, but the ARF6 binding region is less well conserved and may not bind ARF6 (Wilson et al., 2005).
Aims of this study

When this study was commenced, ARF6 had been studied almost exclusively in cell culture. Furthermore, the main tools for assaying ARF6 function were the artifact-prone overexpression of dominant active GTP bound and dominant negative GDP bound mutants. Studies in cell culture hinted that the biochemical and cell biological functions of ARF6 might be used in interesting ways in development, particularly in adherens junction disassembly, cell polarity and neural development. However, virtually no attempt had been made to test whether ARF6 was required for these processes by means of knocking down ARF6 protein levels, leaving open the question of whether these processes specifically depend on ARF6. Additionally, the developmental functions suggested for ARF6 had not been tested in model organisms. Only recently has ARF6 been knocked down in other model organisms: the C.elegans study by Li et al in was published in 2004, and the mouse knockout by Suzuki et al in 2006.

Therefore, this study aimed to test the function of ARF6 in the well-established system of Drosophila development. After the generation of an arf6 null mutant, it soon became apparent that there does not appear to be an absolute requirement for ARF6 in any of the previously proposed developmental events. However, arf6 mutants showed a defect in cytokinesis. Therefore, the aim of the study was refined to characterize the function of ARF6 in Drosophila cytokinesis. As discussed above, a wealth of information is available on the coordination between the microtubules of the central spindle and the actomyosin contractile ring, but very little is known about the temporal and spatial coordination of membrane trafficking events during cytokinesis. It was unclear which molecular machinery connects the central spindle to membrane trafficking during cytokinesis (except Polo binding of the PITP Nir2, published in 2004). The arf6 mutant thus provided a good opportunity to investigate the control of membrane trafficking during cytokinesis.
Results

*Drosophila arf6 null mutants*

In order to study the role of ARF6-dependent endocytic trafficking, deletions were generated in the *arf6* endogenous gene by imprecise excision of the EP2612 transposable element inserted in its first intron (Fig. 4A). *arf6*\(^1\) is a null mutation that corresponds to a 1709 nucleotide deletion in the transcribed region, which entirely removes the open reading frame (Fig. 4A). Consistently, no ARF6 protein can be detected in Western blots from homozygous *arf6*\(^1\) animals using an antibody raised against the *Drosophila* protein (Fig. 4C).

**Figure 4**

(A) Schematic representation of the portion of the genomic region 51f containing *Drosophila arf6* (blue). Protein coding sequences of *arf6* are indicated in red. *arf6*\(^1\), *arf6*\(^2\) and *arf6*\(^3\) shown below are deletions produced by imprecise excision of EP2612 in the presence of transposase.

(B) The flanking sequence on each side of the deletions, with * indicating boundaries of *arf6* sequence, and excision scars in red.

(C) Western blot to detect endogenous ARF6 using a rabbit polyclonal antibody against *Drosophila* ARF6, and rabbit anti Actin as a loading control. ARF6, a 20KDa protein is detected in WT adult flies. ARF6 protein could not be detected in *arf6*\(^1\) zygotic mutant flies.
**Drosophila arf6 is not an essential gene**

ARF6 is not essential for the viability of the fly. *arf6* homozygous progeny from homozygous mutant mothers (i.e. maternal/zygotic mutants) are viable until adulthood and do not present any overt external morphological phenotype.

It has been reported that expression of a GDP-bound dominant negative ARF6 protein (ARF6T27N) impairs myoblast fusion and axon path finding during embryogenesis (Chen et al., 2003; Onel et al., 2004). Both developmental events occur normally in *arf6* null mutant embryos (Fig. 5), indicating that the ARF6TN protein causes secondary defects beyond the loss of ARF6 function.

**Overexpression of ARF6T27N does not result in a phenotype**

Transgenic flies were generated to overexpress ARF6T27N with a carboxy terminal HA tag (UAST:arf6T27N-HA) using the UAS GAL4 system (Brand and Perrimon, 1993), and tested by staining for the HA tag. In contrast to previous reports, no effect could be observed when ARF6T27N-HA was overexpressed in embryos or imaginal discs. The different results might be due to different levels of expression of ARF6T27N in my transgenic flies versus the previously reported transgenic flies. Unfortunately it was not possible to test the level of overexpression, since the UASARF6TN flies produced by Chen et al were no longer available. A second possible explanation is that the HA tag interferes with the folding or interactions of ARF6T27N. Expression of ARF6-HA rescues the *arf6* mutant phenotype, suggesting that it is functional (Figure 6D, 7D). It is however possible that only a small amount of ARF6 protein is required to rescue mutants, whereas a large amount is necessary to act as a dominant negative.
Figure 5: ARF6 is not required for myoblast fusion or midline crossing of axons

Stage 15 embryos derived from arf6¹/arf6¹ mothers. Scalebars: 20μm

(A-B) Lateral view of control (A); (arf6¹/CyO, hb-lacZ) and arf6¹/arf6¹ embryos (B) stained for the muscle marker MHC. Multinucleated muscle cells, but no unfused myoblasts can be seen in arf6 maternal zygotic mutants, as in WT. Dorsal is up and anterior to the left.

(C-D) Dorsal view of control (arf6¹/CyO, hb-lacZ) (C), and arf6¹/arf6¹ embryos (D), stained for the axonal marker BP102. In arf6 mutants, the CNS develops a normal morphology indicating that ARF6 is not required for midline crossing of axons.

*Drosophila arf6* plays a role during chorion formation

arf6¹ females show reduced fertility. Low fertility is due to a partially penetrant requirement for ARF6 during chorion formation in the germ-line (Figure 6, 48
Since the chorion phenotype of arf6 mutants is only partially penetrant, I decided to focus subsequent phenotypic investigations on spermatocyte cytokinesis.

**Table 1**: Reduced fertility and chorion defects or arf6 mutant females

<table>
<thead>
<tr>
<th>Genotype of female</th>
<th>% eggs hatching when crossed to WT males</th>
<th>% eggs with short or fused dorsal appendages *</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT arf6'/arf6'</td>
<td>93% (n=121)</td>
<td>0% (n=100)</td>
</tr>
<tr>
<td>y w Flp;FRTG13 Ovo^2/ FRTG13 arf6'</td>
<td>38% (n=178)</td>
<td>32% (n=203)</td>
</tr>
<tr>
<td>y w Flp;FRTG13 Ovo^2/ FRTG13 arf6^3</td>
<td>ND</td>
<td>71% (n=180)</td>
</tr>
<tr>
<td>P(Ubi)ARF6-HA arf6'/arf6'</td>
<td>79% (n=169)</td>
<td>0.5% (n=182)</td>
</tr>
<tr>
<td>ND not determined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*collapsed eggs were excluded from the analysis

**Table 1**: Reduced fertility and chorion defects or arf6 mutant females

*Drosophila arf6 is required for cytokinesis during spermatogenesis*

arf6' males are completely sterile. Mutant spermatids show a “four wheel drive” phenotype (Fig. 7), diagnostic of a cytokinesis defect during spermatocyte
meiosis. After the second meiotic division the mitochondria in each cell fuse into a phase-dark *Nebenkern* which is roughly the size of the nucleus. If cytokinesis fails after a successful nuclear division, the result is a cell with multiple nuclei but one oversized *Nebenkern* (Fuller, 1993) In arf6 mutant males, over 90% of the spermatids contain more than one nucleus, and 41% have four nuclei per *Nebenkern* (Fig. 6D). This corresponds to a 79% failure rate in cytokinesis during the two meiotic divisions (for determination of the failure frequency, see below and Fig. 9). Occasional 8:1 nuclei-to-*Nebenkern* ratios (in 1.9% of spermatids) suggest that the cytokinesis of the gonial cell mitosis prior to meiosis is also occasionally affected, as previously suggested in the case of other cytokinesis mutants (Brill et al., 2000; Giansanti et al., 2004). Other arf6 mutants (*arf6*2, *arf6*3) show phenotypes indistinguishable from *arf6*1. The male sterility and cytokinesis mutant phenotype are due to the *arf6* mutation, since both an *arf6*+ genomic transgene *P(w*arf6*+) or ubiquitous expression of arf6 (*P(w*Ubi:arf6-HA)) rescues the defects and yields fertile males (Figure 7C-D).
Figure 7: *Drosophila* ARF6 is required for cytokinesis in the testes

(A-C) Phase contrast images of spermatids after the second meiotic division. Nuclei appear white (arrows) and mitochondrial derivatives (*Nebenkerne*) black (arrowheads). (A) WT, (B) *arf6* mutants show ratios of 2 or 4 nuclei per cell and *Nebenkern*. (C) A rescue construct expressing *arf6* from the endogenous promoter restores a 1:1 ratio of nucleus to *Nebenkern* per cell. Scale bars, 20µm.

(D) Frequency of nuclei per cell in WT (black), *arf6* (red), *arf6* rescued by the *arf6* endogenous rescue construct (blue), *arf6* rescued by expression *arf6-HA* under the control of the polyubiquitin promoter (grey), and *arf6* rescued by an *arf6* endogenous rescue construct containing the mutation *Q37E, S38I* (green). The number of cells counted (n) and genotypes are indicated in the key.
A model of spermatocyte cytokinesis failure frequency

The proportion of spermatids with 1, 2 or 4 nuclei after meiosis II can be calculated, assuming that the probability of cytokinesis failure, p, is constant (Figure 8 and appendix 1). Using this model, a predicted proportion of spermatids containing 1, 2 or 4 nuclei can be calculated for each value of p between 0 and 1. The results of this calculation are shown in Figure 8A.

Figure 8

<table>
<thead>
<tr>
<th>lineage probability</th>
<th>proportion of spermatocytes</th>
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<tbody>
<tr>
<td></td>
<td>1:1</td>
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<tr>
<td>(1-p)^3</td>
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<tr>
<td>2p(1-p)^2</td>
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<tr>
<td>p^2(1-p)</td>
<td>1-p</td>
</tr>
<tr>
<td>p^2</td>
<td>p</td>
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</table>

Figure 8: Model of failure frequency during spermatocyte cytokinesis

Possible lineages of a primary spermatocyte undergoing meiosis I and II to generate spermatids. p is the probability of cytokinesis failure. The probability of each lineage is given on the left. The proportion of spermatids containing one, two or four nuclei: Nebenkern is given on the right as a function of p.

In order to test this model of spermatocyte cell division failure, I studied how well the spermatid frequencies from this study and previously reports fit to the model. Let the observed proportions of cells containing 1, 2 or 4 nuclei be designated by A_i, B_i and C_i. a, b and c are the theoretical values of these proportions which all correspond to a certain value of p in the model. If the theoretical values from the model fit the experimental data, then A_i is very close in value to a, B_i to b and C_i to c.

To assess how good the model is, starting from experimental data, it is necessary to:

i) Find the value of p which minimizes the squared differences between the expected (a, b, c) and observed (A_i, B_i, C_i) frequencies for that value of p.
ii) Use the size of the sum of squares to assess the validity of the model. The sum of squares (S) is

\[ S = (A_i - a)^2 + (B_i - b)^2 + (C_i - c)^2 \]

By substituting in the expressions for \( A_i \), \( B_i \) and \( C_i \), and differentiating with respect to \( p \), a cubic equation can be derived (see Appendix 1 for derivation), the solutions of which are the values of \( p \) for which \( S \) is a minimum. This equation was solved numerically, with accuracy 0.001. An EXCEL spread sheet including a script (written by Peter Foster) to calculate the failure frequency from the experimental frequencies of 1:1, 2:1 and 4:1 is included on the accompanying CD.

**Testing the cell division model**

Data on \( A_i \), \( B_i \) and \( C_i \) was taken from this study and for several other mutations previously characterized to affect spermatocyte cytokinesis. Cells containing greater than four nuclei were excluded from the analysis, since the model only considers cells starting meiosis I with one nucleus. Rare cells containing three nuclei were grouped with those containing four nuclei to contribute to the value of \( C_i \).

From the 30 genetic conditions analyzed in this study, the maximum sum of squares was only 0.0938, and the average only 0.0146. The low values of the sum of squared deviations from the model (Figure 9B) suggest that the model describes a wide range of mutant conditions well. This means that in these mutants, the probability of cytokinesis failure might be the same for cytokinesis I and II. This theory could be useful for measuring the magnitude of the effect on cytokinesis of different genetic conditions, as a probability of cytokinesis failure is a more relevant indicator of the requirement of a protein than the raw data \( A_i \), \( B_i \) and \( C_i \).
Figure 9: Testing the failure frequency model

(A) Frequency of cells with one nucleus (blue), two nuclei (red) or four nuclei (black) after meiosis II. Circles, squares and triangles correspond to published data. Theoretical curves are the frequency of the different cells (1:1, 2:1 and 4:1) as a function of p. The curves are derived from the equations given in (A) and appendix 1. Note that the published frequencies fit the theoretical curves well.

(B) Values of p for mutants affecting cytokinesis. The error bars indicate the least sum of squares difference between the observed proportions of cells with one nucleus, two nuclei or four nuclei and those predicted by the model for the corresponding value of p. The alleles and source of frequency statistics (when not from this study) are indicated. For the genotypes generated in this study, the following abbreviations were used:

sib: sibling
arf6 Pr:  w; FRTG13arf6/FRTG13 arf6; Pr/+ 
arf6 pav:  w; FRTG13arf6/FRTG13 arf6; pav/+
ARF6 is required for cleavage furrow progression during cytokinesis in the testes

To characterize the cytokinesis defect in mutant primary spermatocytes, video microscopy of wild type (WT) and mutant cells during meiosis I was performed. Elena Rebollo collaborated in this project, and performed time-lapse imaging of arf6 mutant spermatocytes expressing Histone2A-GFP, gamma Tubulin GFP and alpha Tubulin GFP (see videos on CD). These movies showed that in arf6 mutants, chromosome segregation occurs normally and centrosome behaviour is also normal. The alpha Tubulin GFP movies indicated that the spindle initially forms normally in arf6 mutant spermatocytes. However, it was apparent that in the arf6 mutant, a cleavage furrow is established but later regresses. The kinetics of furrow ingression in wild type and arf6 mutant spermatocytes was therefore examined.

In wild type control cells, cell shape stays constant from prometaphase until anaphase onset (Fig.13, movies on CD). After anaphase onset, the cell elongates, decreasing its diameter at the equator at a slow rate of around 0.4 µm/min (Fig. 10, 14, movies on CD). During anaphase B, which starts 2 minutes after anaphase onset, Pavarotti starts accumulating at the central spindle (Minestrini et al., 2003) (Fig. 10A (black arrowhead), 11A, 14, movie 7). The centralspindlin complex subsequently signals to the cortex and the actomyosin contractile ring forms, as monitored by accumulation of myosin regulatory light chain (Sqh-GFP) at the future cleavage furrow around one minute after the onset of Pavarotti accumulation at the central spindle (Fig. 11B, 14, movie 9) (Adams et al., 1998; Royou et al., 2004; Somers and Saint, 2003). Shortly after accumulation of Pavarotti and Sqh, contraction is accelerated at the equator to around 1 µm/min (Fig. 10A,C 14). 5 minutes after the onset of Pavarotti accumulation, an indentation at the plasma membrane, the cytokinetic “cleavage furrow”, appears (Fig. 10A (black arrow), 11A). Subsequently the progression of the furrow continues at 1 µm/min and finally decelerates to stop around 35 minutes after anaphase onset (min AA) when the furrow is 3 to 5 µm wide (Fig. 10, 11A,C). This narrow opening between the two daughter cells will later differentiate into the ring canal connecting the two daughter cells (Hime et al., 1996).
**Figure 10**

(A) Furrow progression kinetics and timing of Pavarotti accumulation at the central spindle (arrowheads) and appearance of an indented cleavage furrow (arrows) in representative WT, *arf6* mutant “early regressor” and “late regressor” and *chic^13E* cells. Diameter is measured at the furrow tip or future site of furrowing.

(B) Furrow diameter kinetics in control, *arf6* early late regressors. Time zero is the appearance of an indented cleavage furrow.

**Figure 10 Kinetics of cleavage furrow invagination in WT, *arf6* and *chic* cells**

(A) Furrow progression kinetics and timing of Pavarotti accumulation at the central spindle (arrowheads) and appearance of an indented cleavage furrow (arrows) in representative WT, *arf6* mutant “early regressor” and “late regressor” and *chic^13E* cells. Diameter is measured at the furrow tip or future site of furrowing.

(B) Furrow diameter kinetics in control, *arf6* early late regressors. Time zero is the appearance of an indented cleavage furrow.
(C) Furrow ingression rates in control and arf6 late regressors show similar kinetics, whereas ingression rates in chic13E and arf6 early regressors are significantly slower than in wild type. Error bars indicate standard error of the mean. * indicates \( P<0.01 \), Student’s t-test. Number of cells quantified: control; \( \geq 11 \); arf6 early regressors; \( \geq 13 \); arf6 late regressors 5; chic13E, \( \geq 7 \).

In arf6\(^1\) mutants, early events in meiosis I, including chromosome condensation, spindle formation, chromosome congression to the metaphase plate and chromosome segregation during anaphase, proceed normally (movies 1-6 on CD). However, cytokinesis fails in 89% of the cells, consistent with the frequency of spermatids containing 4 nuclei per cell reported above (Figure 8). Cytokinesis fails either early (55% of the failing cells) or late (45%) during the progression of the furrow. In the “late regressors”, cytokinesis proceeds with normal kinetics of furrow formation and progression (Fig. 10A, movie 9). During progression of the furrow, plasma membrane is also added with normal kinetics (see below; Figure 13B-C). In these cells, the furrow proceeds until it is less than 10 \( \mu m \) wide and stays with this diameter for a variable period of time before collapsing (Figure 10).

In the “early regressors”, after anaphase onset, anaphase B cell elongation occurs, with equator contraction at 0.3 \( \mu m/\text{min} \), only slightly slower than in wild type (Fig. 10C, 14). In addition, Pavarotti and Sqh targeting to the central spindle and the contractile ring occur only slightly later than in wild type (Fig. 11B,D, 14, movies 8 and 10). Afterwards, contraction of the equator at fast rates does not take place, but only accelerates to a speed of 0.5 \( \mu m/\text{min} \) and indentation occurs 10, instead of 5 minutes after the onset of Pavarotti accumulation at the central spindle (Fig. 10A, 14). Shortly after the cleavage furrow indentation appears, when it is around 15 \( \mu m \) wide, the furrow regresses and cytokinesis fails. After cleavage furrow collapse, Pavarotti dissociates from the central spindle, although Sqh remains associated with the cortex during regression (Figure 11B,D, movie 10).
Figure 11 ARF6 is not required for central spindle or contractile ring formation

Time-lapse of Pav-GFP (A-B) and Sqh-GFP (C-D) during cytokinesis in control (A, C), and arf6 (B, D) mutant spermatocytes. Times are minutes:seconds AA. Scale bar, 5µm. (A) control, Pav-GFP accumulates at the central spindle during anaphase B (arrowhead, 03:50). Central spindle microtubules labelled with Pav-GFP bundle and compact into a dense midbody (arrowheads, 08:07-22:16). Pav-GFP later remains in the ring canal (arrowhead, 50:02). (B) arf61, anaphase B Pav-GFP central spindle accumulation occurs (arrowhead, 05:42). Pav-GFP labelled microtubules bundle, (arrowhead, 11:54), a cleavage furrow initiates, but central spindle Pav-GFP signal declines (arrowhead, 22:24) as the furrow regresses. (C) Control, Sqh-GFP is targeted to the cortex (arrowhead, 04:55) before accumulating at the future cleavage furrow site (arrowheads, 07:05, 08:11) which then invaginates (arrowhead, 22:14). (D) arf63, Sqh-GFP is targeted 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;; Pav-GFP (A), w; arf61/arf61; Pav-GFP/TM6B (B), y w sqhAX3;; P (w+ Sqh-GFP) (C), y w sqhAX3; arf61/arf61; P (w+ Sqh-GFP) (D).

These observations indicate that ARF6 is not necessary for targeting Pavarotti to the central spindle, or for Actin contractile ring formation. To confirm the observation that the Actin cytoskeletal remodelling functions of ARF6 are not required for cytokinesis, a rescue construct containing the ARF6 with the effector
domain mutations Q37E,S38I was used to make transgenic flies. This rescue construct rescued the cytokinesis defect in arf6 mutant flies (Figure 7).

In summary, the mutant phenotypes reveal two critical phases for ARF6 function during cytokinesis: i) an early crucial role in the progression of the cleavage furrow after the cytoskeleton generates the forces that initiate the indentation of the plasma membrane at the furrow and ii) a later role in establishing a stable ring canal at the end of cytokinesis. The experiments that follow concentrate on the early regressors and the early role of ARF6 in furrow progression.

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

During spermatocyte cytokinesis, the total volume of the two daughter cells equals the volume of the mother (volume change is only 0.8% ± 1.4%, n=5 cells). For spherical cells, this implies that the membrane surface must increase by 26% in the process of cytokinesis. The arf6 phenotype might therefore be 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 membrane recycling through the endocytic pathway (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 in control spermatocytes were examined by measuring the perimeter of the cells in confocal timelapse images (Figure 13) as well as the total surface area calculated for three dimensionally reconstructed cells. The relationship between perimeter and surface area was studied experimentally in control cells. This was necessary since the relationship between volume and surface area is a characteristic of a particular cell shape.
During meiosis I, perimeter increase is proportional to surface area increase (Fig. 12, table 2 and materials and methods). In *arf6* mutants, perimeter is also roughly proportional to surface area, although the $R^2$ values (Table 2) are not as high as in WT, since the data points are clustered around only a small range of surface areas and volumes.
Figure 13 ARF6 is required for rapid plasma membrane growth during cytokinesis

(A) Cell perimeter kinetics during anaphase and cytokinesis. Representative cells are shown for control, *arf6* early regressors and *chic*<sup>13E</sup>.

(B) Kinetics of perimeter length in control, *arf6* early and late regressors and *chic*<sup>13E</sup> cells. *arf6* early regressors have a slower rate of perimeter increase and attain shorter final perimeters.

(C) Rate of perimeter change in control, *arf6* early and late regressors, and *chic*<sup>13E</sup> cells. Perimeter is constant before anaphase. The maximum rate of net membrane growth is achieved after Pavarotti localisation to the central spindle, which happens around 5 min AA, and the appearance of an indented cleavage furrow in controls, and is not attained in the *arf6* cells. Membrane insertion is not significantly impaired in *chic*<sup>13E</sup> mutants until the central spindle is disassembled (cf. 5-10 min AA vs. 10-20 min AA, and see Fig. 15). Error bars represent the standard error of the mean. Membrane insertion is significantly impaired in *arf6* from 5 min AA onwards: * indicates P<0.05, and # indicates P<0.001, Student's t-test.
Plasma membrane growth is negligible prior to anaphase, and starts during anaphase B cell elongation at 0.6 μm/min. This perimeter rate corresponds to membrane addition at around 8 μm²/min. The perimeter rate increases greatly (2.5 fold, corresponding to around 22 μm²/min) directly after the onset of Pav accumulation, peaking around 15 min AA at the time of indented furrow appearance and maximum ingression rate (Fig. 13, 14). Subsequently, the rate decreases until cytokinesis completion. In arf6 mutants, slow membrane addition characteristic of early cytokinesis is maintained after furrow membrane indentation, and the rapid membrane addition phase never occurs (Fig. 13, 14). This data suggests that ARF6 is involved in the rapid membrane addition to the plasma membrane necessary for rapid furrow progression during cytokinesis.

**Figure 14**

<table>
<thead>
<tr>
<th>Anaphase</th>
<th>Cytokinesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>slow constriction 0.4μm/min</td>
<td>fast constriction 1μm/min</td>
</tr>
<tr>
<td>membrane growth 0.6μm/min</td>
<td>membrane growth 1.2μm/min</td>
</tr>
</tbody>
</table>

**WT**

- Pav in CS
- Sqh in CR
- Indentation
- Progression

**arf6**

- slow constriction 0.3μm/min
- slow constriction 0.5μm/min
- regression

**Figure 14 Summary of cytokinesis in control and arf6 early regressor cells**

Mean times of the events of cytokinesis and the rates of cytokinesis furrow ingression and perimeter change ("membrane growth") in control and arf6 spermatocytes, staged with respect to the appearance of Pav-GFP at the central spindle during anaphase B. Black and white bars represent 1minute time intervals. Abbreviations: CS central spindle, CR contractile ring.

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

The arf6 mutant phenotype suggests a link between cleavage furrow progression and rapid increase in membrane surface. Failure in cleavage furrow progression might lead to a defect in surface increase, or *vice versa*. To address this, the rates of furrow progression and membrane addition were examined in mutants for
the profilin chickadee (chic) (Cooley et al., 1992; Giansanti et al., 1998). In chic^{13E} mutants, the central spindle forms normally, Pavarotti is initially targeted properly (Figure 15), but the actomyosin contractile ring usually fails to form (Giansanti et al., 1998). As a consequence, the kinetics of furrow progression are even more affected than in arf6 mutants (Figure 10A, C). However, in these chic^{13E} cells, membrane addition occurs with kinetics similar to control cells until the premature disassembly of the central spindle (Figure 15).

**Figure 15**

![Figure 15 Actomyosin ring constriction is not required for central spindle formation](image)

These results imply that the actomyosin ring contraction and cleavage furrow progression are not essential for the rapid phase of membrane addition during cytokinesis. They also imply that failure of membrane addition is a specific feature of loss of arf6 function and not a trivial consequence of the failure of cleavage furrow ingression. This data leaves open a possible role for the Pavarotti central spindle during rapid plasma membrane addition.

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

The subcellular localisation of ARF6 and its possible association to intracellular endosomal membrane stores and the Pavarotti central spindle was studied using a HA-tagged version of the protein under the control of the polyubiquitin promoter. ARF6-HA is functional, since this expression rescues the arf6 mutant cytokinesis and chorion phenotypes (Fig. 6D, 7D and Table 1). ARF6-HA is present in the cytosol and is enriched at the plasma membrane as well as in intracellular vesicular structures.

Even at early stages of cytokinesis in meiosis I, ARF6 is targeted to the Pavarotti-positive central spindle microtubules (65.7% of punctae ±3.6%, n=41 cells)
including the population of microtubules associated to the cortex where the cleavage furrow forms (Figure 16, 19A-B).

**Figure 16**

![Image](image.png)

**Figure 16 ARF6 punctate structures localise to the central spindle early in cytokinesis**

Primary spermatocyte at the initiation of furrowing showing ARF6-HA (red) already partially localised to the central spindle (arrowheads). Pav-GFP (green) labels the central spindle. Chromosomes are labelled with DAPI (blue). Scalebar: 5 µm

Rabs are small GTPases which are localized to and control traffic through endocytic compartments (reviewed in Deneka et al., 2003). Colocalisation analysis with Rabs indicated that the ARF6 labelled vesicular structures correspond to endosomes as previously reported in mammalian cells (Figure 17, 19), (D'Souza-Schorey and Chavrier, 2006). Some ARF6 endosomes contain E-Cadherin (Figure 18) as previously reported in MDCK cells (Palacios et al., 2002).

The ARF6 label at the central spindle corresponds mainly (87±5.2%, n=17 cells) to recycling endosomes, as defined by localisation of the small GTPase Rab4 (Sheff et al., 1999; van der Sluijs et al., 1992b). ARF6 also shows colocalisation with Rab5 early endosomes, and Rab11 recycling endosomes (Fig. 17B, 19B and 20), consistent with the observation that Rab4 domains exist both on Rab5 and on Rab11 endosomes (Sonnichsen et al., 2000; Trischler et al., 1999).
Figure 17 Endosomal localisation of ARF6 during interphase

(A-D) Primary spermatocytes expressing ARF6-HA and GFP-Rab4 (green), fixed during interphase, and stained for HA (red). (A), GFP-Rab4 (B), GFP-Rab5 (C) GFP-Rab11. During interphase, ARF6-HA colocalises with Rab4 (arrowheads, A) and Rab5 (arrowheads, B) but shows very low levels of colocalisation with Rab11 (C). Scalebars: 10 \( \mu m \)
Figure 18: Cargo of ARF6 endosomes includes E-Cadherin

(A-B) Spermatocytes expressing ARF6-HA and DE-CadherinGFP under the control of the polyubiquitin promotor, stained for HA (red) and Klp3A (blue). Scalebars: 5µm (A) Interphase, Klp3A is nuclear, some ARF6 punctae overlap with DE-Cadherin (dotted circle) or show partial overlap (solid circles). (B) Late cytokinesis, Klp3A labels the midbody (arrowhead) with which ARF6/DE-Cadherin endosomes are associated.

Rab11 recycling endosomes are also recruited to the central spindle and are decorated with ARF6 (Fig.19C, 20). However, in contrast to the ARF6/Rab4 endosomes, Rab11/ARF6 endosomes are not strongly enriched in the central spindle until relatively late in cytokinesis when the cleavage furrow is almost fully invaginated (diameter 4.0±0.4 µm, n=11 cells). This suggests that both Rab4 and Rab11 recycling endosomes may play a role in plasma membrane addition during cytokinesis. However, the late appearance of the Rab11 endosomes at the central spindle in control cells, at a time when furrow collapse had already occurred in arf6 early regressors, instead suggests a role for Rab11 in the ring canal stabilization process, which might be affected in the arf6 “late regressor” mutants.
Figure 19 ARF6 endosomes at the Pavarotti central spindle during cytokinesis

(A-C) Immunostainings of fixed primary spermatocytes. ARF6-HA (red) and Pavarotti (blue) immunostaining and Rab-GFP (green; Rab4 (A) Rab5 (B), and Rab11 (C)). ARF6-HA colocalises with GFP-Rab4 (A) and GFP-Rab5 (B) and GFP-Rab11 (C) at the central spindle (arrowheads). Scale bars, 5μm.

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 Rab4 endosomes at the central spindle (around 73%) are
decorated by ARF6 while only 22% of the Rab4 endosomes not localised to the central spindle contain ARF6 (Figure 20). Similarly 85% of the Rab11 endosomes at late central spindles also contain ARF6, versus 10% elsewhere in the cell (Figure 20). This data indicates that ARF6 targeting to recycling endosomes is specifically biased towards the central spindle endosomal population.

**Figure 20**

Central spindle recycling endosomes are enriched in ARF6, posing the question of whether ARF6 itself targets the recycling endosomes on which it is enriched to the central spindle? To address this question, the distribution of Rab4 and Rab11 endosomes in time-lapse movies of *arf6* mutants was investigated. In *arf6* mutants, Rab4 endosomes are targeted to the central spindle as in wild type (Fig. 21A,B, Movies 11-12). Therefore ARF6 is not required to target Rab4 endosomes to the spindle, but functions downstream of the endosomal targeting event. ARF6 does not seem to play a direct role in Rab11 targeting either. In *arf6* late regressors, the regression occurs around the time when Rab11 accumulation is clearly observed. However, some *arf6* late regressors were observed in which Rab11 endosome localisation does not appear to be affected (Fig.21C,D, Movies 13-14 on CD).
In summary, i) Rab4 and Rab11 recycling endosomes are targeted to the central spindle, ii) ARF6 targeting is biased towards the central spindle population of recycling endosomes, but iii) ARF6 itself does not play a role in the central spindle targeting of recycling endosomes. Instead, another machinery must be involved in targeting ARF6 to the central spindle endosomal population. ARF6 recycling endosomes at the central spindle could provide endosomal membrane stores for rapid membrane recycling, contributing to the membrane addition necessary for fast cleavage furrow progression.

**Physical and genetic interaction of ARF6 with Pavarotti**

Which molecular machinery targets ARF6 to the central spindle endosomes? The central spindle kinesin Pavarotti was identified by Hybrigenics in a yeast-two-hybrid screen on a *Drosophila* embryo cDNA library for proteins interacting with a constitutively active, GTPase-defective mutant of *Drosophila* ARF6. Five clones corresponding to the Pavarotti ORF interacted with ARF6, defining a region (amino acids 727-844) adjacent to the coiled-coil domain of Pavarotti as the ARF6-binding domain (Figure 22).
The interaction between ARF6 and Pavarotti was confirmed by “ex-vivo” binding assays (Figure 23A). These results suggest that Pavarotti might contribute to ARF6 recruitment to the central spindle endosomes during cytokinesis.

To further confirm this proposal, the functional relevance of the molecular binding event was tested by asking whether \textit{arf6} and \textit{pavarotti} mutants show synergistic interactions. In \textit{arf6}^{3} mutants, less than 40\% of the spermatids after meiosis II show more than a 2:1 ratio of nucleus-to-Nebenkern. In \textit{pavarotti} heterozygous mutants only 0.15\% of spermatids show a 2:1 ratio. \textit{arf6}^{3}/\textit{arf6}^{3} ; \textit{pav}^{B200e}/+ males show a synergistic increase in the cytokinetic failure, since more than 65\% of the spermatids show more than the 2:1 ratio (Figure 23B), corresponding to a 12\% increase in the rate of cytokinesis failure during meiotic divisions over siblings with the endogenous \textit{pavarotti} gene (Figure 9B). The frequency of cells containing more than four nuclei was also increased from 2\% to 12.6\%, suggesting that with depletion of Pavarotti levels in \textit{arf6} mutants, cytokinesis of mitotic divisions is also affected. A similar result was obtained using \textit{arf6}^{3}/\textit{arf6}^{3} ; \textit{pav}^{1375}/+ males (Figure 23B), confirming the interaction and ruling out an interaction with some other ethyl methanesulfonate (EMS) induced mutation of the \textit{pav}^{B200} chromosome. This implies that the two molecules work in concert during cytokinesis, suggesting a possible role for Pavarotti in ARF6 recruitment to the central spindle endosomes. The central spindle endosomes decorated with ARF6 might in turn acquire a fast recycling behaviour, which would ultimately contribute to rapid membrane addition during cytokinesis.
Figure 23

(A) "Ex-vivo" binding assay of ARF6 with Pavarotti. Purified GSTPav655-865, but not GST alone (negative control) was able to pull down ARF6-HA and ARF6Q67L-HA from HeLa cell lysates.

(B) Frequency of spermatids following meiosis II with 3 or more nuclei per cell, in sibling males of genotypes FRTG13 arf6<sup>3</sup>/FRTG13 arf6<sup>3</sup>;Pr/+ and FRTG13 arf6<sup>3</sup>/ FRTG13 arf6<sup>3</sup>;pav<sup>B200</sup>/+ (left columns) and FRTG13 arf6<sup>3</sup>/ FRTG13 arf6<sup>3</sup>;Pr/+ and FRTG13 arf6<sup>3</sup>/ FRTG13 arf6<sup>3</sup>;pav<sup>A375</sup>/+ (right columns). In both cases, removing one copy of pav increases the frequency of the arf6 mutant cytokinesis phenotype.

Pavarotti mutant clones in the testes

In order to test whether Pavarotti is necessary to recruit ARF6 to the central spindle, mosaic testes containing clones of homozygous pav<sup>B200</sup> mutant spermatocytes were produced. The rationale was that due to the large size of primary spermatocytes prior to meiosis I, and the lack of cell growth after entry into meiosis I, the perdurance of Pavarotti protein might be sufficient to form a central spindle but insufficient to recruit ARF6. Also, since depletion of the Pavarotti homologue ZEN-4 in C.elegans allows furrow initiation and some central spindle microtubule bundling and does not prevent the Aurora B homologue AIR-2 from localizing to central spindle microtubules, it was possible that the absolute requirement of Pavarotti for microtubule bundling and furrow initiation in Drosophila might be restricted to embryonic divisions (Adams et al., 1998; Powers et al., 1998). However, no central spindles were observed in clones, as assessed by the localisation of the central spindle component Klp3A, and cells lacking Pavarotti failed in cytokinesis, generating multinucleated cells (Figure 24). This is consistent with the essential role of Pavarotti during central spindle organization and cytokinesis as previously reported (Adams et
al., 1998). In the absence of a functional central spindle in *pavarotti* mutants, targeting of the recycling endosomes would not be expected in the first place, hampering the possibility of addressing this way the specific role of Pavarotti in ARF6 targeting.
Figure 24

Clones mutant for pav<sup>Δ200</sup> in testes expressing ARF6-HA. GFP (green) is absent in the clones, outlined in white. Cells in clones are numbered in the ARF6-HA picture.

(A) Clone of primary spermatocytes, stained for Klp3a (blue), ARF6-HA (red). Two multinucleated cells can be seen, whereas cells outside of the clone are mononucleated.

(B) Clone of spermatids stained for DAPI (blue) and ARF6-HA (red). Four multinucleated cells can be seen. The full z stack revealed cells contain 8 or 16 nuclei, suggesting that after two successful mitotic divisions, 2 or more mitotic cytokinesis failed, and all the meiotic cytokinesis failed. Scalebars: 10 µm

Figure 24 Pavarotti is essential for cytokinesis in the testes
Localisation of ARF6 in somatic cells
As a second strategy to test if Pavarotti recruits ARF6 to the central spindle, ARF6-HA localisation was observed upon Pavarotti overexpression, and with respect to endogenous Pavarotti in somatic cells in the embryo, larval brain, and adult sensory organ precursors (Figure 25). In these cells, ARF6 HA colocalises with Pavarotti in cortical structures of diameter smaller than 1µm in both dividing and interphase cells (Figure 25). These structures are likely to correspond to division scars, i.e. remnants of the midbody of previous cell divisions that remain associated to the plasma membrane as recently reported (Gromley et al., 2005).

Figure 25

Figure 25 Colocalisation of ARF6 with Pavarotti in somatic tissues
(A-C) Fixed tissue immunostained for ARF6-HA (red). Scalebars: 5µm
(A) Neuroblast in the brain of a third instar larva, overexpressing PavGFP (green) and ARF6-HA under the control of neurGAL4 and stained for phospho-histone3 (blue) and HA (red). ARF6-HA colocalises with PavGFP in several division remnants (arrowheads) both in the dividing neuroblast (dotted outline) and its progeny (dashed outline).
(B) Sensory organ precursor cell following the first division in a pupal notum, over expressing ARF6-HA under the control of neurGAL4 and stained for endogenous Pavarotti and DAPI. ARF6-HA and Pav colocalise in a division remnant (arrowhead).
(C) Cells of a gastrulating embryo expressing ARF6-HA under the control of the polyubiquitin promoter, and stained for endogenous Pavarotti (green). The majority of Pav punctae are also labelled with ARF6-HA

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In summary, the molecular interaction and the colocalisation data is consistent with the possibility that ARF6 might be recruited by Pavarotti to the central spindle endosomes.

**ARF6 QL overexpression reduces cell number in wings**

To investigate any cryptic function of ARF6 in somatic tissues, UAS:ARF6Q67L-HA was overexpressed in the posterior compartment of imaginal discs, which later give rise to adult organs. The posterior compartment of the wing is generated from a clone of cells, which do not mix with the clone of cells in the anterior compartment during wing imaginal disc development in the larva (Bryant, 1970). If the effects of overexpressing a gene are cell autonomous, this allows the comparison as an internal control between mutant and WT cells in one wing. This internal control is useful in circumstances where the wing size is changed, since the size of the wing is also under environmental control, such as nutrient supply, which differs between individual flies. ARF6Q67L overexpression in the posterior compartment caused wing phenotypes including a reduction in the size of the posterior compartment with respect to the anterior compartment (relative size), fusion of wing veins L4 and L5, and holes in the wing between veins L4 and L5 (Figure 26 A-B, D). Overexpression of a dominant negative version of the Rho GEF Pebble (PblΔDH), which lacks the DH domain which is required for GEF activity, also leads to a reduction in the relative size of the posterior compartment, but not a hole between veins L4 and L5 (Figure 26 C-D, (Echard and O'Farrell, 2003). Wild type wings contain mononucleated cells, each of which produces one wing hair, but overexpression of PblΔDH produces wings containing multinucleated cells with heterogeneous nucleus sizes, and multiple wing hairs (Fig 26 F, (Echard and O'Farrell, 2003). Echard and O’Farrel also observed that the cells density in wings expressing PblΔDH is reduced. Closer examination of wings overexpressing ARF6Q67L revealed the presence of multiple wing hairs, but at frequencies far lower than those observed in PblΔDH expressing wings (Fig 26 E-F). In contrast, the “relative hair density” (density of hairs in the ARF6Q67L overexpressing compartment compared to the density of hairs in the non expressing compartment) was 0.82. This reduction in hair density was significant (P<0.001 in Students T test).

Wild type wings reliably contain similar numbers of cells in anterior and posterior compartments. To estimate the number of cells per wing, I multiplied the
density of wing hairs by the area of each compartment for each wing, using the approximation
relative cell number ≈ relative hair number ≈ relative hair density * relative size
This suggested that the number of cells in the ARF6Q67L overexpressing compartment is less than half the number in the non-expressing compartment (relative hair number = 0.457 +/- standard deviation 0.094, n=11).

In summary, overexpression phenotypes in the wing are consistent with ARF6Q67L either causing a cell cycle delay or causing cytokinesis defects. However, unlike PblΔDH overexpression, ARF6Q67L does not cause many multiple wing hairs but causes hole in the wing. Wing holes suggest that ARF6Q67L might cause an additional developmental defect not caused by PblΔDH. Cytokinesis defects do not necessarily lead to multiple wing hairs. For example, in myb mutant wings, cells have a defect in G2M progression and undergo ectopic endoreduplication. myb mutant wings show a reduction in cell number and increase in cell size, but not multiple wing hairs (Katzen et al., 1998). Also, prehair formation is additionally linked to the planar cell polarity pathway (Wong and Adler, 1993). Detailed analysis of nuclei in pupal wings would be necessary to understand the ARF6Q67L overexpression phenotype.
Figure 26

Overexpression of ARF6 Q67L leads to multiple wing hairs and a reduction in cell number


(A-C) Wing veins L1-L5 are indicated. The posterior compartment is outlined with a dotted red line. A solid red line separates proximal and distal regions. All wings are shown at the same scale. (B) Veins L4 and L5 are partially fused proximally, and there is a hole (dashed red line) in the second posterior cell.

(D) Quantification of the relative size of the posterior compartment compared to the anterior (non-outlined) compartment in wings of the genotypes shown in (A-C). Error bars represent the standard deviation, WT n=12, ARFQ67L n=25, PblΔDH n=14

(E-J) Same scale pictures of (E,F,H,J) posterior compartments and (G,I) anterior compartments. Examples of cells containing multiple wing hairs are circled in red (E-F). (G-H): different areas from the same WT wing, (I-J): different areas from the same ARF6Q67L overexpressing wing.
Discussion

During cytokinesis of *Drosophila* spermatocytes, a nearly spherical cell is divided into two nearly spherical cells. There is no change in cell volume during this process, implying that the surface area of these cells increases by 26% during cytokinesis. In this study, it was found that 500 $\mu$m$^2$ of plasma membrane are added in around 20 minutes during meiosis I cytokinesis, posing a logistic problem of adding plasma membrane at an average rate of over 0.4 $\mu$m$^2$ per second. Potential sources of membrane for the expansion of the plasma membrane include Rab4 and Rab11 recycling endosomes, which were demonstrated to accumulate at the central spindle. Colocalisation analysis revealed that ARF6 is enriched in this central spindle endosome population.

ARF6 is required for cytokinesis, since in the *arf6* null mutants generated in this study, cytokinesis fails in 79% of the two meiotic divisions. During cytokinesis, the progression of the cleavage furrow halts and regresses, and there is a defect in the rapid expansion of the plasma membrane.

The centralspindlin kinesin Pavarotti binds ARF6, and *arf6* and *pavarotti* mutants interact synergistically during cytokinesis in the testes. Pavarotti might therefore be part of the scaffold that recruits ARF6 to recycling endosomes at the central spindle. In the absence of ARF6, recycling endosomes are still targeted to the central spindle, but the surface area of the cell only increases at a slow rate.

These results pose the following questions, which are discussed below:

- How does a cell rapidly expand the plasma membrane during cytokinesis?
- What is the relative contribution of ARF6 to membrane addition during cytokinesis?
- By which mechanisms does ARF6 contribute to membrane addition?
- Is there a purpose in localising membrane trafficking components such as ARF6, Rab4 and Rab11 to the central spindle during cytokinesis?
- Why is *Drosophila* ARF6 only required during male meiosis?

**How does a cell rapidly expand the plasma membrane during cytokinesis?**

The shape changes of a cell during cytokinesis necessitate an expansion of the plasma membrane if the volume remains constant. A cell can solve this problem of plasma membrane expansion in four ways: i) decreasing in volume before or during cytokinesis, ii) stretching the existing membrane to achieve a larger surface,
thereby increasing membrane tension, iii) resolving membrane microvilli and iv) adding membrane to the plasma membrane. There are no previous reports of decrease in the net volume. Volume decrease is indeed not the strategy used by *Drosophila* spermatocytes, since, as shown in this study, their volume remains constant throughout cytokinesis. Under tension, the surface area of biological membranes can stretch only around 2-3% before lysis, which is not sufficient to account for a membrane expansion of around 26% during cytokinesis (Needham and Hochmuth, 1989).

The resolution of folds or microvilli might be used as a strategy by some cell types. In P815Y mastoma cells, the unfolding of microvilli accumulated during interphase is sufficient to account for the apparent increase in surface area during cytokinesis (Knutton et al., 1975). Knutton *et al* suggested that cells have a homeostatic mechanism to maintain a constant surface area to volume ratio throughout the cell cycle, whether it be by growth of microvilli or by spreading during interphase and rounding up prior to division. In contrast, microvilli in some ascidian eggs show the converse behaviour, increasing in number during cleavage furrow progression and disappearing during interphase, suggesting that this mechanism is not conserved (Satoh and Deno, 1984). Cell types that do not produce sufficient extra surface area between G1 and cytokinesis are likely to increase the membrane surface during cytokinesis by delivering membrane to the surface.

At least 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). A third pathway involving trafficking from the Golgi to endosomes represents a mix of these two main pathways (Ang *et al*., 2004). Plasma membrane addition during cytokinesis could in principle involve both pathways. Indeed, a number of cytokinesis functional screens have identified both secretory and endocytic factors (Echard *et al*., 2004; Eggert *et al*., 2004; Skop *et al*., 2004). The relative contribution of the endocytic versus the secretory pathway in a particular cell type may depend on the speed and amount of membrane deposition that each of these pathways can deliver relative to the time window during which cytokinesis must take place, which is defined by the cell cycle regulators. Cytokinesis of meiosis I in *Drosophila* spermatocytes is very demanding, because it requires the expansion of the plasma membrane surface by 500 µm² within
20 minutes. Membrane recycling through the endocytic pathway can make available large stores of previously endocytosed membrane rapidly (Lim et al., 2001; Pelissier et al., 2003; Stinchcombe et al., 2000).

The relative contribution of ARF6 to membrane addition during cytokinesis

The arf6 mutant phenotype reveals two components of the rate of membrane addition: a slow ARF6-independent process and, after central spindle formation, a 2.5 fold faster ARF6-dependent addition process (Figure 13). Consistent with the recycling role of ARF6, the accelerated rate of addition coincides with the positioning of fast recycling Rab4/ARF6 endosomes at the cleavage furrow early during cytokinesis. Slow recycle Rab11/ARF6 endosomes might be involved later for stabilization of the ring canals (Figure 19 and 21). It is tempting to speculate that the slow component corresponds 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 with the arf6 mutant rates. It might also be informative to investigate whether cleavage furrow invagination and plasma membrane expansion occur with normal kinetics in somatic cells in arf6 mutant flies. Cell cycle regulators in somatic cells might put a less stringent time window on the time for cytokinesis completion than in the testes, in which case one might expect slower plasma membrane expansion kinetics in arf6 mutants than in WT. Alternatively, other recycling routes and the secretory pathway may play a relatively greater role in membrane insertion during cytokinesis in these tissues, in which case the kinetics in arf6 mutant cells would resemble those in WT cells.

By which mechanisms does ARF6 contribute to membrane addition?

This study revealed that ARF6 is required for rapid cleavage furrow ingression and rapid expansion of the plasma membrane during cytokinesis. Data on ARF6 function in mammalian cells has shown that ARF6 acts by binding effectors when it is in its GTP bound configuration. At a cellular level ARF6 regulates cortical Actin cytoskeletal remodelling, phospholipid metabolism and membrane trafficking. These three functions are intricately intertwined, and not mutually exclusive, although effector domain mutants suggest that ARF6 control of Actin cytoskeletal dynamics can be uncoupled from its membrane trafficking functions (Al-Awar et al., 2000).
Does ARF6 act on recycling endosomes at the central spindle?

How does ARF6 boost the plasma membrane expansion rate? One possibility is that ARF6 increases the rate of endocytic recycling, by connecting recycling endosomes concentrated at the central spindle with exocyst-defined fusion sites at the plasma membrane of the cleavage furrow. In HeLa cells, the exocyst complex is targeted to vesicular structures at the central spindle and at the cleavage furrow, which would be adjacent to the cortical central spindle ARF6 endosomes as shown in this report (Fielding et al., 2005; Gromley et al., 2005). It has previously been shown that ARF6-GTP interacts with Sec10, a subunit of the exocyst complex that localises to the trans Golgi network as well as to recycling endosomes, and is redistributed to the cell surface after ARF6 activation (Prigent et al., 2003). In this way, ARF6 interaction with the exocyst complex might mediate targeted recycling and insertion of membrane to dynamic regions of the plasma membrane (D'Souza-Schorey and Chavrier, 2006). A second possibility is that ARF6 affects recycling rates by activating its effector PLD, which has frequently been implicated downstream of ARF6 function during regulated secretion and exocytosis (Caumont et al., 1998; Jovanovic et al., 2006; Vitale et al., 2002a).

Does ARF6 act at the plasma membrane?

Another possibility is that ARF6 acts at the plasma membrane to activate its effector PI(4)P5K. In mammalian cells, PI(4)P5K is necessary for cytokinesis, and its product PtdIns(4,5)P2 is required at the plasma membrane for cytokinesis in Drosophila (Emoto et al., 2005; Wong et al., 2005). As discussed in the introduction, PtdIns(4,5)P2 at the plasma membrane may help to tether the actomyosin contractile ring to the plasma membrane. Additionally, the cleavage of PtdIns(4,5)P2 by phospholipase C produces IP3, which regulates the release of intracellular calcium stores by binding IP3 receptors, (reviewed in Berridge, 1993). ARF6 activation of PI(4)P5K could also therefore promote fusion of intracellular vesicles with the plasma membrane, since intracellular calcium release is detected by calcium sensors such as synaptotagmin, stimulating exocytosis in various cell types by promoting SNARE function (Chapman, 2002; Chen et al., 1999; De Blas et al., 2005; Michaut et al., 2000). Intracellular Calcium release may also be used to regulate exocytic events during cytokinesis. IP3 receptor dependent calcium release is essential for the
invagination of the furrow after furrow initiation in *Drosophila* spermatocytes and zebrafish embryos, and for exocytosis at the cleavage plane in sea urchin embryos, (Lee et al., 2003; Shuster and Burgess, 2002; Wong et al., 2005).

**Does ARF6 influence the actomyosin ring?**

An alternative possibility is that ARF6 acts in cytokinesis via its influence on the cortical Actin cytoskeleton. However, several results disfavour this hypothesis. First, no defects in the actomyosin ring formation or association to the cortex were seen in *arf6* null mutants in this study, or by overexpression of ARF6 or its mutants in a previous study (Schweitzer and D'Souza-Schorey, 2002). Second, ARF6Q37E,S38I, an effector domain mutant of ARF6 that in mammalian cells is able to carry out membrane trafficking functions but not promote Actin dependent membrane ruffling, was able to rescue the cytokinesis defects in the *arf6* null mutant. Third, although Actin dynamics are clearly responsible for contractile ring constriction, this does not explain the defect in plasma membrane addition, since data from this study and previous studies has shown that plasma membrane addition and ring constriction are uncoupled (Bluemink and de Laat, 1973; Drechsel et al., 1997; Shuster and Burgess, 2002).

In summary, the data from this study suggests that in the absence of ARF6, Rab4/Rab11 endosomes could still contribute to a basic rate of membrane recycling during cytokinesis. ARF6 may endow the recycling vesicles at the central spindle with a label to perform directed exocytosis using the exocyst complex, or PLD activity, or promote vesicle fusion with the plasma membrane indirectly by activating PI(4)P5K, thereby contributing to more efficient membrane insertion during cytokinesis. Experiments with the ARF6 effector domain mutants unable to interact with PLD, and the quantification of the amount of PtdIns(4,5)P2 at the plasma membrane in WT and ARF6 mutant cells could help to define the mechanism used by ARF6 more precisely.

**Functional significance of membrane trafficking component localisation during cytokinesis**

Is the targeting of the recycling endosomes to the cleavage furrow functionally significant? Does it reflect specific membrane delivery to the furrow? If the requirement of membrane surface increase addresses a purely geometric demand,
then it may not matter in which part of the plasma membrane the new membrane is inserted. However, at least in *Xenopus* embryos, the majority of membrane insertion during the cleavage divisions occurs next to the cleavage furrow (Bluemink and de Laat, 1973). Indeed, similar to our observations of recycling endosomes in the testes, the machinery required for fusion of intracellular vesicles with the plasma membrane, including the exocyst complex and syntaxin, is localised to the cleavage furrow and central spindle in many other cell types (Fielding et al., 2005; Gromley et al., 2005; Jantsch-Plunger and Glotzer, 1999; Low et al., 2003; VerPlank and Li, 2005). Therefore, proteins specifically localised at the central spindle may assemble the relevant endocytic/secretory factors to generate a highly efficient membrane addition machinery. The central spindle might in this way function as a sensor of the cytokinesis event which implements membrane trafficking at the right time and, perhaps, at the right place.

The observation that ARF6 binds to Pavarotti suggests a molecular link between the central spindle and an important part of the trafficking machinery. If they are not at the central spindle, both Rab4 and Rab11 recycling endosomes show low levels of ARF6 colocalisation during cytokinesis, while most of them contain ARF6 when at the central spindle. The results of this study suggest that the binding of Pavarotti to ARF6 ensures this local enrichment in the central spindle endosomes. Mammalian ARFs have previously been shown to bind MKLP1 in Yeast-2-Hybrid and *in-vitro* (Boman et al., 1999). This may reflect a general mechanism to recruit ARF labelled vesicles to the central spindle, from where they may be used as a membrane source for cytokinesis, with Pavarotti being a “pan-ARF recruiter”. At least ARF1 is also required for cytokinesis, although its mechanism of action is unclear. ARF1GTP may be downregulated to allow Golgi fragmentation and dissociation of factors necessary for cytokinesis such as Nir2 from the Golgi, but inhibition of ARF1 GEFs by BFA, which also causes Golgi fragmentation, blocks cytokinesis (Altan-Bonnet et al., 2003; Skop et al., 2001; Skop et al., 2004).

A direct test for the requirement of Pavarotti for the recruitment of ARF6 to the central spindle is still lacking. Since depletion of Pavarotti has such a severe impact on the formation of the central spindle, a more sophisticated approach than looking at *pav* loss of function in mosaics is required. Finer mapping of the residues in Pavarotti required for the interaction with ARF6, or *vice-versa*, would allow the
production of a Pavarotti mutant protein that cannot bind ARF6. Such a Pavarotti protein could then be tested for the ability to recruit ARF6 in spermatocytes lacking endogenous Pavarotti. Such an experiment could potentially answer two questions: is Pavarotti the signal that recruits ARF6 to the central spindle, and is it the central spindle population of ARF6 which is required for rapid membrane recycling during cytokinesis? A potential problem of this approach is that if Pavarotti is a pan-ARF central spindle recruiter, such a protein might not be able to support cytokinesis in somatic tissues. Although technically difficult, it may still be possible to test this mutant Pavarotti protein in clones of cells lacking the endogenous protein.

It has been proposed that it is ARF6 that recruits Rab11 recycling endosomes to the central spindle (Fielding et al., 2005). The data produced in this study shows that in the absence of ARF6, Rab4 recycling endosomes are still targeted to the spindle. Similarly, Rab11 recycling endosomes can also be observed at the central spindle in late arf6 regressors. It therefore seems that in Drosophila, ARF6 and Rab11 can be recruited independently and that ARF6 would act downstream of Rab4/Rab11 recycling endosome localisation to mediate rapid membrane recycling. Rab11 central spindle recruitment happens late and may therefore be responsible for completion of cytokinesis as previously suggested (Fielding et al., 2005; Wilson et al., 2005). The centralspindlin complex can then be viewed like a scaffold that brings together the necessary tools to perform cytokinesis: the actomyosin contractile ring, the basic Rab4/Rab11 endosomal machinery and factors which, like ARF6, contribute to efficient recycling.
Figure 27: Possible mechanisms of ARF6 function during cytokinesis

1. ARF6 is present at the plasma membrane, in the cytosol, and on endosomes. Pavarotti might recruit ARF6 to recycling endosomes at the central spindle

2. Recycling endosomes and the secretory pathway contribute to a slow rate of membrane insertion to the plasma membrane

3. Central spindle recycling endosomes enriched with ARF6 provide rapid membrane insertion to the plasma membrane

A life without ARF6

The data generated during this thesis shows that ARF6 plays an essential role during meiotic cytokinesis in the testes. In addition, the occasional occurrence of spermatids containing >4 nuclei in the arf6 mutant is consistent with cytokinesis failure during the mitosis prior to meiosis in the spermatocytes (Figure 6). Furthermore, the arf6 phenotype reveals an incompletely penetrant maternal ARF6
requirement during morphogenesis of the chorion (Figure 7). Otherwise mitosis of the somatic line and other developmental processes occur normally in individuals completely lacking ARF6: morphologically normal arf6 mutant adults are observed. The overexpression phenotype of ARF6Q67L-HA in the wing is consistent with a cytokinesis defect, despite the observation that ARF6 is not essential for cytokinesis in this tissue. Interaction of ARFs with GAPs, GEFs or effectors are thought to be specific in vivo. However, at high levels of overexpression, ARF6Q67L might be able to sequester proteins that normally interact with other ARFs, thus causing a non-specific cytokinesis phenotype. ARF6Q67L might additionally cause a partial block in cell cycle progression as previously observed (Schweitzer and D'Souza-Schorey, 2002).

In arf6 mutants, cytokinesis defects are seen only in the testes, but arf6 is not unique in this regard, since many other mutants that affect cytokinesis in Drosophila (e.g. four wheel drive, giotto, kinesin like protein 3A, four way stop) preferentially affect spermatocyte cytokinesis, and have little or no effect on the somatic line (Brill et al., 2000; Farkas et al., 2003; Giansanti et al., 2006; Williams et al., 1995). It is however surprising that many of the previously proposed ARF6-dependent processes are not affected when ARF6 is completely absent (in the maternal/zygotic null mutants). For example, the GTP exchange factor Loner/Schizo, which plays a role during myoblast fusion and axon path finding in Drosophila, has been proposed to catalyze GTP exchange of ARF6 (Chen et al., 2003; Onel et al., 2004). This was based on the specific GEF activity of Loner on ARF6, but not ARF1 in-vitro, as well as the phenotype of overexpression of the dominant negative, GDP-bound, ARF6 mutant. The lack of myoblast/neuronal phenotypes in arf6 null mutants shows that either the real target of Loner/Schizo is another GTPase different to ARF6, or that in the absence of ARF6 Loner/Schizo can recruit a second target that can fully perform the functions of ARF6. Furthermore, the overexpression of ARF6T27N-HA in this study did not result in a visible or viability phenotype when overexpressed in the embryo or imaginal discs, which is difficult to reconcile with the previous published results.

In mammalian cultured cells, ARF6 has been implicated in a number of essential processes including cell migration, cell-cell adhesion and phagocytosis (D'Souza-Schorey and Chavrier, 2006). The mouse arf6 knockout shows impaired
cell migration during hepatic cord formation (Suzuki et al., 2006) Those processes are, however, not affected in the arf6 null condition in Drosophila. In principle, one possibility could be that a second redundant arf6 gene acts to carry out those ARF6-dependent functions. No second arf6 gene has been found in Drosophila (Lee et al., 1994), and with the complete sequencing of the Drosophila genome such possibility can be excluded. In addition, the closest homolog to ARF6 encoded in the genome is the Golgi protein ARF1 (68% identical), which is specifically involved in trafficking through the secretory, but not the endocytic pathway, (reviewed in D'Souza-Schorey and Chavrier, 2006), and thus is unlikely to compensate for a specific requirement for ARRF6 in the control of endocytic trafficking. However, in the case of rapid membrane expansion during cytokinesis, it may be that a sufficient rate of membrane insertion is all that is required by the cell. If membrane insertion during cytokinesis is only an issue of supply and demand, any source which provides membrane at the right place and time would be sufficient for successful cytokinesis. The phenotype of an arf6 knock out in mammalian systems will tell us in the future whether those functions of ARF6 are specific to vertebrates, but dispensable in insects.

In summary, it is likely that many membrane trafficking components act degenerately to ensure sufficient membrane insertion during cytokinesis. This serves as a repetition of the lesson in biology that natural selection acts to ensure that processes such as cytokinesis work reliably, but not to ensure that they are simple or even necessarily efficient. After over 25 years working on cytokinesis, its high level of degeneracy led Ray Rappaport to comment “When I began working on cytokinesis, I thought I was tinkering with a beautifully made Swiss watch, but what I was really working on was an old Maine fishing boat engine: overbuilt, inefficient, never-failed, and repaired by simple measures.” (keynote address at the ASCB cytokinesis meeting, 2004).
Materials and Methods

_Drosophila stocks and genetics_

_**arf6** mutants_

_arf6_ alleles _arf6^1_, _arf6^2_ and _arf6^3_ were generated by the imprecise excision of EP(2)2612 in the presence of transposase. PCR and sequencing of flanking regions confirmed that only the _arf6_ gene was affected. The chromosome carrying EP(2)2612 carried a linked lethal mutation, which was cleaned by recombination. A homozygous viable EP(2)2612-containing chromosome was subsequently used to remove the linked mutation from _arf6^1_. _arf6^2_ and _arf6^3_ were cleaned by recombination with the chromosome _P(w+FRTG13) L^2 sp_

_Cloning and transgenics_

_P(w^+Ubi:arf6-HA)_

_P(w^+Ubi:arf6-HA)_ construct: a PCR product containing _arf6_ coding sequence from the LD22876 clone (from the Berkeley _Drosophila_ Genome Project Expressed Sequence Tag Project) with SacI and XbaI-sites in primer overhangs and including a C-terminal HA epitope tag was inserted between the SacI and XbaI site of pSRalpha. To express _arf6-HA_ under the control of the polyubiquitin promoter, the SacI to XbaI fragment containing arf6-HA was excised from the PSRalpha construct and ligated between the SacI and XbaI sites of the TOPO vector. A Kpn1 to Xba1 fragment from the resulting TOPOarf6-HA vector was cloned into the polyubiquitin vector between the Kpn1 and Spe1 sites and used to generate transgenic flies.

_P(UASTarf6Q67L-HA) and P(UASTarf6T27N-HA)_

The T27N mutation in _arf6_ was generated by insertional mutagenesis in TOPOarf6-HA with pfu using the complementary primers CGCGGCTGGAAAAAACACGA TTCTG and CAGAATCGTGTTTTTTCCAG CCGCG, and confirmed by sequencing. ARF6Q67L-HA in the pSRalpha vector was a gift from Philippe Chavrier. Sac to Xba1 fragments from from the TOPOarf6-HA vectors between the Kpn1 to Xba1 sites in the pUAST vector. The resulting _P(UAST:arf6Q67L-HA)_ and _P(UAST:arf6T27N-HA)_ vectors were used to make transgenic flies. _arf6T27N-HA_
was overexpressed using Da:GAL4, en:GAL4, GMR:GAL4 and ey:GAL4 (Freeman, 1997.3.15; Hazelett et al., 1998; Wodarz et al., 1995). For the experiments on the wing, arf6Q67L-HA was overexpressed in the posterior compartment using en:GAL4.

P(w′arf6+)

For P(w′arf6+), a 3.8Kb fragment containing the arf6 gene and flanking sequences was amplified from genomic DNA and cloned into the TOPO-XL vector. The TOPO-XL arf6rescue vector was digested with Xba1, Not1 and Sph1, and the resulting 3.8Kb fragment cloned into the pCasper4 vector between the Not1 and Xba1 sites and used to generate transgenic flies expressing arf6 from the endogenous promoter.

P(w′arf6Q37E,S38I)

For P(w′arf6Q37E,S38I), the TOPO-XL vector containing the arf6 gene and flanking sequences was mutagenised by PCR with pfu, using the complementary primers GTATCGTTGTAACAATTTGCCAAGTTTC and GAAACTTGGCGAAATTGTTACAACGATA. After sequencing to confirm the presence of this mutation and the lack of mutations elsewhere, the new sequence was cloned into pCasper4 as above, and used to make transgenic flies.

P(UbiGFP-Rab5), P(UbiGFP-Rab11) and P(UbiGFP-Rab4)

P(UbiGFP-Rab5) and P(UbiGFP-Rab11) were generated from the previously described pUAST-GFP-Rab5 (Wucherpfennig et al., 2003), and pUAST-GFP-Rab11 (Emery et al., 2005). For the generation of P(UbiGFP-Rab4), a PCR product containing rab4 coding sequence from pOT2-GH18176 with Xho1-sites in primer overhangs was cloned into pEGFP-C3 at the Xho1 site. The Nhe1-Xba1 fragment from pEGFP-C3-rab4 containing EGFP-rab4 was cloned into the Xba1-site of pUAST. In all cases, Not1-Xba1 fragments from the pUAST vector containing GFP-Rab were cloned into the polyubiquitin vector between the Not1 and Xba1 cloning sites and used to generate transgenic flies.
Previously described stocks used for video microscopy and genetic interactions

The pav\textsuperscript{B200}, pav\textsuperscript{A375} and chic\textsuperscript{13E} alleles have been described previously (Adams et al., 1998; Giansanti et al., 1998; Salzberg et al., 1994). Pav-GFP, GFP–alpha-tub84B, His2AvD-GFP, and DE-cad-GFP expressed under the control of the polyubiquitin promoter (Lee et al., 1988) have previously been described (Clarkson and Saint, 1999; Minestrini et al., 2003; Oda and Tsukita, 2001; Rebollo et al., 2004). Sqh-GFP under the control of the sqh promoter has also been previously described (Royou et al., 2004). Flies expressing γ-Tubulin GFP were a gift from S. Llamazares (S. Llamazares, personal communication).

Clones of pav\textsuperscript{B200} cells in the testes

To generate clones lacking Pavarotti in the testes, the flippase (flp)/ Flippase Recombination Target (FRT) system was used (Golic and Lindquist, 1989). Males of genotype \textit{yw hsflp; p(ubi:arfHA)/+ ;P(ubi-GFP)61EFP(neoFRT)80B/pav\textsuperscript{B200}} \textit{P(neoFRT)80B}, raised at 25˚C, were heat shocked 4-5 days after egg laying two times for one hour at 38˚C, and eclosed adults were dissected 5-7 days later. \textit{pav\textsuperscript{B200}} clones were identified by the lack of the nuclear GFP signal.

Germ Line Clones of \textit{arf6} mutants

The germline cells contributing to each egg consists of 16 cells, one of which is selected to become the oocyte, the other 15 becoming nurse cells. Germline cells are surrounded by an epithelial layer of somatic follicle cells. To determine whether the requirement for ARF6 in the production of eggs is in somatic cells or germline cells, the flp/ FRT system was used to generate germ line clones as described in (Chou and Perrimon, 1992). Briefly, females of genotype \textit{yw hsflp; FRTG13 Ovo\textsuperscript{D1}/FRTG13 arf6\textsuperscript{l}} raised at 25˚C were heat shocked for two hours at 38˚C as third instar larvae to activate the flippase, and generate germ line clones of genotype \textit{yw hsflp; FRTG13 arf6\textsuperscript{l}/FRTG13 arf6\textsuperscript{l}}. \textit{Ovo\textsuperscript{D1}} is a dominant female sterile mutation which, when present in germline cells, prevents them from developing into vitellogenic eggs, so only \textit{arf6\textsuperscript{l}} homozygous germ line cells developed into eggs that were laid. Adults were collected and crossed for three days to wild type males (strain Oregon R) in vials supplemented with fresh yeast. Eggs were collected from yeast
supplemented apple juice plates in laying cages. The same procedure was used for arf62 and arf63.

**Protein extraction from *Drosophila* adults**

15 flies per sample were frozen at -80°C in an Eppendorf tube. Flies were ground in 200µl ice-cold PBS containing 1% SDS (sodium dodecyl sulphate) and protease inhibitor cocktail tablets (Roche). The resulting mixture was sonicated twice for 20 seconds at 20% power with a microtip sonicator (Bandelin Sonoplus), and a 10µl aliquot reserved for the Bradford protein quantification assay. 40µl 6* loading buffer containing β-mercaptoethanol was added, and the sample incubated for 5 minutes at 95°C. Samples were centrifuged for one minute at 13,000 RPM in a benchtop centrifuge, and the supernatant transferred to a clean 1.5ml Eppendorf and stored at -20°C.

**ARF6 antibody and detection of ARF6 by Western blotting**

A polyclonal antibody was raised (Eurogentec) in rabbit against the peptide ARTELHRIINDREM, corresponding to amino acids 99-112 of *Drosophila* ARF6. In Western blotting analysis, this antibody binds a 20KDa protein, which is ARF6, since this band is eliminated in extracts from arf61 mutant flies. Several other proteins of >20KDa are also bound, which are unaffected in extracts from arf61 mutants. Anti-ARF6 antibody was not suitable for immunofluorescence.

For Western blotting, rabbit anti Actin (Sigma A2066) was used 1:400 and rabbit anti ARF6 (described above) was used at 1:50. Proteins were separated using sodium dodecylsulfate-polyacrylamide gel electrophoresis in a 14% acrylamide gel, and transferred to PVDF membranes (Millipore) using a semi dry transfer cell (Biorad). Membranes were blocked overnight in PBS containing 0.1% Tween-20 and 5% dried milk powder (“Western buffer”). Primary antibodies were incubated with the membranes for 90 minutes at room temperature. Membranes were washed three times 20 minutes in PBS with 0.1% Tween 20. Primary antibodies were detected by incubating for 45 minutes at room temperature with HRP-conjugated anti Rabbit at 1:15000 (Dako) in Western buffer. After three twenty minute washes, HRP was detected using the ECL plus western blot detection kit (Amersham Biosciences).
Immunofluorescence Staining

Embryos aged 12-24 hours were collected on apple juice plates from laying cages. Embryos were collected on a metal sieve, rinsed with water and dechorionated in bleach:water (1:1 mix) for three minutes. Embryos were rinsed thoroughly with water and blotted dry on a tissue. For examination of myoblast fusion and midline crossing of commissural axons, embryos were transferred to a scintillation vial containing 0.5ml 37% paraformaldehyde (PFA), 4.5ml fix buffer (100mM HEPES, 2mM MgSO$_4$, 1mM EGTA, pH 6.9) and 5ml heptane. After 20 minutes of shaking, the fix buffer and PFA were removed and 5ml of methanol added. After 30 seconds of vortexing, devitellinized embryos were removed and washed three times in methanol, and stored at -20°C. For preservation of Pavarotti and ARF6-HA, a “strong fix” protocol was used: after blotting dry, embryos were fixed for four minutes in 5ml heptane saturated with 5ml 37% PFA, and devitellinized as described above. This fixation was poor for preserving the nuclear Pavarotti signal, but very effective for cytoskeletal components and division scars. For staining, embryos were rehydrated for 10 minutes in 1:1 methanol:PBT (PBS containing 0.1% Triton X), and three times for 20 minutes in PBT. Embryos were blocked for one to two hours in PBT containing 0.5% BSA (bovine serum albumin). Embryos were incubated with primary antibodies at 4°C overnight in PBT containing 0.5% BSA. Mouse antibody BP102 from the Hybridoma bank was used at 1:30. A rabbit polyclonal antibody against β-Galactocidase (Cappel) was used at 1:5000 to identify heterozygous arf6 embryos zygotically rescued by the CyO, hb-lacZ balancer. Rabbit anti MHC (Kiehart and Feghali, 1986) was used at 1:500. To detect primary antibodies, after three 20 minute washes in PBT, embryos were incubated with secondary antibodies in PBT containing 2% normal goat serum (NGS) for two hours at room temperature. The following secondary antibodies from Molecular Probes were used at 1:500: Alexa 488 conjugated goat anti rabbit (MHC stainings) or Alexa 546 conjugated goat anti rabbit and Alexa 488 conjugated goat anti mouse (BP102 stainings). Finally, embryos were washed three times for 20 minutes in PBT, and transferred to 20%, 40% and finally 80%, Glycerol in PBS for mounting.
**Larval neuroblasts**

Brains were dissected from late third instar larvae in PBS, and fixed for 20 minutes in freshly defrosted PBS containing 3.7% PFA. After washing in PBS, brains were blocked in blocking buffer (PBS containing 0.3% Triton X and 10% BSA). Primary antibodies were incubated with the brains in blocking buffer overnight at 4°C at the following concentrations: rat anti HA (Clone 3F10, Roche) 1:500, rabbit anti-acetyl and phospo Histone H3 (Sigma, cat H9161) 1:1000. After three twenty minute washes in PBS containing 0.3% Triton X, primary antibodies were detected using Alexa 546 conjugated goat anti rat (Molecular probes) and Cy5 conjugated Goat anti rabbit (Jackson ImmunoResearch Laboratories) at 1:500. After three washes in PBS containing 0.3% triton X, brains were mounted with the ventral side towards the coverslip in Moviol. This procedure was effective for preserving the ARF6-HA in the ventral ganglion, but not for the central brain.

**Sensory organ precursors**

To fix sensory organ precursors during the division of the P1 cell, pupariating larvae of genotype w; */GAL80TS; UAS:arf6-HA/Neur:GAL4 (Venugopala Reddy et al., 1999) were collected and incubated at 29°C 13.5 hours before dissection. The dorsal cuticle was isolated in PEM and fixed for 20 minutes in PEM containing 4% PFA. After separation from the abdominal cuticle, nota were fixed for a further 20 minutes in 4% PFA in PEMT (PEM containing 0.2% Triton X). Nota were washed twice for five minutes in PEMT, once for ten minutes in PEM containing 5mM NH₄Cl and twice for five minutes in PEMT before blocking for two hours in blocking buffer (PEMT containing 0.5% BSA and 1% NGS). Primary antibodies were incubated with nota overnight at 4°C in blocking buffer at the following concentrations: rat anti HA (Clone 3F10, Roche) 1:500, rabbit anti Pavarotti 1:250 (Adams et al., 1998). Nota were washed three times for 20 minutes in PEMT, and primary antibodies were detected with Alexa 488 conjugated goat anti rat and Alexa 546 conjugated goat anti rabbit (Molecular probes). Nota were washed for 20 minutes in PEMT, 20 minutes in PEMT containing DAPI to detect DNA and 20 minutes in PEMT, and mounted ventral side to the coverslip in Moviol.
Testes

Testes dissected from adult males in PBS, fixed for 20 minutes PBS containing 4% paraformaldehyde and then for another 20 minutes after the addition of 0.2% TritonX-100. Fixative was removed by washing with PBS and then with PBS containing 0.1% TritonX-100. All subsequent staining and washing steps were carried out in PBS containing 0.1% TritonX-100. Blocking was performed for at least 2 hours with 0.5% BSA. Testes were incubated overnight at 4°C with 0.5% BSA and primary antibodies at the following concentrations: rat anti HA (clone 3F10, Roche), 1:500, rabbit anti Pavarotti 1:250 (Adams et al., 1998), rabbit anti Klp3A 1:500 (Williams et al., 1995). Following 3* 20 minute washes, primary antibodies were detected using Alexa546 conjugated anti rat (Molecular probes) and Cy5 conjugated anti rabbit (Jackson ImmunoResearch Laboratories) at 1:500, in the presence of 2% NGS for 2 hours at room temperature. Finally, testes were washed three times for 20 minutes. Confocal images were acquired using a Zeiss LSM 510 microscope, and with 63X (N.A. 1.4) or 100X (N.A. 1.4) objective lenses.

Live spermatocyte imaging
Oil Protocol

Primary spermatocytes were prepared for confocal time-lapse imaging as described in (Rebollo and Gonzalez, 2004), except that coverslips for the culture chambers were immersion sonicated for 15 minutes in 1% 7X PF detergent (MP Biomedicals) using a Branson 2510 sonicator instead of the first boiling step. The second boiling step was also replaced with a 10 minute sonication in absolute ethanol.

Schneider’s Protocol

Culture chambers were prepared as described above. Males collected within one day of hatching were anaesthetised and the testes dissected and washed in Schneider’s medium containing 10% foetal calf serum (complete Schneider’s). Testes were transferred to a 20μl drop of complete Schneider’s in the culture chamber, and cut near the tips using syringe needles (BD microlance 3, gauge 0.3mm). Cysts of cells were gently pushed out of the testes and separated using the syringe needles. The culture chamber was filled with complete Schneider’s containing 8μM FM4-64 (Molecular Probes) to visualize cell membranes. A second coverslip was added to the
top of the culture chamber and fixed in place with a drop of mineral oil to prevent evaporation.

**Image Quantification**

**Colocalisation**

Colocalisation (given as % colocalisation ± standard error of the mean) was quantified in the Zeiss LSM image browser by manually counting punctae. In dividing cells, punctae within 3µm of Pavarotti staining were classified as “central spindle” localised, other punctae as “non central spindle”. Images were processed with Adobe Photoshop 7.0 (Adobe Systems).

**Cell diameter and Perimeter**

Cell perimeter and diameter were measured in ImageJ (http://rsb.info.nih.gov/ij/). Rates given for furrow ingression, perimeter and surface area changes are the slope of the linear regression line through the data points between the given time points. Fluorescence images are the maximal intensity projection between 4 and 6 confocal sections except for Sqh-GFP, where a single section is shown.

**Volume Measurement**

To calculate total cell volume, confocal z-stacks of 0.51µm optical slices through spermatocytes expressing DE-Cadherin-GFP before and after cytokinesis, were obtained. Volume was calculated as the area encompassed by the cell in each slice, multiplied by the slice thickness.

**Surface area calculations**

For the controls (n= 6 cells) to test the suitability of perimeter measurements to determine cell surface area, spermatocytes were cultured using the Schneider’s protocol. 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 cones of which the radii are the distances between the points on the outline and the axis of symmetry. On average, WT cells increased in surface area by over
20% during cytokinesis. As a control for the accuracy of the surface area measurements using midplane confocal sections, it was tested whether the volume stays constant. For three cells the volume was also calculated from an outline using the same ‘cones approximation”. The standard deviations of the calculated volume were 4.5%, 2.7% and 2.6%, and the next volume change over the course of the experiment ranged from -4% to +6%. This volume change is not sufficient to account for the observed surface area increase.

The software used to calculate the surface area and volume from cell outlines is on the CD. For each cell, the relationship between perimeter and surface area could be described by the linear function

\[ P = mS + c \]

where \( P \) is perimeter, \( S \) is surface area, \( m \) is the slope of the linear regression line, and \( c \) the intercept on the perimeter axis. \( m \) was similar for different cells, whereas \( c \) was variable (Fig. 13, table 2). This means that 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 over time is a good indicator of the rate of surface area change.

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 change perimeter between \( t_1 \) and \( t_2 \) is

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

And the rate of perimeter change for the time period \( t_1 \) to \( t_2 \) is therefore

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

So the rate of perimeter change and the rate of surface area change are proportional by the factor \( m \).

Wing compartment quantification

The distal area of the wing (distal to a line separating the proximal and distal regions, as shown in Figure 26) was measured using ImageJ. Within this area (\( A_t \)), the posterior compartment was approximated by the area between vein L4 and the posterior wing margin (\( A_p \)), as there is no clear morphological structure in the adult wing to indicate the anterior/posterior compartment boundary. In wings with a hole in
the second posterior cell, the area of the hole was subtracted from the measurements
to give the true values of $A_t$ and $A_p$. The anterior compartment area $A_a$ was then
calculated as $A_t - A_p$, and the ‘relative size’ of the posterior compartment as $A_p/ A_a$.
For hair density, the number of hairs was counted inside an area of known size in the
submarginal cell for the anterior compartment, and in the second posterior cell for the
posterior compartment.

**Pavarotti Binding Assay**

The sequence encoding Pavarotti amino acids 655-865 was cloned into
pGEX4T1 at the carboxy terminus of GST. pGEX4T1Pav655-865 and pGEX4T1
were transformed into E.coli strain B21. Expression was induced by 1mM isopropyl
β-D-thiogalactopyranoside for 5 h at 20°C. GST proteins were affinity purified using
glutathione sepharose beads (Amersham Biosciences). GST proteins were eluted using
glutathione and dialyzed against a buffer containing 20 mM Tris, pH 7.4, 150 mM
NaCl, 2 mM EDTA, 2 mM β-mercaptoethanol, and 10% glycerol, and stored at -80°C.
pSRalpha(ARF6-HA) and pST(alpha)ARF6Q67L-HA was transfected into HeLa cells
using Effectene (Qiagen). Cells were harvested 20-24 hours after transfection and lysed
in 50mM Tris pH5.5, 137mM NaCl, 1% TritonX-100, 10mM MgCl₂, 10% glycerol
(Buffer B) with complete protease inhibitor cocktail tablets (Roche), followed by a 15
minute centrifugation at 13,000rpm at 4°C. Supernatants were incubated with 20µg of
GST fusion protein for 15 minutes at 4°C in the presence of 0.5% BSA, and then for 1-2
hours at 4°C after the addition of glutathione sepharose beads. Beads were washed
three times in Buffer B, once in Buffer B containing 0.1% SDS and once in PBS.
Bound proteins were eluted using 4x NuPage LDS sample buffer (Invitrogen). The
presence of ARF6 proteins and the amount of GST proteins bound to the beads was
assayed by Western blot analysis using rabbit anti HA antibody diluted 1:500 (clone
3F10, Roche) and affinity purified polyclonal rabbit anti GST antibodies diluted
1:10,000 (prepared by the Protein Expression and Purification Facility, MPI-CBG,
Dresden). Western blot analysis was performed as described above, except that for the
GST Western, a 10% acrylamide gel was used, and to detect rat anti HA, HRP
conjugated anti Rat was used at 1:500 (Jackson). The binding assay was repeated in
three independent experiments with the same results.
Publication

Part of this thesis has been submitted for publication:
References


cell-cell contacts and specifies transport vesicle delivery to the basal-lateral membrane in epithelial cells. *Cell* 93, 731-40.


Appendix 1: Mathematics of the cell division model

Proportions of multinucleated cells after meiosis II cytokinesis

The proportion of cells with 1, 2 or 4 nuclei after meiosis II can be calculated, assuming that the probability of cytokinesis failure, P is constant (Figure 8)

1:1 \[4(1-p)^3 + 4p(1-p)^2\]
\[= 4 - 8p + 4p^2 - 4p + 8p^2 - 4p^3 + 4p - 8p^2 + 4p^3\]
\[= 4(1-p)^2\]

2:1 \[2p(1-p) + 2p(1-p)^2 + 2p^2 (1-p)\]
\[= 2p - 2p^2 + 2p - 4p^2 + 2p^2\]
\[= 4p(1-p)\]

4:1 \[p^2\]

The sum of these ratios is
\[4(1-p)^2 + 4p(1-p) + p^2\]
\[= 4 - 8p + 4p^2 + 4p - 4p^2 + p^2\]
\[= 4 - 4p + p^2\]
\[= (2-p)^2\]

Therefore the actual proportions of cells of types 1:1, 2:1, 4:1 are given respectively by a, b, and c in

\[(2-p)^2 a = 4(1-p)^2 \quad (1)\]
\[(2-p)^2 b = 4p(1-p) \quad (2)\]
\[(2-p)^2 c = p^2 \quad (3)\]

Where a is the proportion of cells with one nucleus, b is the proportion of cells with two nuclei and c is the proportion of cells with 4 nuclei.

Differentiating (1) with respect to p, using the notation a' for the derivative of a, we have

\[-2(2-p) a + (2-p)^2 a' = -8(1-p)\]
\[-8(1-p)^2 + (2-p)^3 a' = -8(1-p)(2-p) \quad \text{[multiply by (2-p) and substitute]}\]
\[(2-p)^3 a' = 8(1-p)^2 - 8(1-p)(2-p)\]
\[\quad = 8(1-p)(1-p - 2+p)\]
\[
\frac{-8(1-p)(2-p)^2}{(2-p)^3 b'} = 4(1-p)(2-p)
\]

Similarly from (2),
\[
\frac{-8p(1-p)(2-p)^3 b'}{8p(1-p) + 4(1-2p)(2-p)} = \frac{4(2p-2p^2 + 2-5p+2p^2)}{4(2 - 3p)}
\]

and
\[
\frac{-2(2-p) c + (2-p)^2 c'}{2p(2-p)} = \frac{2p^2 + 2p(2-p)}{4p}
\]

Least sum of squares

In order to test this model of spermatocyte cell division failure frequencies, it was necessary to test how well real data fits to the model. Let the observed proportions of cells containing 1, 2 or 4 nuclei be designated by \( A_i, B_i \) and \( C_i \). \( a, b \) and \( c \) are the theoretical values of these proportions which all correspond to a certain value of \( p \) in the model. If the theoretical values from the model fit the experimental data, then \( A_i \) is very close in value to \( a \), \( B_i \) to \( b \) and \( C_i \) to \( c \). To assess how good the model is, starting from experimental data, it is necessary to:

i) Find the value of \( p \) which minimises the squared differences between the expected \((a, b, c)\) and observed \((A_i, B_i, C_i)\) frequencies for that value of \( p \).

ii) Use the size of the sum of squares to assess the validity of the model.

The sum of squares of differences is
\[
S = (A_i - a)^2 + (B_i - b)^2 + (C_i - c)^2
\]

For least sum of squares \( S \) has a minimum value, and the derivative \( S' = 0 \). Hence differentiating with respect to \( p \) gives
\[
2(A_i - a) A_i' + 2(B_i - b) B_i' + 2(C_i - c) C_i' = 0
\]
\[
A_i A_i' + B_i B_i' + C_i C_i' = a A_i' + b B_i' + c C_i'
\]

Multiplying by \((2-p)^5\) and substituting for \( A_i, A_i' \) etc,
\[
-32(1-p)^3 + 16p(1-p)(2-3p) + 4p^3 = -8a(1-p)(2-p)^2 + 4b(2-3p) (2-p)^2 + 4cp(2-p)^2
\]

Dividing by 4 and expanding terms
\[-8+24p-24p^2+8p^3 + 8p-20p^2+12p^3 + p^3 = -2a(4-8p+5p^2-p^3) + b(8-20p+14p^2-3p^3) + c(4p-4p^2+p^3)\]
\((-8+8a-8b) + (32-16a+20b-4c) p + (-44+10a-14b+4c) p^2 + (21-2a+3b-c) p^3 = 0\]
\(-8(1-a+b) + 4(8-4a+5b-c) p - 2(22-5a+7b-2c) p^2 + (21-2a+3b-c) p^3 = 0 \quad (4)\]

The least sum of squares fit to given values a, b, c is therefore a value of p, which is a root of this cubic equation.

**Existence of at least one solution for cubic equation (4) in the Cell Division Model**

In the cubic function (4)
\[y = -8(1-a+b) + 4(8-4a+5b-c) p - 2(22-5a+7b-2c) p^2 + (21-2a+3b-c) p^3\]
we are expecting a root between 0 and 1 to give the value of p for the least sum of squares fit for the data a, b, c with the cell division model, where
\[a + b + c = 1, \quad a \geq 0, \quad b \geq 0, \quad c \geq 0\]

First, we can show there is a root between 0 and 1, by showing \(y \leq 0\) when \(p = 0\) and \(y \geq 0\) when \(p = 1\):

At \(p = 0\),
\[y = -8(1-a+b) \leq 0\]
since \(1-a+b \geq 1-a \geq 0\).

At \(p = 1\),
\[y = -8(1-a+b) + 4(8-4a+5b-c) - 2(22-5a+7b-2c) + (21-2a+3b-c) = 1 + b - c \geq 1 - c \geq 0\]
Appendix 2: Legend to movies on CD

All movies are time-lapses of *Drosophila* spermatocytes in meiosis I. The rate of image collection is not constant within each video, so in movies 1-6, times are given in hours:mins:seconds and anaphase onset is indicated, and in movies Fig7-15, times relative to anaphase onset (min AA) are indicated in mins:seconds. Frames are shown for 0.2 seconds except frames with annotations such as “Anaphase onset” or arrowheads, which are shown for 0.4 seconds.

**Movie1 His2AvD-GFP in control**

Left panel: condensed chromosomes labelled with His2AvD-GFP congress to the metaphase plate before segregating at anaphase. Right panel: the same cell imaged using phase contrast. Chromosomes initially appear as phase dark structures in a pale spindle-envelope surrounded by phase dark membranes. The cleavage furrow forms after the chromosomes have reached the poles and have started to decondense.

Genotype *w; FRTG13 arf6^3/CyO; P(UbiHis2AvDGFP)/TM6B*

**Movie2 Histone2AvD-GFP in arf6^3**

Left panel: GFP, right panel: phase contrast. Chromosomes behave as in controls, showing no obvious segregation defects, and the cytokinesis furrow is not initiated until after chromosomes have reached the poles.

Genotype *w; FRTG13 arf6^3/FRTG13 arf6^3; P(UbiHis2AvDGFP)/TM6B*

**Movie3 γ-Tubulin GFP in control**

At anaphase onset, centrosomes appear as single objects labelled with γ-Tubulin GFP at the spindle poles. During telophase, when cleavage furrow ingression is almost complete, the centrosome can be seen splitting. Genotype *w; FRTG13 arf6^3/CyO; P(Ubi γ-Tubulin GFP)/MKRS*
Movie 4 $\gamma$-Tubulin GFP in \textit{arf6} 

Centrosomes are localised as in controls, and separate normally during cleavage furrow invagination. Genotype \textit{w; FRTG13 arf6^{1}/FRTG13 arf6^{1}; P(Ubi $\gamma$-Tubulin GFP)/MKRS}

Movie 5 GFP–alpha-tubulin84B in control

The metaphase spindle, labelled with alpha Tubulin GFP, consists of two populations of microtubules: those in the centre of the cells inside the partially broken down nuclear envelope, and an outer population in close contact with the cortex. After anaphase onset, the central spindle consists of both inner and outer populations of non-kinetocore microtubules, which start to become bundled, appearing brighter. Bundles of the outer microtubule population concentrate at the position where the cleavage furrow will form. As the cytokinesis furrow invaginates, the populations appear to meet as a dense midbody is formed (arrowheads, time 20min AA). Genotype \textit{P(Ubi-GFP-alpha-Tub84B);arf6^{1}/CyO}

Movie 6 GFP–alpha-tubulin84B in \textit{arf6^{1}}

The spindle appears similar to the control throughout metaphase and anaphase. 20min AA, some bundling of central spindle microtubules can be seen (arrowhead), but these microtubules are later lost from the central spindle and the furrow regresses. \textit{P(Ubi-GFP-alpha-Tub84B);arf6^{1}/arf6^{1}}

Movie 7 Pav-GFP in control

During metaphase, Pav-GFP is cytosolic. After anaphase onset, Pav-GFP accumulates on central spindle microtubules during anaphase B cell elongation, before the initiation of the cytokinesis cleavage furrow. Pav-GFP remains on the increasingly
bundled central spindle microtubules during cleavage furrow invagination. Genotype w; arf6^+/CyO; P(UbiPav-GFP)/TM6B

Movie 8 Pav-GFP in arf6^1

Pav-GFP is localised as in control cells until after cleavage furrow initiation. As the cleavage furrow stops invaginating, Pav-GFP signal fades from the central spindle microtubules, and eventually the cleavage furrow regresses. Genotype w; arf6^1/arf6^1; P(UbiPav-GFP)/TM6B TM6B

Movie 9 Sqh-GFP in control

During metaphase Sqh-GFP is localised in the cytosol and several punctae. After anaphase onset Sqh-GFP is transferred to the cortex, where it concentrates at the site of the future cleavage furrow. Sqh-GFP remains concentrated at the cleavage furrow during invagination. Genotype y w sqh^{AX3}; P(w+ sqh-gfp)

Movie 10 Sqh-GFP in arf6^3

Sqh-GFP is localised as in the control until cytokinesis. As the cleavage furrow regresses, Sqh-GFP remains associated to it. Genotype y w sqh^{AX3}; FRTG13 arf6^3/FRTG13 arf6^3; P(w+ sqh-gfp)

Movie 11 GFP-Rab4 in control

GFP-Rab4 is localised to endosomes and cytosol. Rab4 endosomes can be seen concentrating at the central spindle as the cytokinesis furrow starts to ingress (arrowheads) and remains concentrated at the central spindle until furrow ingression is complete. Genotype w P(Ubi-GFP-Rab4); +/+ 

Movie 12 GFP-Rab4 in arf6^3

Rab4 GFP is localised as in control cells, and concentrates at the central spindle during cytokinesis (arrowheads). Genotype w P(Ubi-GFP-Rab4); FRTG13 arf6^3/FRTG13 arf6^3
**Movie 13 GFP-Rab11 in control**

Rab11 is localised to endosomes and the cytosol. When the furrow is deeply invaginated, Rab11 endosomes concentrate at the central spindle (arrowheads).

Genotype w; P(UbiGFP-Rab11)/SM6B

**Movie 14 GFP-Rab11 in arf6^1 late regressor**

Prior to cytokinesis GFP-Rab11 is localised as in controls. Some accumulation of GFP-Rab11 at the central spindle can be seen (arrowhead) before the furrow regresses. Since the recording of this cell started after anaphase onset, the min AA shown were estimated on the basis of the furrow ingression.

Genotype w; P(UbiGFP-Rab11)arf6^1/arf6^1

**Movie 15 arf6^3 late regressor**

This cell dividing in Schneider’s medium and FM4-64 shows furrow kinetics similar to controls, reaching a minimum diameter of 3µm (arrowheads, 46:04 minAA.

Genotype w; FRTG13 arf6^3/FRTG13 arf6^3; P(UbiHis2AvD-GFP)/TM6B
## Appendix 3: Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>AA</td>
<td>After anaphase onset</td>
</tr>
<tr>
<td>ACAP</td>
<td>Arf GAP with coiled coil, ANK repeat and PH domains</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP Ribosylation Factors</td>
</tr>
<tr>
<td>Arl</td>
<td>Arflike</td>
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<tr>
<td>ARNO</td>
<td>ARF nucleotide-binding site opener</td>
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<tr>
<td>BFA</td>
<td>Brefeldin A</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>Ca$^{2+}$</td>
<td>Calcium ion</td>
</tr>
<tr>
<td>CDK</td>
<td>Cyclin dependent protein kinase</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
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<tr>
<td>CHO</td>
<td>Chinese Hamster Ovary</td>
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<td>Chickadee</td>
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<td>Carboxy terminal</td>
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<td>Diacylglycerol</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>duf</td>
<td>Dumbfounded</td>
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<td>E</td>
<td>Glutamic acid</td>
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<tr>
<td>EFA6</td>
<td>Exchange factor for ARF6</td>
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<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
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<tr>
<td>FIP</td>
<td>Family of Rab11 interacting proteins</td>
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<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
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<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GIT</td>
<td>G-protein-coupled receptor kinase-interacting target</td>
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<td>GTP</td>
<td>Guanosine triphosphate</td>
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<td>GST</td>
<td>Glutathione S-transferase</td>
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<td>Hemagglutinin</td>
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<td>HGF</td>
<td>Hepatocyte growth factor</td>
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<td>Horseredish Peroxidase</td>
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<td>Amino terminal</td>
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<td>NGS</td>
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<tr>
<td>NSF</td>
<td>N-ethylmaleimide-sensitive factor</td>
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<td>Nuf</td>
<td>Nuclear Fallout</td>
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<td>PA</td>
<td>Phosphatidic acid</td>
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<td>Pav</td>
<td>Pavarotti</td>
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<td>Abbreviation</td>
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<tr>
<td>PC</td>
<td>Phosphatidylcholine</td>
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<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PE</td>
<td>Phosphatidylinositolamine</td>
</tr>
<tr>
<td>PH domain</td>
<td>Pleckstrin Homology domain</td>
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<tr>
<td>PI(4)K</td>
<td>Phosphatidylinositol (4) Kinase</td>
</tr>
<tr>
<td>PtdIns(4)P</td>
<td>Phosphatidylinositol phosphate</td>
</tr>
<tr>
<td>PtdIns(4,5)P2</td>
<td>Phosphatidylinositol (4,5) bisphosphate (PtdIns(4,5)P2)</td>
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<td>PtdIns(3,4,5)P3</td>
<td>Phosphatidylinositol (3,4,5) triphosphate</td>
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<td>PI(4)P5Kα</td>
<td>Phosphatidylinositol (4) Phosphate 5 Kinase α</td>
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<td>PITP</td>
<td>Phosphatidylinositol transfer protein</td>
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<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>Q</td>
<td>Glutamine</td>
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<tr>
<td>rMLC</td>
<td>Myosin regulatory light chain</td>
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<tr>
<td>RNAi</td>
<td>RNA (ribonucleic acid) interference</td>
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<tr>
<td>Rst</td>
<td>Roughest</td>
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<tr>
<td>S</td>
<td>Serine</td>
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<tr>
<td>SAR</td>
<td>Secretion-associated and Ras related</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>siRNA</td>
<td>Short interfering RNA</td>
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<tr>
<td>SNARE</td>
<td>Soluble NSF Attachment Protein Receptors</td>
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<tr>
<td>Sqh</td>
<td>Spaghetti squash</td>
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<tr>
<td>T</td>
<td>Threonine</td>
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</table>
Declaration

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than those specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

The thesis work was conducted from 01.10.2002 to 15.07.2006 under the supervision of Dr. Marcos A. González-Gaitán at the Max Planck Institute of Molecular Cell Biology and Genetics,

Signed:
Naomi Foster
Date: