The role of membrane trafficking proteins during cell division in mouse oocytes

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I, Roberta Dale, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

Mouse oocytes undergo two successive meiotic divisions to give rise to one large functional oocyte and two small polar bodies. These divisions are crucial as anomalies in this process would preclude normal fertilization and regular development of the embryo. How this asymmetric cytokinesis is controlled is still poorly understood. The purpose of my study is to focus on the cellular events occurring during cell division (cytokinesis) in mouse oocytes, and in particular on the role of specific membrane trafficking proteins which have been found to play a role during cell division in other model organisms. My study has focused on a lipid transport protein, phosphatidylinositol transfer protein–beta (PITPβ) that can bind and transfer phosphatidylinositol and regulate the synthesis of phosphoinositides. In this study I show that PITPβ, a membrane trafficking protein which localises to the Golgi in somatic cells, does not localise to the Golgi in mouse oocytes; instead it is found in the early endosome compartment. Over-expression of PITPβ in oocytes at the germinal vesicle stage (GV) causes an abnormal accumulation of early endosomes compared to the controls. In addition, I have investigated the role of another membrane trafficking protein, the small GTP binding protein, Rab11. I have found that this protein localises strongly at the cleavage furrow of oocytes undergoing the first meiotic division. When injected with a Rab11S25N dominant-negative mutant, more than half of the oocytes remain arrested at metaphase I and do not extrude a polar body. These results suggest that Rab11 may regulate cytokinesis during the first meiotic cell division in mouse oocytes.

Table of contents

List of figures

List of tables

Abbreviations

BP	Band Pass
BSA	Bovine Serum Albumin
cAMP	cyclic Adenosine Monophosphate
CDK1	Cyclin Dependent Kinase 1
CSF	Cytostatic Factor
dsRNA	double-stranded RNA
EEA1	early endosome antigen 1
ER	Endoplasmic Reticulum
FSH	Follicle Stimulating Hormone
GFP	Green Fluorescent Protein
GM130	Golgi Matrix Protein of 130 kDa
GV	Germinal Vesicle
GVBD	Germinal Vesicle Breakdown
hCG	human Chorionic Gonadotrophin
IBMX	3-isobutyl-1-methylxanthine
LAMP1	Lysosomal-associated-membrane protein 1
LH	Luteinising Hormone
MI	Meiosis I
MII	Meiosis II
MO	Morpholino oligonucleotide
MPF	Maturation Promoting Factor
MTOC	Microtubule Organising Centre
O/N	Overnight
Pb	Polar body

PITPβ	Phosphatidylinositol transfer protein beta
PI4KIIIβ	Phosphatidylinositol-4-kinaseIII beta
PBS	Phosphate Buffered Saline
PIP2	Phosphatidylinositol (4,5) bisphosphate
PLC	Phospholipase C
PMSG	Pregnant Male Serum Gonadotrophin
PVP	Polyvinylpyrrolidone
RNAi	RNA interference
RT	Room Temperature
SAC	Spindle Assembly Checkpoint
ZP	Zona pellucida

1. General Introduction

Mouse oocytes undergo two successive meiotic divisions to give rise to one large functional oocyte and two small cytoplasmic bodies, the polar bodies. Anomalies in this process would preclude normal fertilization and regular development of the embryo. How this mechanism is regulated is still poorly understood. The experiments presented in this thesis were designed to investigate the cellular events occurring during cell division (cytokinesis) in mouse oocytes, and in particular on the role of membrane trafficking proteins which have been found to play a role during cell division in other model organisms. To date no work has been undertaken in mouse oocytes. In order to introduce this topic I will first provide a broad overview of gametogenesis and oocyte development before progressing to discuss the mechanisms of cytokinesis and the role of PITPβ and Rab11.

1.1 Overview of oogenesis

Gametogenesis is the first phase in the sexual reproduction of animals. This process, which is called spermatogenesis in the male and oogenesis in the female, leads to the formation of the two highly specialised sex cells: the spermatozoon and the oocyte. These cells are morphologically diverse but both ultimately become haploid cells. The process of haploidization is called meiosis. Diploidy is restored at fertilization, and with the mechanism of egg activation, a new individual may develop. Oocytes and spermatozoa are very different in size; nevertheless the oocyte interacts with the spermatozoon before the completion of meiosis, whereas the spermatozoon acquires the facility to fertilize the oocyte after the

completion of meiosis. During meiosis in the male one primordial germ cell gives rise to four equal spermatozoa, whereas in the female it leads to the formation of one large oocyte and two small polar bodies. Asymmetrical cell division during oogenesis is a unique phenomenon and is the subject of this thesis.

1.2 Formation of primordial germ cells

Vertebrate embryos contain cells whose fate early in development is to become the progenitors of gametes. These cells, called primordial germ cells (PGCs), migrate to the developing gonads, which will then give rise to the ovaries in females and the testes in males. In the female, these PGCs migrate to the developing gonad to become *oogonia*; here they proliferate by mitosis for a while before initiating meiosis I, at this stage they are called *primary oocytes.* In mammals this stage occurs before birth (O W and Baker, 1976). Before the initiation of meiosis I the DNA replicates. Each chromosome is composed of two sister chromatids. At prophase I the duplicated homologous chromosomes pair on their axes and crossing-over takes place between non sister chromatids. Following these events, the oocyte is then arrested in prophase I for a time ranging from a few days to many years, depending on the species (Alberts et al., 2010).

In the mouse, primordial germ cells may be observed in the 8-day-old embryo. The germ cells migrate to the ovary to become *oogonia* and from there they reach the gonads where they become *primary oocytes*. By day 14 after fertilization the oocytes undergo meiosis and remain arrested in prophase of the first meiotic division until they are stimulated to resume meiosis just prior to ovulation. At the time of birth the ovary is now populated with thousands of small oocytes of 12-20μm in diameter (Pedersen 1986). A few days after birth, the arrested

oocytes begin to grow. During the growth phase they increase in size from 12µm to around 80µm in diameter. At puberty, hormonal stimulation triggers meiosis and the oocytes undergo *meiotic maturation*. This phase is characterized by the breakdown of the nuclear envelope (GV), the condensation of the chromatin into bivalents, the separation of the homologous chromosomes and the extrusion of the first *polar body* (PB1). At this point the oocytes are arrested at metaphase II (MII) with the chromosomes aligned on the spindle. At ovulation, the oocytes migrate to the ampulla region of the oviduct; in this region fertilization takes place and this triggers the resumption of meiosis with the emission of the second polar body (Alberts et al 2010; Pedersen 1986).

1.3 Oocyte growth

1.3.1 Oocyte – somatic cell interactions

The growth phase of oocytes is quite long and the increase in size is often remarkable; an example is the frog oocyte, which grows from a diameter of 50µm to 1500µm over a period of 3 years. Mammalian oocytes are smaller; the mouse oocyte for example, grows from around 12µm up to 80µm in diameter. The size of a fully grown oocyte depends mainly on the amount of stored yolk in the cytoplasm, even though the nucleus also enlarges to some degree. Yolk contains lipids, proteins and polysaccharides. Mammalian oocytes store relatively small amounts of yolk. Another distinguishing characteristic of oocytes is the extracellular matrix formed of glycoproteins. In most species, it surrounds the oocyte plasma membrane and in mammalians it is called the *zona pellucida.* It serves to protect the developing embryo from mechanical damage and pathogens (Chouinard, 1975; Alberts et al 2010).

The growth of an oocyte takes place mostly after DNA replication, throughout the long arrest after diplotene in prophase I. Oocytes need to produce a large quantity of ribosomes in order to produce the proteins necessary for embryogenesis. Growth of an oocyte needs help from other cells. Nurse cells for example, are found in some invertebrates and are part of the progeny of oogonia; these cells are connected to the oocyte via cytoplasmic bridges and macromolecules can pass immediately from the nurse cells to the cytoplasm of the oocyte. Sometimes these cells can produce ribosomes and other products, as for example in the insect oocyte (Alberts et al 2010). Follicle cells help the growth of some invertebrate and vertebrate oocytes. Oocytes are surrounded by a layer, or layers, of follicle cells which communicate with the oocyte via gap junctions which allow the exchange of ions and small molecules. Follicles contain several cell types and tissue: the granulosa, theca, endothelial cells and supporting connective tissue. The primordial follicle is a small non-growing oocyte enclosed by somatic 'pregranulosa' cells. In the mammalian ovary the granulosa cells communicate with the oocyte through gap junctions (Anderson and Albertini 1976).

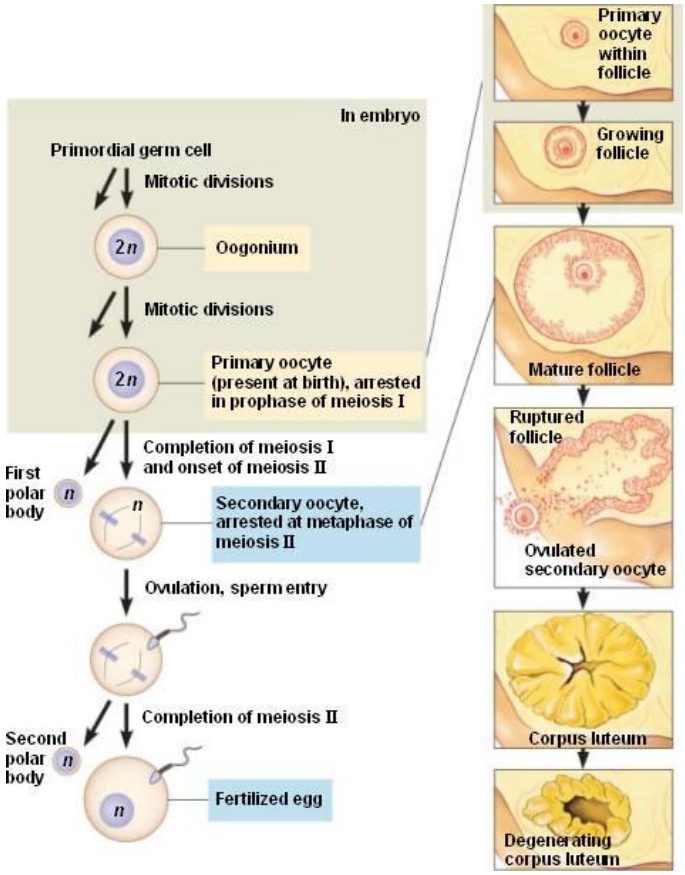

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Figure 1.1. The stages of oogenesis

Early in embryogenesis primordial germ cells migrate to the developing gonad to become oogonia. They undergo a series of mitotic divisions after which they are called primary oocytes and remain arrested in prophase until the female becomes sexually mature. At this point a few oocytes mature periodically upon hormone stimulation and they complete the first meiotic division to become secondary oocytes, which in turn undergo the second meiotic division to become mature eggs. In most vertebrates the maturation of the oocyte is arrested at this stage and completes meiosis II only after fertilization.

1.3.2 Accumulation of maternal reserves during oocyte growth

1.3.2.1. mRNA and protein

The oocyte goes through a series of prematuration changes in order to become competent for further development. By doing this it accumulates specific RNA macromolecules, which are essential later on for the control of embryogenesis. Mammalian oocytes store less RNA than other species, for example the amphibian *Xenopus*. Transcription increases as the follicle begins to grow; the nucleolus increases in size dramatically as RNA accumulates in the nucleus. Transcription of new RNA stops almost entirely when the germinal vesicle breaks down and starts again with zygotic genome activation (ZGA) with the new embryonic genome. Maternal mRNA disappears after the activation of the zygote genome at the 2-cell stage in the mouse and the 4-6 cell-stage in humans. During oogenesis many proteins are synthesized and stored in the cytoplasm of the oocyte for use later on in development (Longo, 1987).

1.3.2.2 Organelles

Accumulation of RNAs and proteins are not the only major changes occurring during oogenesis. Cytoplasmic organelles, for example, multiply and redistribute during oocyte maturation. This process is fundamental for the fertilization and the future embryo development. Mitochondria, for example, are organelles which supply energy and their functional integrality is important for the survival and the development of the cell. In oocytes, mitochondria can act as stores of intracellular calcium (Ca^{2+}) and they provide adenosine trisphosphate (ATP) for fertilization and the development of the preimplantation embryo (Torner et al., 2004). Intracellular calcium, which is essential for the maturation of the mammalian oocyte, derives not only from mitochondria but also from the endoplasmic reticulum (ER) (Krisher 2004). The changes occurring in the organization of the ER in mice,

hamsters and *Xenopus* oocytes are based on the development of cortical clusters of ER. Their formation depends on the ability of the oocyte to produce Ca^{2+} transients in response to sperm and $InsP_3$ (Kline 2000). It has been shown that in mouse oocytes ER clusters disappear on completion of meiosis II (Fitzharris et al., 2003). The dispersal of the ER from the MII spindle to the oocyte cortex is important to provide a source of Ca^{2+} in the cortex, which is the site of sperm-oocyte fusion (Saunders et al., 2002).

1.4 Oocyte maturation

1.4.1 Meiosis

Meiosis is two successive cell divisions, termed Meiosis I (MI) and meiosis II (MII). In the first division (MI), both the chromosome number and the DNA content is halved; this event occurs with the segregation of the two replicated homologs into separate daughter cells. During the second meiotic division (MII), the sister chromatids separate and this time only the DNA content is halved (Alberts et al, 2010). Meiosis can be divided into the following phases: Prophase I, Metaphase I, Anaphase I, Telophase I and Meiosis II (MII). During Prophase I the homologous chromosomes pair (or synapse) and recombination (or crossing over) occurs. The replicated chromosomes are named bivalents or tetrads as they have two chromosomes and four chromatids. At this point cross-over of the non-sister chromatids occurs and this process is called *chiasmata*. At Metaphase I the homologous chromosomes move along the metaphase plate; at Anaphase I the chromosomes are pulled toward separate poles of the spindle; finally, at Telophase I each daughter cell gains half the number of chromosomes, but each chromosome still consists of two sister chromatids. Meiosis II (MI) is a similar process to MI, but here the sister chromatids are segregated into a different haploid cell (Alberts et al, 2010).

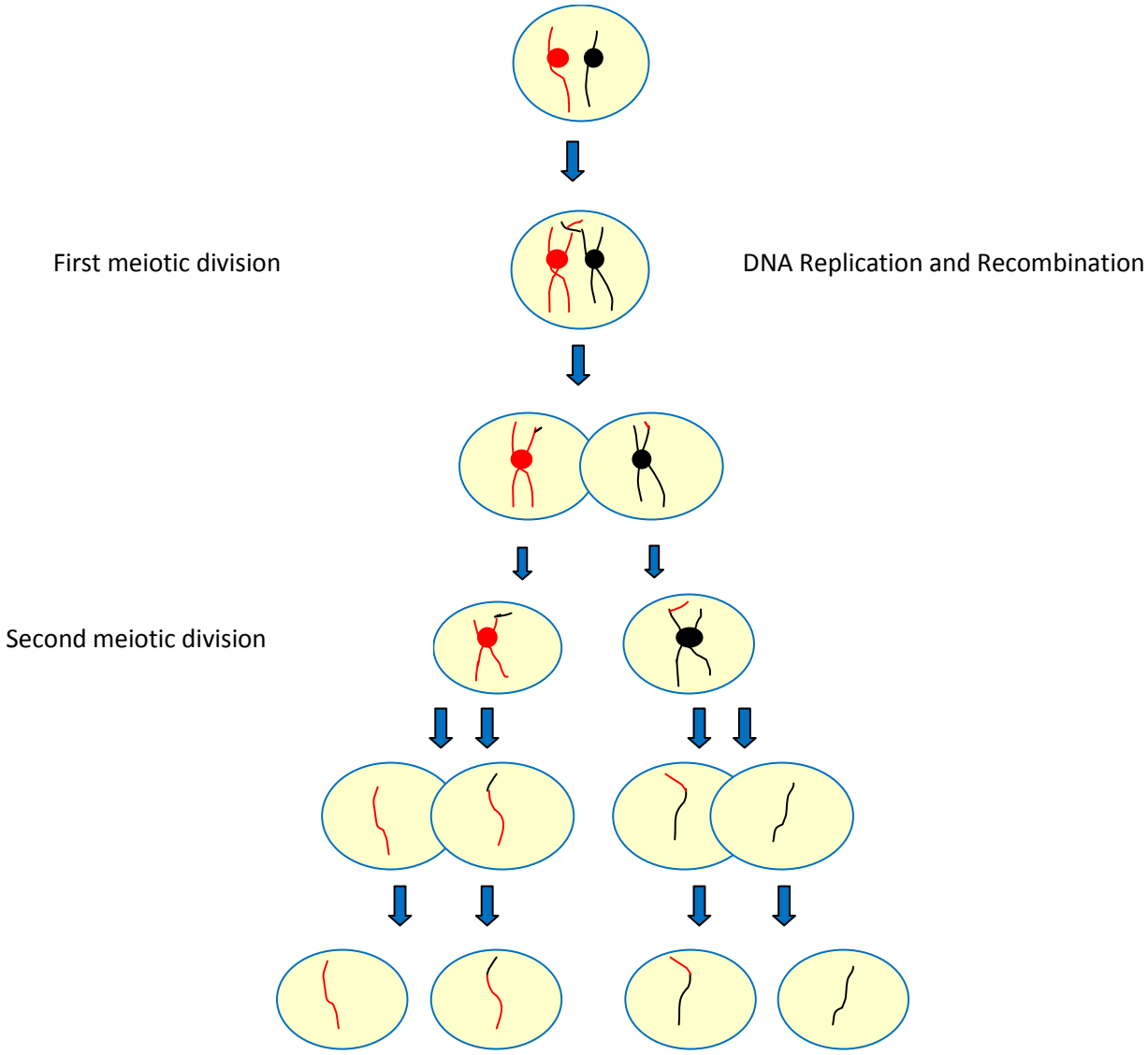


Figure 1.2. Meiosis

The first step in meiosis is the replication of the DNA of the diploid cell. This event is followed by two divisions of the cell, called meiosis I and meiosis II. During the first division the homologous chromosomes separate into two daughter cells. At this stage recombination and crossing over occur. In the second division, the sister chromatids separate and segregate and the result is the formation of haploid germ cells.

1.4.2 Control of meiotic maturation by MPF and CSF

Oocytes arrest at metaphase of the second meiotic division due to an increase in Cytostatic Factor (CSF). Cells remain arrested until the sperm enters the oocyte and induces a rise in Ca^{2+}which leads to completion of meiosis II. CSF maintains this arrest by preventing the degradation of Maturation-Promoting Factor (MPF). MPF also regulates the first meiotic cell cycle and releases the oocytes from the GV arrest. Meiotic maturation including the formation of the first meiotic spindle is controlled by MPF, which is a heterodimeric protein made of a regulatory cyclin B subunit and a catalytic Cyclin Dependent Kinase 1 (CDK1) subunit (Murray and Kirschner 1989). The activity of MPF can be regulated by the phosphorylation of CDK1 and by the degradation of Cyclin B. Cyclin B includes any B-type cyclin degraded at metaphase, in mammals this comprises B1 and B2 (Chapman and Wolgemuth 1993). Cyclin B1 is the type of cyclin responsible for the MPF activity in mammals, whereas B2 is non-essential. The activity of MPF is essential for a cell to enter mitosis. In mammalian oocytes an increase in MPF activity causes exit from prophase I arrest. The rise continues with the first meiotic division and decreases with the extrusion of the first polar body and returns to the previous levels when the egg arrests at MII. CSF keeps the mature eggs arrested at MII. Fertilization or experimental activation causes spindle rotation and the extrusion of a second polar body (Maro and Verlhac 2002; Maro et al., 1984). This is due to the degradation of cyclin B through the Anaphase-Promoting Complex/Cyclosome (APC/C). Emi2 has been discovered recently as an inhibitor of the APC/C complex in eggs and its degradation is Ca^{2+} dependent. Metaphase II arrest is maintained by Emi2 and the c-Mos/MAPK pathway (SAC proteins), which maintains MPF stable. SAC proteins function by arresting cells in metaphase through inhibiting the APC/C (Tung et al., 2005).

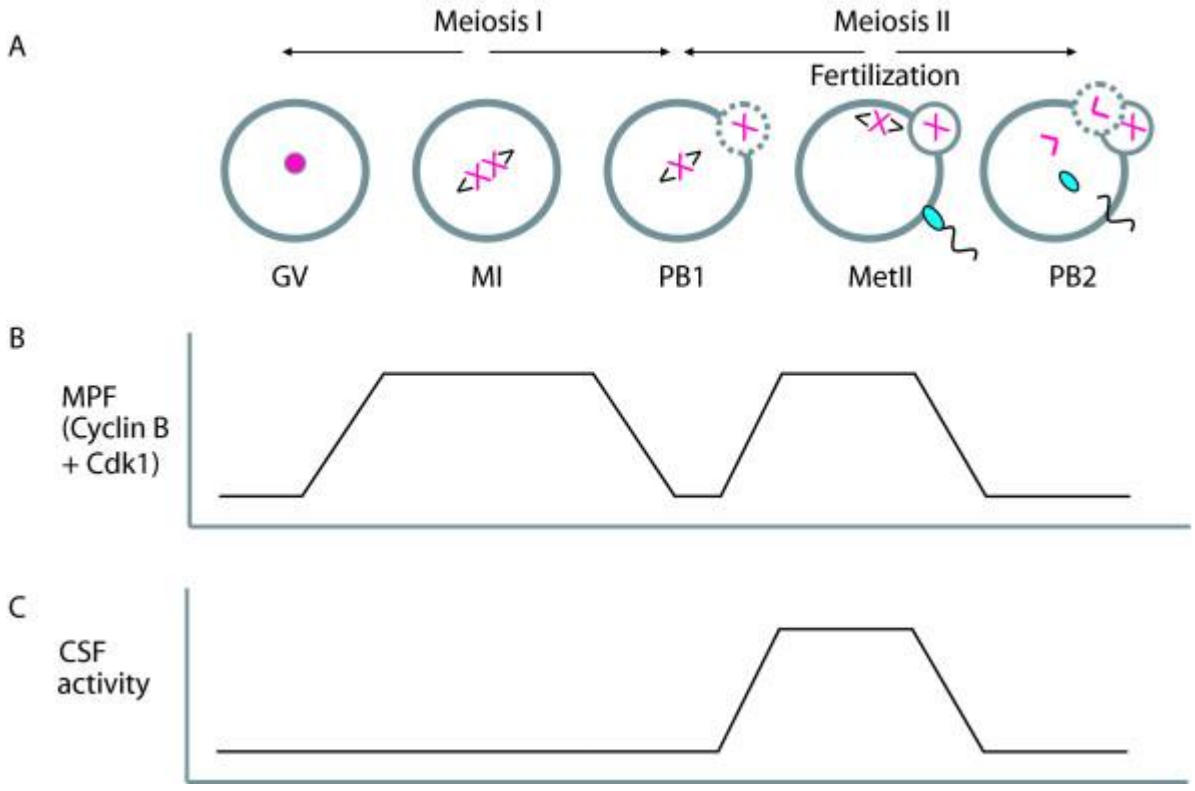


Figure 1.3. Regulation of meiosis by MPF and CSF

(A) A schematic representation of meiosis in oocytes showing only one set of homologous chromosomes. During MI, homologous chromosomes segregate between the oocyte and the first polar body. The cell then arrests at MII until fertilization. (B) MPF activity is highest at MI and MII. (C) High CSF activity blocks the oocyte at MII, until sperm activates the oocyte (Madgwick and Jones 2007).

1.4.3 Establishing asymmetry in oocytes

1.4.3.1 Migration of mitotic spindle

The position of the spindle within the cell is crucial in asymmetric divisions. This position is driven by interactions between the spindle poles and the cell cortex. Astral microtubules polymerize from the poles to the cortex (Cowan and Hyman 2004). Vertebrate oocytes lack centrosomes and astral microtubules and therefore other mechanisms are involved in determining the position of the spindle. In fact in mitosis, spindle assembly is directed by centrosomes, the main sites of microtubule polymerization (Ou and Rattner 2004). It should be pointed out that in most animals, except the mouse and hamster, the centrosomes are derived from the spermatozoon and delivered at fertilization.

The unequal cell division in oogenesis is also regulated by a series of events that take place in the cytoskeleton of the oocyte. The cytoskeleton is composed of microtubules and microfilaments. The microtubules form the spindle and segregate the homologous chromosomes during MI and sister chromatids during MII. Just after GVBD, during metaphase I, different areas in the cytoplasm referred to as Microtubule Organising Centre's (MTOCs) start to polymerise microtubules (Maro et al., 1986). These MTOCs migrate to the chromosomes so that the growing microtubules can be organised into a 'bipolar array' around the chromosomes (Brunet and Maro 2005). However, it has been shown in mouse oocytes by Brunet et al in 1998 (Brunet et al., 1998) that microtubules have the ability to polymerize and arrange in the absence of chromosomes into bipolar structures due to the action of motor proteins and MAPs (Microtubule Associated Proteins).

In addition, in mouse oocytes actin microfilaments and spindle microtubules are responsible for the asymmetry of the two meiotic divisions (Brunet and Maro 2005). Spindle migration and anchoring depend on actin microfilaments and when the microtubules are depolymerised

the chromosomes can still migrate to the cortex (Longo and Chen 1985; Maro et al 1986) through the microfilament network (Azoury et al 2008). In addition, spindle migration depends on the activity of FORMIN2, an actin filament nucleator (Leader et al 2002; Dumont et al 2007). Anomalies in microtubule and actin microfilament-dependent processes in meiosis can cause fertility problems since the oocytes do not mature normally. Before spindle migration microfilaments are present in the whole cytoplasm; upon spindle migration this network of microfilaments becomes denser around the spindle and in the cortex. They surround the microtubules giving rise to a tight sheet. This network could drive spindle migration until anaphase takes place (Azoury et al., 2008; Schuh and Ellenberg, 2008).

Furthermore, bipolar spindles which have formed upon the ablation of chromosomes localise to the periphery of the oocyte (Brunet et al., 1998). Spindle migration starts when MPF has reached a high level. The high level of MPF together with the presence of the chromosomes controls the arrangement of the microtubules and actin microfilaments followed by the migration of the spindle to the cortex. If the cytoskeleton is disorganized or there is a loss of symmetry in the meiotic division, gametes of inferior quality are produced (Brunet and Maro 2005). Spindle relocation depends on filamentous actin (F-actin), but the mechanisms involved during this process are unknown. Furthermore, it has been demonstrated that spindle relocation in mouse oocytes is also dependent on myosin (Schuh and Ellenberg 2008). In fact, the spindle poles are enriched in activated myosin and were pulled by this network. When they inhibited the activation of myosin, spindle elongation and pulling stopped, suggesting that myosin pulling creates the force to drive the movement of the spindle. In addition, spindle migration is also inhibited when the Golgi is disorganised (Wang et al., 2008).

The other factor which contributes to the asymmetry of mammalian oocytes is cortical reorganization. The actomyosin ring is the main component of this mechanism. Microfilaments accumulate under the plasma membrane forming the actin cap and they

control the redistribution of cortical granules (Connors et al 1998; Sun et al 2001). The other components which contribute to the formation of the cortical reorganization are the chromosomes. In fact, in the absence of microtubules, the chromosomes are still capable to migrate and induce cortical reorganization (Maro and Verlhac 2002). This is dependent on the small GTPase RAN. Alterations to the RAN-GTP gradient can stop cortical reorganization around the chromosomes (Deng et al 2007). A second small GTPase downstream of RAN could be RAC. This protein is activated in the vicinity of chromosomes and its inhibition leads to detachment of the spindle and loss of the actin cap in meiosis II mouse oocytes (Halet and Carroll 2007). In conclusion, RAC-GTP, under the influence of RAN-GTP could function in the regulation of the actomyosin ring to induce cortical reorganization. When asymmetry is lost oocytes of low-quality are produced which is a sign of pre-and post-ovulatory ageing.

1.4.3.2 Polarisation of oocytes

A feature of the cell divisions in oogenesis is that they are highly asymmetric. Each division is characterised by a precise segregation of the maternal genome and an asymmetric division of the cytoplasm in order to generate a large oocyte and two small polar bodies. The polar body is not capable of sperm binding due to its tiny size and absence of microvilli on its surface (Motosugi et al., 2006) and therefore cannot be fertilized (Fisk et al., 1996). Asymmetry is needed to generate only one cell which is able to bind to sperm and be fertilized and give rise to an embryo. A consequence of this asymmetric division is the generation of a 'polarized' oocyte. The oocyte possesses a cortical domain which contains the chromosomes, it lacks microvilli and determines the site of emission of the second polar body. Loss of this cortical domain leads to a failure in polarization and therefore in the production of low-quality gametes or ageing gametes. In mammals polarization is needed to ensure a correct formation of distinct cortical domains which separate the introduction of the

paternal genome from the elimination of a set of maternal chromosomes into the polar body. In fact, sperm entry occurs in an area distinct to the area of the second polar body emission.

Another important aspect of oocyte polarization concerns the expansion of the perivitelline space. The emission of the first polar body leads to the expansion of the perivitelline space. This expansion increases the probability that the sperm is found in the polar body half before binding to the oocyte (Motosugi et al 2006).

The oocyte surface is covered by microvilli. The cortical cytoplasm contains small vesicles called cortical granules which are derived from the Golgi apparatus. As the spindle migrates to the periphery at meiosis I the cortex is reorganized. This is a conserved mechanism in all mammalian species. Microvilli start to disassemble and filaments accumulate under the plasma membrane to form the actin cap (Longo and Chen 1984; Tremoleda et al 2001). This event leads to the decrease of the cortical granules in this area by exocytosis or distribution into the cytoplasm (Ducibella et al 1988; Carniero et al 2002; Ferreira et al 2009). This reorganization may be crucial for the shape and the formation of the polar body. In contrast to the mitotic spindle, the anaphase I spindle of oocytes does not elongate and this could contribute to the generation of a small polar body (Verlhac et al 2000). In mitotic cells the metaphase spindle delineates the cleavage plane of the cell which is needed to define the cytokinetic furrow (Glotzer 2009). But in oocytes this process is not valid as anaphase and polar body emission take place at the same time. In meiosis II a second spindle is formed and remains arrested in the oocyte cortex while the cortical area around it stays modified.

1.5 Mechanisms of cytokinesis in somatic cells and oocytes

1.5.1 Introduction

Cytokinesis is the division of one cell into two daughter cells. This event has been studied for more than one hundred years but the mechanisms which drive this process are still poorly understood. Most animal cells divide by forming a constriction in the middle of the cell which pinches the cell into two daughters. In plants, for example, cytokinesis occurs differently. In fact, plant cells assemble a septum in the middle of the cell (Glotzer 1997). Here I will focus on animal cytokinesis, in particular on cell division during mouse oogenesis.

Cytokinesis can be divided into five different steps:

Cleavage plane specification

Furrow assembly

Furrow ingression

Midbody formation

Cell separation

In order to undergo cytokinesis the cell needs to make sure that all the other events in the cell cycle are occurring properly. In fact, at anaphase the cell needs to assure that the chromosomes separate so that the genetic material is evenly distributed. The events which occur during the cell cycle depend on the activation and the inactivation of cyclin kinase complexes (Glotzer 1997). The first event in cytokinesis is the specification of the cleavage plane where the cell will decide to divide. This plane is determined by the position of the spindle and therefore of the microtubules, during late metaphase or early anaphase. It is still not known how microtubules create this division plane.

The second phase of cell division is the assembly of a cleavage furrow. A contractile ring forms in the cortical region of the cell, against the plasma membrane. This ring is composed of actin and myosin, which are two crucial components in cytokinesis; in fact, inhibition of these two proteins leads to a failure in cytokinesis (De Lozanne et al., 1987; Fishkind and Wang 1993). The third stage is the ingression of the cleavage furrow. The cell membrane is pulled inwards to divide the cell into two daughter cells. Myosin slides actin filaments against each other to keep the contractile ring tighter. As the furrow moves inwards, new membrane is added behind its boundary.

The next step is the formation of a midbody. The midbody is the centre of the intercellular bridge; it contains microtubules and an area of high protein density (Eggert et al., 2006). The spindle plays a central role during cell division in animal cells. In fact, when the microtubules reach the furrow they form a structure known as the midbody. At this point the contractile ring starts to interact with the microtubule bundles and possibly the microtubules could carry the components necessary for the addition of new membrane to the furrow. The last step in cytokinesis is the separation of the cell into two. The plasma membrane of the cell divides forming two plasma membranes. The force generating this mechanism could come from the contractile ring or from the migration of the two daughter cells (Glotzer 1997).

1.5.2 Specification of cleavage plane

The term ‘C phase’ was used for the first time by Canman et al. (Canman et al 2000) to describe the period during the cell cycle in which cytokinesis occurs. In particular, it is the time (1 hour) in which the cortex remains able to contract after the onset of anaphase. There are different models which try to explain how the mechanism of the specification of a cleavage plane occurs. All of them rely on signals emanating from microtubules to the cell

cortex. *Polar relaxation* is the term used to indicate a negative signal from microtubules to the cortex at the poles, which prevents the assembly of a furrow in that region. *Equatorial stimulation* is referred to a positive signal from the microtubules to the cortex at the equator, which gives rise to the assembly of a furrow there. A second model comprises the identity of the microtubules delivering the signals and the dominance of asters versus midzones. Asters are microtubule arrays nucleated by centrosomes outside the spindle, whereas midzones are antiparallel arrays of microtubules that form between the separating chromosomes during cytokinesis. This debate has been solved by an experiment in which they separate the two arrays and see that they both lead to the formation of a furrow and send signals to the cortex (Bringmann and Hyman 2005). Regarding the other hypothesis it seems that in conclusion all of them can exist in a single cell (Eggert et al 2006). Astral microtubules elongate at anaphase in many species and often they touch the cortex. This elongation is fundamental as it plays a role in the determination of microtubule signalling to the cortex (Shuster and Burgess 1999; Strickland et al 2005).

1.5.3 Cleavage furrow and abscission

The assembly of the furrow is driven by the contraction of the actomyosin ring. The biochemistry of the ring, composed of actin and myosin II, is known. But how exactly this ring organizes itself is still not clear. There are different models: the purse-string model, in which the sliding of the filaments shortens the ring and this force leads to the ingression of the furrow; the second hypothesis is that the filaments are orthogonal to the ingressing membrane and this contraction would lead to furrow ingression; the third hypothesis states that the filaments are found parallel to the axis of chromosome segregation and the contraction of the filaments would stop ingression in theory but this is the most common orientation observed in cells. The last model is the anisotropic orientation of filaments; in this

model the constriction occurs by gelation-contraction. In conclusion, there are several mechanisms which could lead to the contraction of the ring and therefore furrow assembly in cells.

The ingression of the furrow is generated by the force from the cytoskeleton together with an increase in plasma membrane surface area. One model explaining this mechanism is the 'equatorial relaxation' model, which states that the cortex is under tension and the ingression of the furrow happens at the equator because there the cortex is softer (Wang 2001).

It has also been known for years that furrow ingression could be due to the addition of new membrane (Bluemink and de Laat 1973). This phenomenon has been observed in sea urchin oocytes and in *Xenopus* oocytes (Eggert et al 2006). Oocytes are larger than most cells and require more membrane to be inserted owing to their large surface area.

Completion or abscission is the final step of cytokinesis. When furrow ingression is terminated, the intercellular bridge is about 1-1.5um in diameter. Just before the separation of the daughter cells the bridge is reduced to 0.2um. Microtubules become compacted and start to disappear across the bridge (Echard et al 2004; Piel et al 2001).

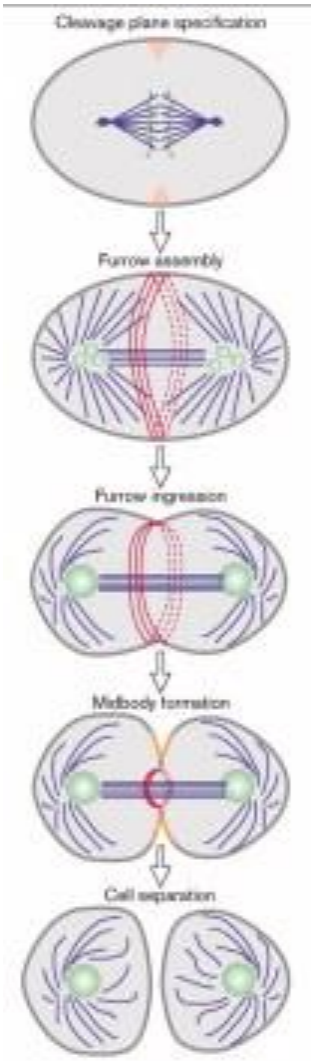


Figure 1.4. The five steps of cell division

Cytokinesis can be divided into five different steps: cleavage plane specification, furrow assembly, furrow ingression, midbody formation and cell separation (Glotzer 1997).

1.5.4 Control and regulation of cytokinesis

The main components which regulate cytokinesis may be grouped into five categories: components of the central spindle, RhoA and its regulators, non-muscle myosin II, actin and its regulators and factors needed for trafficking and fusion of membrane vesicles (Glotzer 2005).

The destruction of cyclins leads to the inactivation of mitotic kinases and to dephosphorylation and activation of different proteins that are essential for the assembly of the central spindle, which is composed of a set of microtubules that at anaphase become concentrated with key components of cytokinesis. One of these proteins is PRC1, which is a microtubule associated protein (MAP) required for the organization of the central spindle and cytokinesis in most animal cells (Glotzer 2005). The assembly of the central spindle is dependent on MKLP1, a member of the kinesin 6-family and CYK4, a Rho family GTPase activating protein. (Mishima et al 2004). Rho A is a key regulator in the assembly of the contractile ring. Depletion of RhoA blocks the formation of the furrow. RhoA GTP leads to actin polymerization and myosin II activation. Non-muscle Myosin II is one of the key regulators of cytokinesis because it is necessary for furrow formation (Mabuchi and Okuno 1977; Straight et al 2003). Actin is the second major component necessary for the formation of the contractile ring in cytokinesis. This protein interacts with other proteins such as the formins which are involved in filament growth (Kovar et al 2003). The last components which contribute to cytokinesis are those involved in membrane addition. These are: syntaxins, rab family GTPases, subunits of the exocyst complex and coatomer complex members (Echard et al 2004; Murthy and Schwarz 2004).

1.5.4.1 Microtubules

Microtubules of the bipolar spindle play a major role in cytokinesis. In fact, they determine the position of the cleavage plane. There are two theories on how microtubules determine the position of the cleavage furrow. The first one suggests that the formation of the cleavage furrow is induced by a local minimum of microtubule density below the cortex (Dechant and Glotzer 2003). The other model suggests that the furrow is formed by the interaction of the asters with the spindle (Bringmann and Hyman 2005). Both studies were carried out in early embryos of *C.elegans*. Oocytes lack centrosomes, therefore the spindles lack astral microtubules. In mouse oocytes, spindle migration and anchoring necessitate actin microfilaments but not microtubules. These processes are based on interactions between the microfilaments and the chromosomes (Maro and Verlhac 2002), but the mechanisms which drive these interactions are unknown. PARD6A, a member of the PAR family (PARtitioning defective; Ahringer 2003) may be involved in the process linking the chromosomes to the actin network. In addition, in mouse oocytes the position of the spindle is related to a local reorganization of the oocyte cortex. This cortical domain starts to form during spindle migration and actin microfilaments form under the plasma membrane (Longo and Chen 1985). Chromosomes are responsible for cortical actin reorganization by an 'at distance' effect (Glotzer 1997).

1.5.4.2 Proteins localising to the midzone

The successful completion of cytokinesis requires the interaction of different proteins that localise to the midzone, which is a bundled microtubule array found between separating chromosomes, formed at anaphase and it is sometimes called central spindle (Eggert et al 2006). There are four classes of these proteins: microtubule motor proteins, kinases, GTPases and components of the telophase disc. The small GTPases of the Rho, Rac and Cdc42

families have important roles in cytokinesis (Straight and Field 2000). The small GTPase Rho plays a crucial role in the induction of the cleavage furrow. In fact, depletion of Rho leads to failure of furrowing at telophase. Rho is also the main regulator of actin dynamics in interphase and mitotic animal cells (Piekny et al 2005). Rac activity becomes polarized during meiotic development and controls the stability and the anchoring of the spindle to the cortex, and as a result asymmetrical cell division (Halet and Carroll 2007).

Kinesins of the CHO1/MKLP1 and KLP3A families are important for the creation of the midzone and the completion of cytokinesis (Straight and Field 2000). Kinesins might be necessary for the stabilization of the microtubule bundles that are required for furrow ingression and completion. The chromosomal passenger proteins have the ability of relocating from chromatin to the central spindle at anaphase (Andreassen et al 1991). These are called telophase disc proteins and the most important proteins are: INCENP and TD-60. Mutant versions of INCENP produced a failure in cytokinesis. In fact, the furrows formed but regressed (Mackay et al 1998; Eckley et al 1997). The last group is composed of the kinases of the Polo and Aim-1 families. These proteins are significant for the assembly and preservation of the central spindle and the midbody (Ohkura et al 1995; Carmena et al 1998).

1.5.4.3 Addition of new membrane at the midbody

A study in sea urchin zygotes revealed that the addition of new membrane in the cleavage furrow is a separate event in cytokinesis (Shuster and Burgess 2002). These authors found that new membrane addition takes place in the cleavage furrow late in telophase and it is independent of contractile ring constriction. The study was carried out by following the secretion of the extracellular matrix protein hyalin, which was used as a marker to follow the addition of new plasma membrane (Shuster and Burgess 2002). Hyalin and new membrane were driven to the cleavage plane by the astral microtubules. The cell needs the addition of

new membrane at the midbody in order to divide into two daughter cells. New membrane could originate from excess membrane which is stored within the plasma membrane and from internal membrane derived from endocytic, secretory and recycling pathways (Albertson et al 2005). Secretion involves the trafficking of the vesicles from the ER to the Golgi and then to the plasma membrane. The vesicles fuse through the membrane specific t-SNARE and v-SNARE protein interactions. Endocytosis takes place when the endocytic vesicles budding from the plasma membrane 'pinch off' from the plasma membrane migrating to the early endosomes. From here the vesicles return to the plasma membrane through the (RE) recycling endosomes, or go to the late endosomes and then to lysosomes for degradation (Albertson et al 2005). The vesicles delivered to the cleavage furrow provide new membrane in order to elongate the furrow. How do vesicles migrate to the cleavage furrow? This mechanism needs more investigations but one possibility could be that these vesicles migrate within the central spindle and its associated proteins. They might be derived from the Golgi directly or from other trafficking organelles like the endosomes (Albertson et al 2005). Studies in *C.elegans* embryos indicate that the RE is fundamental for membrane addition in cytokinesis (Skop et al 2001).

Brefeldin A is a drug known to inhibit post-Golgi trafficking and to disassemble the Golgi (Fujiwara et al., 1988). It is a specific inhibitor of ARF-mediated Golgi-based vesicles (Donaldson et al 1992). BFA binds the boundary between ARF-GDP and Sec7 catalytic domain of a class of high mw GEFs, forming a complex unable of GDP dissociation or GTP exchange. In early *C.elegans* embryos BFA interferes with the final phase of cytokinesis; in fact, in these embryos a cleavage furrow forms but then it regresses (Skop et al 2001). Similar abscission failure has been observed in Meiosis I mouse oocytes treated with BFA (Wang et al 2008). In the experiment half of the oocytes exhibited a failure in abscission during cell division. More interestingly, BFA disrupted the asymmetric positioning of the spindle in the

oocytes, resulting in the spindle positioned parallel to the cortex instead of being perpendicular to it (Wang et al 2008). In addition, the BFA-treated oocytes formed two half-size metaphase II oocytes, instead of a full-sized oocyte and a small polar body.

1.5.5 The importance of membrane traffic in cytokinesis

Over the past few years many studies have revealed that membrane traffic plays an important role in animal cytokinesis. The addition of new membrane during the formation of the cleavage furrow is a conserved mechanism in animal cytokinesis and it has been widely studied in the past years (Albertson et al 2005). This additional membrane could be derived internally in the cell from secretory, endocytic or recycling pathways or from excess membrane which is stored in the plasma membrane. For example, microvilli might provide additional membrane in amphibian embryos (Denis-Donini et al 1976). Different studies have also revealed that membrane addition could derive from the traffic of internal vesicles (Albertson et al 2005). The most relevant study comes from *Xenopus* embryos which showed large clusters of exocytic fusion pores in close proximity to the invaginating furrow (Danilchick et al 1991). The first vesicles shown to be important for membrane addition in cytokinesis were the golgi-derived vesicles. The delivery of Golgi-based vesicles is required during the final stages of cytokinesis in *C.elegans* embryos (Skop et al 2001). A quarter of the proteins linked to the midbody are Golgi derived and many of these proteins are necessary in the early and late stages of cytokinesis in *C.elegans* embryos (Skop et al 2004).

The Golgi compartment plays two main roles in cytokinesis: one is to provide proteins which are required for cytokinesis through the release of Golgi-associated proteins and the second role is to supply membrane through the addition of golgi-vesicles (Leaf et al 1990). In many cell types the Golgi disassembles as the cells enter mitosis (Colanzi et al 2003). Arf1 is the

protein which regulates this disassembly (Altan-Bonnet et al 2003). Therefore, as the cells enter mitosis Arf1 is inactive, the Golgi disassembles releasing Golgi-associated proteins into the cytoplasm. By using Arf1 inhibitors the Golgi cannot disassemble leading to defects in chromosome segregation and a failure of the ingression of the cleavage furrow. The proteins released into the cytoplasm by the Golgi during mitosis comprise COPI, spectrin, MyosinIIa and Cdc42 (Altan-Bonnet et al 2003). Some of these proteins, such as Cdc42, play a role during cytokinesis (Drechsel et al 1997). In conclusion, the disassembly of the Golgi is required for the release of important proteins required during cytokinesis. Furthermore, it has been shown that the inheritance of a continuous Golgi ribbon upon cell division is dependent on the mitotic spindle (Wei and Seemann 2009).

Endocytosis is also required during cytokinesis. Clathrin and dynamin are two membrane-associated proteins that initiate vesicle budding from the plasma membrane and they have been found to regulate cytokinesis in *Dictyostelium discoideum* (Gerald et al 2001). Dynamin is also required for the completion of cytokinesis in *C.elegans* (Thompson et al 2002). The largest study has been carried out in zebrafish embryos, in which it was shown that endocytosis occurs from the early to the late stages of cytokinesis (Feng et al 2002). How endocytosis contributes to the regulation of cytokinesis remains unclear. It might contribute to the recycling of components which have been delivered previously to the cleavage furrow by exocytosis (Danilchik et al 2003).

In vivo and in vitro studies have shown that endocytosis takes place via clathrin-coated pits which separate from the plasma membrane to become coated vesicles. These coated pits contain clustered receptors, solutes and membrane lipids. Presumably the coated vesicles fuse forming early endosomes which are found in the cell periphery. Once in the early endosomes, internalized molecules are either recycled back to the plasma membrane, transported to the lysosomal compartment for degradation, or transcytosed in polarized cells. There are two

models to explain the mechanisms occurring during membrane traffic in endocytosis; Palade in 1975 proposed that early and late endosomes as well as lysosomes exist already in the cell and are connected by carrier vesicles that separate from one compartment and transport their content to the next compartment and then recycle. According to this model, these compartments are expected to enclose resident proteins. The second model is the maturation model, which states that early endosomes are continually being made by the fusion of the incoming vesicles with each other. Then, while being transported into the cell, the early endosome matures receiving components from the Golgi compartment and becoming a late endosome and eventually a lysosome. Early endosomes are different from the late endosomes morphologically, biochemically and functionally. In fact, one example is that membrane traffic from and to the early endosomes does not necessitate intact microtubules, compared to later stages (Gruenberg and Howell 1989).

Therefore, endocytosis occurs within the trafficking of membrane through different organelles such as the early, late and recycling endosomes (REs). The RE is important for the addition of membrane during cytokinesis (Riggs et al 2003). This compartment delivers specific proteins to the plasma membrane and it associates with microtubules and localizes to the microtubule-organizing center (MTOC), which is a fundamental component in the positioning of the contractile ring. The first studies which showed the implication of RE in cytokinesis were done on *C.elegans* embryos through the inhibition of Rab11 by RNAi. (Skop et al 2001). Rab11 is a small GTPase protein which localises to the recycling endosomes compartment and its role in cytokinesis will be discussed in detail in the following paragraphs.

Vesicle fusion is an important mechanism occurring in many cellular processes including cytokinesis. Membrane fusion can be either heterotypic or homotypic. The former indicates the fusion of membrane from diverse sources, one providing the target (t)-SNARE and the

other providing the vesicle (v)-SNARE. The latter comprises the fusion of membrane from the same source and is done by symmetrical interactions of v-SNARE and t-SNARE (Albertson et al 2005). Even though it is known that SNAREs provide heterotypic fusion, studies have shown that syntaxin might also provide homotypic vesicle fusion in plants (Lauber et al 1997). In mammalian cells, overexpression and dominant-negative studies have shown that syntaxins are essential in the final stages of cytokinesis (Low et al 2003). In conclusion, syntaxins in mammalian cells seem to work in the same way as those in plants, therefore mediating the homotypic vesicle fusion required to complete cytokinesis.

1.6 Phosphatidylinositol transfer proteins (PITPs)

Phosphatidylinositol transfer proteins (PITPs) are proteins which bind and transport phospholipids, in particular PtdIns (phosphatidylinositol) and PtdCho (phosphatidylcholine) and transfer them to specific membrane compartments in vitro (Helmkamp et al., 1974; van Paridon et al., 1987). These proteins have been shown to be involved in membrane traffic and in the regulation of cytokinesis (Giansanti et al., 2006; Carvou et al 2010).

The main component of biological membranes are lipids. There are two mechanisms that regulate the movement of lipids between membranes. The first involves budding from one membrane and fusing to the other, while the second is the result of specific lipid transporters that bind to lipids. Several lipid transporters (lipid transport proteins, LTP) specific for different hydrophobic ligands have now been identified. In humans there are different LTPs which may be subdivided by sequence similarity of their lipid binding domain. One of these families is the PITP domain proteins (Cockcroft, 2007).

In 1974 the first mammalian PITP protein was purified from brain cytosol and shown to be present as two types. It was demonstrated that these two species were dependent on the

presence of either PC or PI bound to the protein and the two species could be inter-exchanged when incubated with appropriate lipid vesicles (Helmkamp et al., 1974; van Paridon et al., 1987). PITP was cloned in 1989 (Dickeson et al., 1989).

Proteins with the PITP domain may be sub-divided into two classes. Class I PITP, containing PITPα and PITPβ, and class II PITP which contains the RdgB proteins (Allen-Baume et al., 2002). Of the five genes in the human that encode PITP proteins, two code for single domain proteins (Class I: PITPα and PITPβ) and their crystal structure is known. They have a hydrophobic pocket that can receive a single phospholipid molecule, either PI or PC. Whereas the Class II RdgB family share 40% sequence identity, PITPα and PITPβ share 77% sequence identity.

Eight β-strands that form a large concave sheet flanked by two long α-helices form the lipid-binding cavity. The cavity is closed by a 'lid' composed of a C-terminal α-helix (the G-helix) and an 11 amino acid extension. The phospholipid is closed within the protein and the polar headgroup interacts with amino acid residues found at the end of the cavity distant to the lid.

Only when the lid is displaced the phospholipid has access to the membrane interface. The *sn*-1 and *sn*-2 fatty acyl chains of the ligand are found in distinctive sites with the methyl ends close to the opening of the lipid binding cavity. The protein now in its 'closed' conformation for transport through the aqueous compartments has to 'open' in order to exchange lipids. When the protein is found in its 'open' conformation (no lipid attached) the lipid binding cavity faces the membrane interface and the G-helix is dislodged. The lipid exchange loop, a loop which contains helix B, is now out of position. At this point the protein can remain membrane-associated (Wirtz et al., 2006). Thiol-modifying reagents e.g N-ethylmaleimide (NEM) are useful tools to study PITPs in their lipid free form. This reagent links to Cys95 of the class I PITPs, which is found in the lipid-binding pocket and prevents the binding of the lipid due to steric hindrance (Shadan et al.,2008). Class I PITPs are

proteins which interact continuously with the membrane interface to exchange their lipid cargo in cells.

The conformational change in PITP starts when the protein reaches the membrane interface and this is due to the presence of two tryptophan residues (WW) found at the tip of the loop that faces the membrane interface. Mutation of these two residues causes a loss of membrane docking and lipid transfer (Tilley et al 2004; Shadan et al 2008). The different residues required for the binding of the lipid in the hydrophobic cavity can be grouped into three kinds: residues required for the binding of the phosphate moiety which are Q22, T97, T114 and K195; residues required for the binding of inositol (PI) which are T59, N90, E86 and K61 and finally residues essential for the binding of PC (C95 and F225). In all species with a PITP domain the conservation of residues essential for the binding of the phosphate moiety and the inositol head group of PI is high.

Proteins with a PITP domain bind phosphatidylinositol, which is the most versatile of lipids. Lipid kinases can phosphorylate three hydroxyls of PI either individually or in combination producing seven phosphorylated derivatives of PI. PI is an insoluble amphiphilic lipid synthesised at the endoplasmic reticulum. In order to transfer PI from the ER to other organelle membranes across the aqueous cytosol there are different mechanisms in place. PITPs work together with the PI kinases which may be subdivided into the 3-kinase and the 4-kinase families. Every derivative of PI which is phosphorylated can bind to protein modules within effector proteins at target membranes, like the PH domain, FYVE domain. Phosphoinositides participate in cell signalling, endocytosis, membrane trafficking, modulation of ion channels, dynamics of the actin cytoskeleton and many other functions. The specific function of PITPs lies in their C-terminal region which interacts with different proteins and lipids.

1.6.1 PITPβ

Tanaka and Hosaka in 1994 screening for gene products that were capable of rescuing the *SEC14* mutant in *Saccharomyces cerevisiae* first discovered PITPβ. The mutant cells showed a defect in secretion from the Golgi to the plasma membrane. Rat brain cDNA was used to identify genes able to rescue this defect. They identified a gene which encoded a protein that had a similar sequence to PITPα and therefore it was called PITPβ. PITPNB is the gene which encodes PITPβ and it can be alternatively spliced. The first splice variant is termed PITPβ-sp1 and the second is PITPβ-sp2 (Morgan et al., 2006). The C-terminal region of the two variants differ by 17 amino acid residues. These variants are expressed in cultured cells but also in cells obtained from animal tissues. The study of the lipid binding properties of these two variants has confirmed that both variants bind and transfer PI and PC, similar to PITPα. It has been demonstrated that the C-terminus of splice variant 1 of PITPβ has got a serine residue (Ser262) that is constitutively phosphorylated by protein kinase C (van Tiel et al., 2002). But phosphorylation has no effect on the transfer acitivity of splice variant 1 *in vitro*. The two variants localise to the Golgi, the endoplasmic reticulum and the nuclear envelope (Morgan et al., 2006; Carvou et al., 2010). This study is in disagreement with another study in 2006 which reports that PITPβ localises only at the *trans*-Golgi network (TGN) (Phillips et al., 2006).

1.6.2 The role of PITPβ in membrane traffic and cytokinesis

Depletion of the gene for PITPβ in murine embryonic cells was shown to be embryonically lethal (Alb, Jr. Et al., 2002).

Studies concerning its cellular role are more recent and show that both splice variants of PITPβ were depleted by RNAi (Carvou et al., 2010). The knockdown cells showed a distorted nucleus with a compacted Golgi compartment compared to control cells. In

addition, these cells had a defect in retrograde traffic from the Golgi to the ER. This traffic is mediated by COPI-coated vesicles and one of the main cargo which is transported by these vesicles is the KDEL receptor which retrieves escaped ER proteins. This receptor migrates between these two compartments and in knockdown cells it remains arrested in the Golgi compartment. The retrograde defect could be rescued by re-expressing the wild type PITPβ (Carvou et al., 2010).

In addition, the expression of mutants lacking PI or PC transfer were not able to rescue the defect indicating that the features of PITPβ studied *in vitro* represent the major activities required *in vivo*.

PITPs bind and transport phospholipids, in particular PtdIns (phosphatidylinositol) and PtdCho (phosphatidylcholine) and transfer them to specific membrane compartments in vitro (Helmkamp, Jr. et al., 1974; van Paridon et al., 1987). Target membranes can then convert PtdIns by phosphorylation into different kind of phosphoinositides such as PtdIns(4,5)P_2 known as PIP2. Local changes in phospholipid composition at the cleavage furrow are fundamental for the completion of cytokinesis (Emoto et al 2005). In addition, it has been demonstrated that PtdIns(4,5)P_2 is required for normal cytokinesis (Field et al 2005). In fact, PtdIns(4,5)P_2 is present at the cleavage furrow and has a role in adhesion between the contractile ring and the plasma membrane.

1.6.3 PITPβ and *Drosophila* spermatogenesis

Drosophila has three PITP proteins which represent the members of Class I and Class IIa and b of the PITP family in mammals. These are: Dm-PITP (which correspond to mammalian PITPα/β), Dm-RdgBα (corresponding to mammalian RdgBαI/II) and Dm-RdgBβ (which correspond to mammalian RdgBβ). It has been shown that Dm-PITP is required for meiotic cytokinesis in Drosophila spermatocytes (Giansanti et al., 2001).

In fact, *Gio* and *fwd* are two genes which encode for Dm-PITP and Dm-PI 4-kinase respectively and were found to be essential for the regulation of the constriction of the actomyosin ring and for the ingression of the cleavage furrow (Giansanti et al., 2006; Gatt and Glover, 2006). The process which separates the two daughter cells at the end of cell division is termed cytokinesis. This process is regulated by the constriction of an actomyosin ring which forms beneath the equatorial cortex leading to furrow ingression and to the final step of cytokinesis termed abscission; at this stage the actomyosin ring disassembles and new membrane is added.

The single class I PITP (Gio) is enriched at the furrow membrane in *Drosophila* spermatocytes and in its absence the actin ring inside the furrow is disorganized (Giansanti et al 2006). Mutations in Gio cause an abnormal localisation of Golgi-derived vesicles at the equator of the cell. In wild type spermatocytes the golgi-derived vesicles lie at the poles and are not found in the centre of the cells. The invaginating furrow is not able to fuse with these vesicles causing defects in cell division. The same phenotype is present in *fwd* mutants. Mutations in *gio* in neuroblasts in the brain show the same cytokinetic defect. In *Drosophila* spermatocytes mutations in *gio* causes a failure in the acroblast assembly and a dispersion of the vesicles in the cytosol. The acroblast forms at the end of the second meiotic divison due to the aggregation of Golgi vesicles.

Thus it can be speculated that PITP is specifically responsible for maintaining the actin cytoskeletal ring inside the furrow and also plays a role in membrane addition to the cleavage furrow. The actin cytoskeleton is attached to the membrane via PtdIns(4,5)P_2 and possibly, in the absence of PITP, local levels of PtdIns(4,5)P_2 are perturbed at the membrane furrow. In addition, PI4Ks are essential regulators of the secretory trafficking pathway (Simonsen et al 2001). PI4KIIIBeta is found in the cytoplasm and also at the Golgi complex (Wong et al 1997; Godi et al 1999). The recruitment of PI4KIIIBeta to the Golgi complex depends on the

small GTP-binding protein ARF1, which possibly enhances the synthesis of PI4P which in turn can be synthesised to $PI(4,5)P_2$ (Godi et al 1999).

1.7 Rab proteins

Rab proteins, the biggest family of monomeric small GTPases, function as molecular switches between GTP (active) and GDP (inactive) bound conformations. It is known that Rabs function in the tethering/docking of vesicles to their target compartments, causing membrane fusion. But these proteins also control the movement of vesicles and organelles along cytoskeletal elements (Zerial and McBride 2001). They have been shown to be involved in membrane traffic and in the regulation of cytokinesis (Skop et al., 2001; Fielding et al., 2005; Giansanti et al., 2007).

Human cells contain more than 60 Rab proteins which are localized to different compartments in the cells. In the active conformation (GTP) these proteins interact with specific effectors (Zerial and McBride 2001; Segev 2001). Prenylation of C-terminal cysteine motifs links Rab GTPases to membranes (Leung et al 2006). The transfer between the membranes is carried out through a complex of GDP-bound Rab proteins with RabGDI, which is a GDP-dissociation inhibitor protein. This exchange is then facilitated by GDI displacement factors (GDFs) (Goody et al 2005; Pfeffer and Aivazian 2004). In addition, GEFs (guanine exchange factors) catalyze the exchange of GDP into GTP; following this exchange RabGTPases start to interact with their effector proteins (Zerial and McBride 2001; Grosshans et al 2006; Segev 2001). GAPs (guanine activating proteins) activate the hydrolysis of GTP (Bernards 2003).

1.7.1 The role of Rab11 in cytokinesis

Rab11 is a small GTPase which has been localized to both the Trans-Golgi Network and the recycling endosomes. The Rab11 subfamily comprises Rab11a and Rab11b, which share 90% of amino acid identity, and Rab25. The crystal structure of Rab11a has been studied. The GDP-bound form is a dimer with the switch I and switch II regions involved in monomer interaction (Pasqualato et al 2004). Rab11b, compared to Rab11a, has a different oligomerization state for the inactive form of Rab11. Each Rab11 isoform possess different interactions in the nucleotide binding site. These differences demonstrate that they might have different GTP binding rates or hydrolysis rates (Scapin et al 2006).

The Rab11 family is involved in the targeting and movement of recycling endosomes to the plasma membrane, in the transport of molecules of the trans-Golgi network to the plasma membrane, in phagocytosis and in polarized transport in epithelial cells (Chen et al 1998; Cox et al 2000, Ullrich et al 1996). Rab11 in particular is required for Trans-Golgi network to plasma membrane transport. Expression of a dominant-negative form of Rab11 (S25N) leads to the inhibition of the cell surface transport of vesicular stomatitis virus (VSV) G protein causing this protein to accumulate in the Golgi compartment (Chen et al 1998). In CHO and BHK cells Rab11 colocalises with internalised transferrin in the pericentriolar recycling compartment. Rab11 mutants change the morphology of this compartment. In fact, the expression of Rab11-GTP in these cells causes an accumulation of labeled elements in the perinuclear area of the cell; Rab11-GDP causes a scattering of the transferrin labeling (Ullrich et al., 1996).

Studies in *C.elegans* embryos first demonstrated that Rab11 is required for cytokinesis (Skop et al 2001). Later on, it was shown that Rab11 and its effector protein Nuclear-fallout (Nuf), a homologue of arfophilin-2, an ADP ribosylation factor effector that binds Rab11, are both

required for cellularization of *Drosophila* embryos (Pelissier et al 2003; Riggs et al 2003). Inhibition of Rab11-expression by RNAi in *C.elegans* embryos gives rise to defects in the syncytial germ cell in the ovary. The nuclei of the syncytial germ cell are dispersed throughout the cell instead of being at the periphery. In the three-cell and four-cell stage embryos the furrow ingresses but then regresses suggesting that these final stages of cytokinesis require Rab11. The same phenotype happens in the two-cell stage embryos (Skop et al., 2001). Injection of Rab11S25N mutant protein during cycle 12 and 13 of *Drosophila* embryos causes an inhibition of membrane invagination during slow phase (the phase in which membrane ingression is dependent on the microtubule cytoskeleton in *Drosophila* cellularization). The nuclei are disorganized and sometime 'fall' inside the embryo instead of being aligned at the cortex (Pelissier et al., 2003). In addition, Rab11 colocalises with its effector protein Nuf. These proteins are equally required for their localization to the recycling endosome compartment. *Drosophila* embryos with low levels of Rab11 give rise to membrane recruitment and actin remodelling defects similar to nuf-derived embryos. These observations show that Nuf and Rab11 play similar roles at the recycling endosome compartment in membrane trafficking and actin remodelling during the first stages of furrow formation in *Drosophila* embryos (Riggs et al., 2003).

Rab11 has been shown to arbitrate the movement of the recycling endosomes to the furrow during late telophase, thus regulating the last step of cytokinesis (Fielding et al 2005; Wilson et al 2005). Fielding et al in 2005 suggested that some of the new membrane added into the plasma membrane during furrow expansion comes from recycling endosomes. They identified a family of Rab11 interacting proteins called FIPs. Rab11-FIP3 and Rab11-FIP4 also play a role in cytokinesis. In fact, Rab11 is responsible for the recruitment of FIP3 to endosomes that accumulate in the furrow region at telophase. Knock down of FIP3 with RNAi results in defective cytokinesis (Fielding et al 2005). Rab11-FIP3 and FIP4 interrelate

with Arf6 and the exocyst in order to control membrane trafficking in cell division (Fielding et al 2005). ADP-ribosylation factor (Arf)6 is a member of the family of ARF small GTP-binding proteins and it controls membrane movement between the plasma membrane and early endocytic compartments (Chavrier and Goud 1999). Arf6 localizes at the plasma membrane and it regulates post-endocytic recycling through its downstream exocyst complex effector (Prigent et al 2003). Arf6 binding to FIP3 is required for Arf6 recruitment to the midbody and controls the delivery of recycling endosomes to the cleavage furrow (Schonteich et al 2007).

1.7.2 Rab11 and *Drosophila* spermatogenesis

In prophase of *Drosophila* spermatocytes, Rab11 localises in a subcompartment of the Golgi stacks, which corresponds to the trans-Golgi network (TGN). As meiotic division starts, the Golgi disassembles and Rab11 becomes concentrated on the endoplasmic reticulum (ER) compartment. At early anaphase/telophase this protein associates with different vesicle-like structures which are found at the cell poles. At late telophase these vesicles move towards the cell equator where they fuse with the cleavage furrow. The origin of these vesicles is not clear and whether or not they are recycling endosomes. Rab11 mutant spermatocytes show two cytokinetic defects: they show an abnormal accumulation of Golgi-derived vesicles (Lva) at the cell equator during telophase and the actomyosin ring fails to constrict completely leading to a failure in cytokinesis (Giansanti et al 2007).

These cytokinesis phenotypes are identical to those elucidated by mutations in *gio* (PITP) and *fwd* (PI4KIIIβ). Interestingly, studies using double mutants analysis and immunostaining for PITP and Rab11 indicate that PITP, PI4KIIIβ and Rab11 function in the same cytokinetic pathway, with PITP and PI4KIIIβ acting upstream of Rab11 (Giansanti et al., 2007).

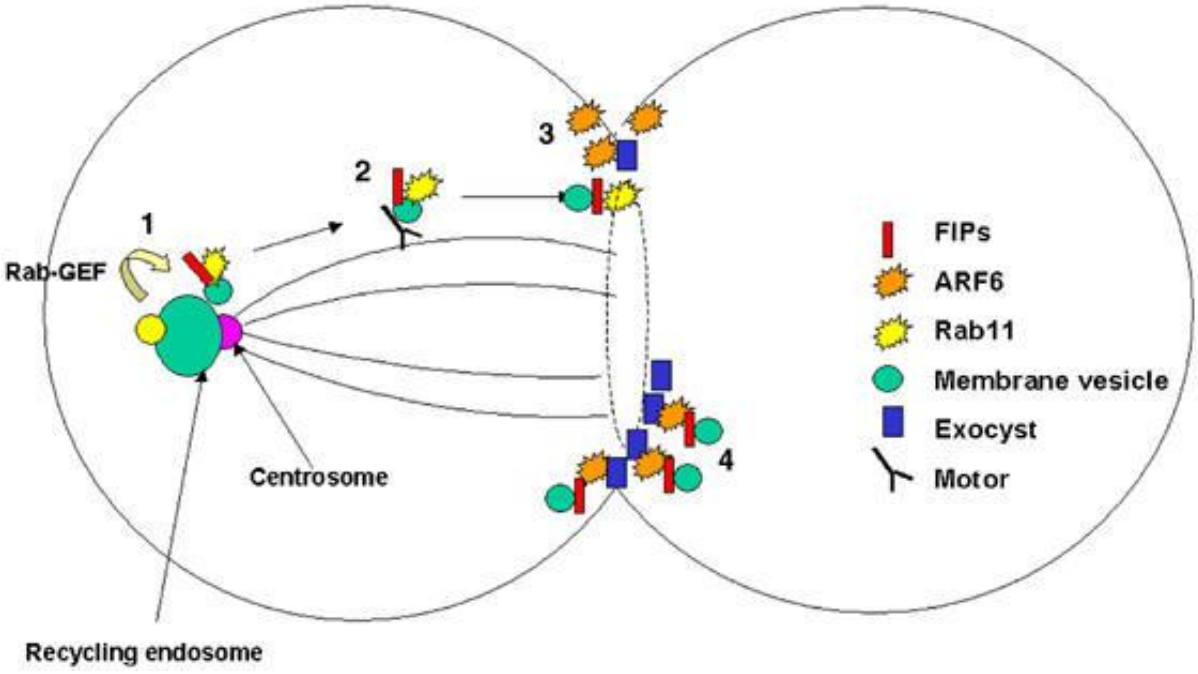


Figure 1.5. Rab11 and its interacting proteins in cytokinesis.

Rab11-GTP helps the recruitment of FIP3 to vesicles derived from recycling endosomes found in the centrosome. The vesicles, by interacting with a motor protein, move along microtubules into the furrow, where they active Arf6, which is found on the plasma membrane. The interaction of the three proteins may serve to help the transport of the vesicle to the plasma membrane by interacting with the Exocyst complex (Fielding et al., 2005).

1.8 Aims/Objectives

The overall aim of the current project was to study aspects of polarization in mouse oogenesis. In particular, to investigate the role of membrane trafficking proteins during cytokinesis and compare these mechanisms to those operative in other systems such as *Drosophila* spermatogenesis. One specific objective was to study the localisation of PITPβ and its relation to the Golgi compartment. A further objective was to study the role of Rab11 in cytokinesis and in particular its association with other proteins such as Cdc42, PI4Kβ and Arf6. The final aim was to determine whether the behaviour and role of these proteins changes in meiosis I and meiosis II.

2. Materials and Methods

2.1 Mice

Oocytes of MF1 mice were collected and manipulated in M2 medium from Sigma, unless otherwise stated.

2.2 Oocyte collection and culturing

Germinal vesicle (GV) oocytes were collected from 4-6 week old MF1 mice. To increase the oocyte yield, mice were primed by intraperitoneal injection of 7.5IU pregnant mare serum gonadotrophin (PMSG) (Calbiochem). Mice were culled by cervical dislocation at 46-48 hours after PMSG injection. The abdominal area of the mice was rinsed with ethanol. A small cut was made with scissors in the bottom third of the skin layer. The ovaries were removed and transferred to dissection medium (M2 medium) containing cyclic AMP (cAMP) (250µM) in order to keep the oocytes arrested at the GV stage. 10mls of M2 medium was poured into a needle-less syringe and pushed through a (0.22µm) microfilter into a_ml test tube. A 10µl aliquot of cyclic AMP (cAMP) was pipetted into the filtered medium and mixed by several inversions of the tube. The tube was placed on the hot block to keep the oocytes at 37ºC, and then a small volume was transferred into a smaller tube to proceed with the collection of the ovaries. The cumulus-enclosed oocytes were isolated by mechanical perforation of the ovaries with a needle. GVs adequate for the experiments had a central germinal vesicle and a big amount of cumulus cells surrounding the GVs. A large pulled glass pipette was used to transfer GVs from the dish into a fresh drop of M2 medium with cAMP under oil. The oil was used to prevent evaporation of the medium whilst being maintained at 37ºC. The

cumulus masses were removed by repeated mouth-pipetting, using a narrow glass pipette. The oocytes were then washed few times in drops of dissecting medium ready to use.

To obtain MII stage oocytes mice were injected with human Chorionic Gonadotrophin (hCG) 48 hours after PMSG injection. Mice were culled by cervical dislocation and oviducts were dissected 12-14 hours after hCG injection. Cumulus cells were removed by adding the enzyme hyaluronidase (3mg/ml) in 2 ml of M2 medium. The solution was prepared in a petri dish for the oviducts to be placed into. In order to release the ovulated MII oocytes, the oviduct was held down with small forceps and using a needle, a sharp cut was made across the tube where the mass of oocytes produced a distortion. The cluster would then release itself from the oviduct simultaneously. The dish was placed back onto the hot block because the enzyme needed to stay for two minutes at 37ºC in order to work properly. MII oocytes were collected with a large pulled glass pipette and then washed with M2 medium for 2-3 times. For in vitro maturation, oocytes were cultured in M16 media (SIGMA) in a CO_2 incubator. For parthenogenetic activation, MII oocytes were cultured for 7 minutes in M2 medium containing 7% ethanol and then washed twice in normal M2 medium.

2.3 Immunostaining

Oocytes were fixed with paraformaldehyde (3.7% in PBS) for 40 min, permeabilized with Triton X-100 (0.25% in PBS) for 10 min, then washed in PBS containing 3% BSA for approximately one hour at room temperature. Fixed oocytes were incubated with the primary antibody for 1 hour in the dark at 37C, washed once for 1 hour with PBS containing 3% BSA, then incubated with the secondary antibody for 1 hour in the dark at 37ºC and washed with PBS-BSA (3%) for 1 hour. Finally, the oocytes were washed with 1000 µl of PBS-BSA (1%) containing bisBenzimide (Hoechest 33342) (5µg/ml) for 5 min to label chromosomes. The

following primary antibodies were used: Alpha-tubulin (1:100; mouse monoclonal, AbCam Cambridge, UK); 4A7 anti-PITPβ (1:100; mouse monoclonal IgG raised in Professor Shamshad Cockcroft's laboratory); anti-Rab11 (1:100; rabbit polyclonal, AbCam Cambridge, UK); GM130 anti-golgi(1:500; rabbit polyclonal, AbCam Cambridge, UK); anti-GM130 (1:100; rabbit polyclonal, AbCam Cambridge, UK); anti-LAMP1 (1:100; rabbit polyclonal, AbCam Cambridge, UK); anti-EEA1 (1:100; rabbit polyclonal, Abcam Cambridge, UK); anti-PI4KIIIβ (1:100; rabbit polyclonal, Millipore); anti-Arf6 (1:100; rabbit polyclonal, gift from Professor Shamshad Cockcroft). The secondary antibodies were: Alexa Fluor 488 goat anti-mouse (1:1000, Santa Cruz), and Alexa Fluor 546 goat anti-rabbit (1:1000, Santa Cruz).

2.4 Image Acquisition and Analysis

Confocal microscopes allow the observation of small samples in cross-sections. Here we used them to scroll through the different planes of the oocytes to get a comprehensive picture of what was occurring within each region. A confocal microscope is formed of an objective which focuses a laser beam onto the specimen which emanates fluorescence. The fluorescence is then collected by the objective and sent to the detector via a dichroic mirror. The pinhole (confocal aperture) is found in front of the detector. Light which comes from planes below or above the focal plane is out of focus when it hits the pinhole so it does contribute to forming the image. The advantage of this microscope is that it is possible to image a thin 'optical slice' out of a 'tick' specimen (up to 100µm).

Confocal images (3.5 µm thick) were acquired with an LSM510meta confocal microscope (Carl Zeiss Microimaging, Inc.) with the following band pass emission filters (nm):

- Excitation of Hoechst staining, which gives a blue fluorescence, was at 364nm by a UV laser, and the emission was picked up via a 385-470nm band pass (BP) filter.

- Excitation of the Alexa Fluor 488 goat anti-mouse, which gives a green-yellow fluorescence, was via an Argon laser at 488nm wavelength and peak light emission was collected using a band-pass (BP) filter of 505-550nm.

- Excitation of red Alexa Fluor 546 goat anti-rabbit was produced by a 543nm HeNe laser. The emission was picked up by a 585-615 BP filter.

Images displayed in the figures were analyzed with MetaMorph (Molecular Devices) and are representative of at least 10-15 similar observations from 3 experiments.

2.5 Microinjection

Oocytes were injected using a micropipette and Narishige manipulators mounted on a Leica DM IRB inverted microscope (Leica, Wetzlar, Germany). Oocytes were placed in a drop of M2 medium containing 3-isobutyl-1-methylxanthine (IBMX) covered with mineral oil to prevent evaporation. They were immobilized with a holding pipette while the injection pipette was pushed through the zona pellucida to make contact with the oocyte plasma membrane. A short overcompensation of negative capacitance caused the pipette tip to penetrate the cell. Microinjection was carried out using a fixed pressure pulse through a picopump (WPI, Sarasota, FL). The oocyte volume is ~250 pl. The volumes of the injections were estimated at 3-5% of total cell volume by cytoplasmic displacement. After

microinjection, the oocytes were removed in fresh drops of M2 + IBMX under oil and allowed to recover for a few minutes before any further manipulation.

2.6 Protein knockdown using antisense morpholino

Morpholino antisense oligos (Gene Tools, USA) were used in order to knockdown PITPβ and Rab11 proteins. Usually, in order to study the function of a specific protein in mouse oocytes the mRNA is degraded and this is done by double-stranded RNA (dsRNA) which is called RNA interference (RNAi) (Svoboda et at., 2000; Wianny and Zernicka-Goetz, 2000). But we have used Morpholinos instead of RNAi because they have been proved to be very effective for mouse oocytes and non-toxic (Lefebvre et al., 2002). Morpholinos are very stable, water soluble and specific; an antisense oligo binds to a complementary sequence of the selected mRNA. This prevents the translation of the mRNA and therefore the protein cannot be synthesised by the mRNA. Morpholinos are composed of nucleotides in which the riboses are transformed into morpholines by introducing an amine (www.gene-tools.com).

GV oocytes were microinjected with 1.5mM antisense oligonucleotide morpholino designed against the start region of the single-copy mouse PITPβ (PITPβ MO: 5'-ATTCCTTAATCAGCACCATCTTCCG -3'). For Rab11 the sequence was Rab11 MO: 5'-ATATGCACTGTCTGTCACCTCGTTG-3').The oocytes were cultured in M16 medium (SIGMA) containing IBMX in a 5% CO_2 humidified incubator at 37ºC for 24 hours to keep them arrested at the GV stage. After 24 hours they were released in M16 only to resume maturation. A control Morpholino was used to check for possible non-specific effects of the PITPβ or Rab11 Morpholinos. This control Morpholino has no target and no essential biological activity except for reticulocytes in thallasemic humans. In fact, in these cells the

oligo corrects a splicing error forming a correctly-spliced mRNA which codes for normal beta-globin chains (www.gene-tools.com).

2.7 Transformation of plasmids into E.coli

Rab11S25N dominant negative mutant was a generous gift from Prof Gwyn Gould (Glasgow University, UK) and was subcloned into pcDNA3.1. The original vector was pEGFP-C1. The time required for transformation is 1 hour 45 minutes. The growth requires approximately 16 hours for visible colonies. The reagents used are: L-broth, LB-agar plates with antibiotics and chemically competent E.coli cells stored in 50µl aliquots in -80 degrees. The first day we verified the selection sequence of our plasmid (ampicillin resistance 100mg/ml). We used LB-agar plates with this antibiotic. To promulgate a plasmid carrying the gene of interest 5-10ng of plasmid DNA was placed into a labelled sterile eppendorf tube. The competent E.coli cells were placed directly on ice after removing from -80 degrees storage. As the cells thaw 100µl was added to the tube containing the plasmid DNA. The content was mixed and the tube placed on ice for 30 minutes. The tube was then removed from ice and incubated for 30sec-2minutes in a 42ºC waterbath for a heat shock. 900µl of sterile 1X L-broth was added to the tube and incubated at 37ºC for 1 hour in a waterbath. 50µl and 100µl of the culture were placed on LB-agar + Ampicillin plates using a glass spreader. We plated two dilutions of the culture to ensure isolated colonies. We flamed the spreader between plates and allowed to cool before using. We waited 5 minutes to let the plates absorb the inoculum, we inverted the plates and incubated them at 37ºC for 16 hours. The day after we examined the plates and determined the efficiency of the transformation. We picked isolated colonies to prepare miniprep DNA. The plasmid DNA was sent for sequencing.

2.7.1 Mini prep DNA

1-5ml of overnight cultures of E.*coli* were placed in LB (Luria-Bertani) medium (Sigma). The bacterial cells were span at 500 x g for 10 minutes. The bacterial cells were resuspended in 250µl of Buffer P1 (containing RNAse) and transferred to a microfuge tube. 250µl of Buffer P2 (lysis buffer) was added and the tube was inverted gently 4-5 times to mix. 350µl of Buffer N3 (neutralizing buffer) was added and the tube was inverted immediately and gently 4-6 times. It was centrifuged for 10 minutes at 10,000 x g to collect the cells by centrifugation. The plasmid DNA was purified using QIAprep Spin Miniprep kit (QIAGEN). During centrifugation a QIAprep spin column was placed in a 2-ml collection tube. The supernatants were added to the QIAprep column and centrifuged for 30-60 seconds. The flow-through was then discarded. The QIAprep spin column was washed by adding 0.5ml of Buffer PB and centrifuged 30-60 seconds. The flow-through was discarded. It was washed again by adding 0.75 ml of Buffer PE and centrifuged for 30-60 seconds. The flow-through was discarded and the column was centrifuged for an additional 1 minute to remove residual wash buffer. The QIAprep column was then placed in a clean 1.5ml microfuge tube. To elute DNA we added 50µl of sterile Milli-Q H_2O to the centre of the column, we let it stand for 1 minute and then centrifuged for another 1 minute. The concentration of the plasmid DNA was quantified using a Nano Drop spectrometer (ND 1000, Thermo Scientific) and its presence was confirmed by agarose gel electrophoresis.

2.8 Preparation of in vitro mRNA

In vitro mRNA was made by using the Ambion mMESSAGE mMACHINE T7 Ultra Kit. This kit was used to produce large amounts of mRNA for microinjections. As a template we have used linearized plasmid DNA which had a T7 RNA polymerase promoter site for in vitro transcription. We did a DNA miniprep to produce a high quality template. The plasmid

DNA was then linearized with a restriction enzyme downstream of the insert which needed to be transcribed. The template was then analyzed on agarose gel to make sure that cleavage occurred. For the assembly of the transcription reaction different reagents were thawed, assembled at room temperature and mixed properly. They were then incubated at 37ºC for 1 hr. Then, 1µL of TURBO DNase was added, mixed well and incubated again for 15min at 37ºC. This was to remove the template DNA. The second step was the Poly (A) Tailing. At this point the tailing agents were added and 2.5µL of the reaction mixture was put on the side. 4µL of E-PAP was added and mixed gently. The mixture was incubated at 37ºC for 45min. The RNA was purified by using RNeasy Mini kit (QIAGEN). The reaction was precipitated with lithium chloride. The column was span down. Then for the extraction phenol chloroform was used and isopropanol for precipitation. A small aliquot of the final product was run on a gel to check the expression of the RNA before being stored at -20ºC. Also, on the gel we put a small aliquot of all the tailing reagents and the minus enzyme control that we left on the side during the reaction. As a control, an RNA size marker was also added. The gel was examined on a UV transilluminator (TFX-20M, Vilber Lourmat). The RNA was quantified by using a Nano Drop spectrometer (ND 1000, Thermo Scientific) before being aliquoted into 1µl aliquots and stored at -80ºC.

2.8.1 Agarose gel electrophoresis

This technique was used to check the size and quantity of purified DNA or RNA. 0.8% agarose gel was made by adding 0.4g of agarose powder into 50ml of 1x trisacetate-ethylemide acid (EDTA) (TAE buffer) (Promega). The mixture was boiled for few minutes in a microwave oven to dissolve the agarose powder completely. Ethidium bromide (10mg/ml) was then added to the agarose TAE buffer and the mixture was poured into a gel casting apparatus (BioRad). After the gel has set (approximately 15-20 minutes), it was put into a

BioRad mini-sub filled with 1x TAE buffer. The samples of DNA and RNA were mixed with loading buffers (RNA formaldehyde loading buffer and DNA blue loading dye) and loaded onto the gel together with a 1kb DNA ladder (Promega) as an indicator of the molecular weight. The gel run for 20-30 minutes at 100V using a BioRad Power Pac 300 and analysed on a UV transilluminator (TFX-20M, Vilber Lourmat).

2.9 Western blotting

In order to prepare the oocytes for western blotting, GVs were washed in 1% PVP twice, transferred into 2µl of blotting buffer and then frozen at -20 Cº. The 12% gel was prepared according to the following tables:

- Mini Resolving gels (15 ml) for 1.5mm gels-using 40% Acrylamide/Bis

% Acrylamide	**12%**	**12%**
Number of gels	**1**	**2**
Distilled water (ml)	3.25	6.50
Acrylamide/Bis (ml)	2.25	4.50
1.5M Tris pH 8.8 (ml)	1.9	3.75
10% SDS (µl)	75	150
10% APS (µl)	60	120
TEMED (µl)	6	12

- 4% Stacking gels (6.0 ml) – using 40% Acrylamide/Bis

Number of gels	**1**	**2**
Distilled water (ml)	1.90	3.80
Acrylamide/Bis (ml)	0.30	0.60
1.0M Tris pH 6.8 (ml)	0.75	1.5
10% SDS (µl)	30	60
10% APS (µl)	12	24
TEMED (µl)	3	6

The first lane of the gel was loaded with 10µl of a Pre-stained molecular weight marker. The samples were heated up at 95 Cº for 5 minutes and then span and loaded carefully into the

wells. The gel was then run for 1.5hr with a constant voltage of 130V. At this point a PVDF membrane was wet in 100% methanol and then rinsed with distilled water and left in blotting buffer to equilibrate. After completion of electrophoresis, the gel was removed and transferred into a box containing blotting buffer and placed onto a rocker for 5 minutes. The western blot sandwich was assembled in the following order: sponge, filter paper, gel, membrane, filter paper, sponge. The sandwich box was then placed into a transfer tank which was filled with blotting buffer. Everything was then placed onto a stirrer and the transfer was run at 100V for 1-2 hours. For detection, 1L of PBS-tween buffer and 50ml of 5% milk blocking buffer were made. After the transfer the cassette was removed from the tank and tweezers were used to place the membrane gel-facing side up into some distilled water for a few minutes on the rocker. The water from the membrane was poured off and the membrane was immersed in Indian Ink/PBST (1:500 dilution) solution for 10 minutes on the rocker. The ink was then washed off a few times with PBST. The membrane was incubated in 5% milk blocking buffer and blocked for 1hr at room temperature on the rocker. The primary antibody was diluted in blocking buffer in a 50ml centrifuge tube in a volume of 3ml. The membrane was then transferred into the 50ml tube and placed onto a roller mixer for 1-3 hrs at room temperature (or overnight) to incubate. After incubation, the membrane was washed 3 x 5 min with 10-20ml of PBST in the same tube. At this point the membrane was incubated with the secondary antibody diluted in 5% milk blocking buffer for 1 hour at room temperature. Afterwards, it was washed 3 x 5 min with PBST. To detect the blot ECL solution was used and to analyse and take images the blot was exposed to an LAS3000 imaging machine.

2.10 Statistical analysis

All t-tests are two-tailed and calculated using two samples (unpaired) which have a similar variance. Error bars shown on figures indicate the standard deviation.

P value	**Symbol**	**Significance**
>0.05	NS	Not significant
0.01-0.05	*	Significant
0.001-0.01	**	Very significant
<0.001	***	Extremely significant

Table 2.1 Composition of culture media (Sigma-Aldrich) :

Inorganic salts	**M16 (Liquid) g/L**	**M2 (Liquid) g/L**
CaCl2 • 2H2O	0.251	0.251
MgSO4 (anhyd)	0.165	0.165
KCl	0.356	0.356
KH2PO4	0.162	0.162
NaHCO3	2.101	0.35
NaCl	5.532	5.532
Sugars		
D-Glucose	1	1
Other		
Albumin, bovine Fraction V	4	4
Lactic acid • Na	4.35	4.35
Phenol red • Na	0.01	0.01
Pyruvic acid • Na	0.036	0.036
HEPES	-	5.43

Table 2.2 Reagents used in treatments

Loaded agent	Concentration	Incubation time	Function
Hoechst	2μM	5min	Chromatin dye
IBMX	44μg/ml	*	GVBD inhibition
Ethanol	7%	8min	Parthenogenetic activation

Table 2.3 Final concentrations of agents microinjected inside the oocyte

Injected agent	**Concentration inside the oocyte**
Morpholinos	30-50μM
Rab11 S25N mRNA	20-30pg/oocyte
PITPβ WT mRNA	20-30pg/oocyte
Cdc42 mRNA	20-30pg/oocyte

3. The localisation of PITPβ during meiosis in mouse oocytes.

3.1 Introduction

The overall aim of this study was to investigate the role of proteins involved in membrane trafficking and cytokinesis. Phosphatidylinositol transfer protein beta (PITPβ) is a protein that binds and transfers the phospholipids phosphatidylinositol (PtdIns) and phosphatidylcholine (PtdCho) between different membrane compartments in vitro (Helmkamp, Jr. et al., 1974; van Paridon et al., 1987). PITPβ was first purified from bovine brain in 1995 (De Vries et al., 1995). A few years later it was shown that ablation of the gene for PITPβ was embryonically lethal in murine embryonic cells (Alb et al., 2002).

In somatic cells PITPβ localises to the Golgi compartment and the Endoplasmic reticulum (Shadan et al., 2008). Recently, it has been shown that depletion of PITPβ in HeLa cells by RNAi leads to the formation of a distorted nucleus and a compacted Golgi in comparison to control cells. Furthermore, these cells also showed a defect in retrograde traffic from the Golgi to the Endoplasmic reticulum compartment (Carvou et al., 2010).

The present chapter investigates the dynamics of PITPβ distribution and its possible role during meiosis in mouse oocytes. The first study to identify a role for a class I PITP in meiosis was performed in *Drospohila* spermatocytes (Gatt and Glover 2006; Giansanti et al., 2006). It was demonstrated that a *Drosophila* gene, giotto (*gio*), which encodes the class I PITP, was required for mitotic and meiotic cytokinesis. In spermatocytes gio was enriched at the membrane furrow and when mutated, the actomyosin ring became disorganised around the cleavage furrow and could not constrict entirely leading to a failure of abscission, the last

step of cytokinesis (Giansanti et al., 2006). The same phenotype was observed in *Drosophila* neuroblasts. Moreover, this mutation caused an abnormal accumulation of Golgi-derived vesicles at the equator of spermatocytes during the telophase stage, suggesting that the gene may be involved in the regulation of membrane-vesicle fusion during *Drosophila* male meiosis (Gatt and Glover 2006; Giansanti et al., 2006).

This model system (*Drosophila spermatocytes*) is used extensively for the study of cytokinesis since mutants defective in cytokinesis can be easily identified by looking at the morphology of the spermatids (Fuller, 1993). Furthermore, due to the large size of meiotic spindles, these defects can be easily analyzed with good cytological resolution (Giansanti et al., 2001). To date no studies on the role of PITP in oogenesis have been reported. The data available in somatic cells suggest that the phenotype is different in *Drosophila* and mammalian cells. We have targeted PITPβ because deletion of this gene is embryonically lethal in mice (Alb et al., 2002) whereas PITPα is mainly expressed in the brain and localised in the axons (Cosker et al., 2008). In this Chapter, I investigate the distribution and localization of PITPβ in mouse oocytes during maturation as an initial attempt to determine whether it plays a role in female meiosis.

3.2 Results

3.2.1 Localisation of PITPβ during meiosis of mouse oocytes.

As a first step to understand possible roles for PITPβ in female meiosis we have used a PITPβ-specific antibody (anti-mouse monoclonal against PITPβ Ab 4A7) to localise the protein in mouse oocytes. The existence and localisation of the protein was analysed by immunocytochemistry. This antibody has been widely characterised and used for immunofluorescence of PITPβ in somatic cells (Shadan et al., 2008).

To examine the specificity of 4A7 in mouse oocytes I used Western blotting to determine the oocyte proteins that immunoreact with the antibody. The blot shows a single band of molecular weight 35kDa, which is the expected size for PITPβ, suggesting that the antibody is highly specific (Figure 3.1A). Immunocytochemistry was used to determine the distribution of PITPβ in GV-stage oocytes. The antibody shows homogeneous staining of the cytoplasm plus some vesicular staining (indicated by the arrows) (Figure 3.1Bi). The staining of PITPβ is variable. Control oocytes in which the oocyte was not exposed to primary antibody showed no labelling, suggesting that the staining required the presence of the primary antibody.

We next examined the localisation of PITPβ during oocyte maturation. Interphase GV stage mouse oocytes present a vesicular pattern of localisation of PITPβ in the cytoplasm (Figure 3.2Aii). In prometaphase, after GVBD, PITPβ is still localised in a vesicular pattern throughout the cytoplasm (Figure 3.2B). At metaphase I, when the spindle is formed and the chromosomes are aligned on the metaphase plate, the protein is present in a more punctuate pattern (Figure 3.2C). At telophase I, the spindle has already migrated to the cortex of the oocyte, the homologous chromosomes have separated and the first polar body is being formed. At this stage PITPβ localisation is still present in the cytoplasm in a vesicular pattern similar to the GV stage (Figure 3.2D) but there is no enrichment of the protein at the cleavage

furrow of the oocyte. This result is different to that in *Drosophila* primary spermatocytes at the same stage, where *gio* localises both at the cleavage furrow region and at the cell poles in late anaphase/early telophase while in metaphase I and anaphase I it is present at the cell poles and the spindle envelope (Giansanti et al., 2006). Finally, in MII arrested eggs, PITPβ is present in the cytoplasm but in a less vesicular pattern compared to the MI stage (Figure 3.2E). This cytoplasmic localisation is similar to that observed for *gio* in *Drosophila* Neuroblasts during prophase. Even though, in Neuroblasts the localisation of *gio* changes in later stages of mitosis accumulating at the spindle envelope until telophase and around the nuclei in late telophase (Giansanti et al., 2006).

3.2.2 PITPβ does not localise to the Golgi compartment in mouse oocytes.

Experiments performed in somatic cells have revealed that PITPβ localises to the Golgi compartment and the Endoplasmic Reticulum (Morgan et al., 2006; Shadan et al., 2008). Therefore, to examine whether the same localization occurs in mouse oocytes we labelled the oocytes with an anti-PITPβ antibody (4A7) and an anti-Golgi antibody (GM130) at different stages of maturation. We found that there is no colocalisation of the protein to the Golgi compartment (Figure 3.3A). We found that PITPβ does not localise to this compartment even at earlier stages of maturation. Studies in our laboratory have mapped a localisation to the ER (Mehlmann et al., 1995; FitzHarris et al., 2007). The pattern is not consistent with what is shown by the PITPβ antibody. In contrast to somatic cells PITPβ does not appear to localise to the Golgi or the ER during meiosis.

3.2.3 PITPβ localisation to lysosomes and recycling endosomes in mouse oocytes.

Since PITPβ does not localise to the Golgi compartment, we wanted to identify the structure it was found to be associated with. The punctuate distribution was thought to be a membrane bound vesicular distribution so we examined the structure of the lysosomal compartment by using an anti-LAMP1 antibody. The structure of this compartment has never been analysed before in mouse oocytes. We found that at the GV stage there is a partial colocalisation of PITPβ with this compartment (Figure 3.4A). A higher magnification of the overlay shows a colocalisation of the two antibodies only on some large vesicles but not on the smaller vesicles that make up the majority of the staining (Figure 3.4B). Therefore, PITPβ appears to localise to large LAMP1 positive vesicles that are thought to be lysosomal in origin.

To further investigate the compartment associated with PITPβ localisation we labelled the oocytes with anti-Rab11a, which is known to be associated with the recycling endosome compartment (Ullrich et al., 1996) (Figure 3.5A). The oocytes were fixed and labelled at the GVBD stage. The pattern of distribution of PITPβ was very different from the Rab11a distribution, the first being vesicular whereas the latter not. Thus, there was no clear colocalisation of PITPβ with this compartment (Figure 3.5Aiii).

3.2.4 PITPβ localises to the early endosome compartment in mouse oocytes.

After having analysed various compartments and not having found a clear result concerning the localisation of PITPβ in mouse oocytes, we next examined whether the early endosome compartment could be the site of PITPβ localisation. We labelled GV oocytes with an antibody, anti-EEA1, which labels the early endosome compartment and found that the two antibodies provided a very clear colocalisation (Figure 3.6A). The colocalisation is present throughout the oocyte, in the cytoplasm, the cortex and on the PITPβ positive vesicles

(Figure 3.6Aiii). This result shows that PITPβ behaves differently in oocytes compared to all other cells that have been studied.

3.2.5 PITPβ cannot be depleted with morpholino oligonucleotides.

Although PITPβ did not localise to the cleavage furrow or accumulate on Golgi vesicles as in *Drosophila*, it was decided to investigate what the role of PITPβ in this novel cell compartment may be. We have designed and used morpholino oligonucleotides in an attempt to deplete this protein (see Chapter 2). PITPβ MO-injected oocytes were cultured in the presence of IBMX for 24 hours before being released from arrest and the effects on the first meiotic division were observed. We found that there was no significant difference in the rate of Pb1 extrusion between the PITPβ MO-injected oocytes (78%; 62/79 oocytes) and the control MO-injected oocytes (80%; 66/82 oocytes $P>0.05$) (Figure 3.7A). Moreover, MO-injected and control-injected oocytes were fixed for immunocytochemistry at the GV stage (Figure 3.7B) or 15-16 hours after release from meiotic arrest (Figure 3.7C) at the MII stage and labelled with anti-PITPβ and Hoechst to determine whether the protein had been depleted by the treatment with morpholino oligonucleotides. Some oocytes were fixed at the GV stage to see if there was any difference/reduction in the amount of vesicles present in the PITPβ MO injected oocytes and the control oocytes. In fact, at the MII stage the protein is present in a less vesicular pattern throughout the oocyte and therefore it is more difficult to see a difference but the data show that there is no obvious decrease in the level of fluorescence in the treated oocytes compared to the control oocytes. To confirm that the protein had not been depleted we performed a western blot of PITPβ MO-injected oocytes versus control MO-injected oocytes at the GV stage and the MII stage. Tubulin was used as a loading control (55kDa). The size of PITPβ was 35kDa. Extracts from 130 oocytes were loaded in each lane and it is clear that PITPβ is similar in PITPβ MO-injected oocytes and the control MO-

injected oocytes (Figures 3.7D-3.7E). Thus, PITPβ appears to be stable in mouse oocytes and therefore longer periods would be required to deplete it. Unfortunately, oocytes do not remain in a reliably physiologically normal state in periods of in vitro arrest for more than around 24 hours, which was the duration we tested.

3.2.6 Over expression of PITPβ causes an increase in the number of PITPβ positive vesicles.

In the event that we were unable to deplete PITPβ, an alternative strategy of over-expression was examined to see whether this could reveal a role for PITPβ on the early endosome compartment. To examine the effect of exogenous PITPβ, GV-stage oocytes were microinjected with PITPβ WT mRNA and allowed to mature overnight. Oocytes were fixed and labelled with the antibody 4A7 to examine the distribution of PITPβ. Remarkably, oocytes injected with PITPβ mRNA showed a dramatic increase in the appearance of vesicles, predominantly in the cortex of the oocyte (Figure 3.8A). No such increase was observed in control oocytes injected with water (Figure 3.8B-3.8C) ***P<0.001. To determine whether the vesicles were part of the early endosome compartment oocytes were co-labelled for PITPβ and EEA1.We found that the EEA1 antibody completely colocalised with the PITPβ-positive vesicles induced by PITPβ over-expression (Figure 3.8Aiii). In addition, we noticed that the EEA1 positive vesicles were already present in the control oocytes but in the over expressed oocytes they increased in number and their size increased considerably (Figures 3.8Aii-3.8Bii). To quantify the effect of PITPβ over-expression the number of large vesicles were counted on an image of the oocyte that sectioned the equator of the oocyte. Despite such a dramatic effect on early endosome dynamics, over expression of PITPβ did not have any effect on the rate of Pb1 extrusion compared to control oocytes. In fact, the percentage of Pb1 extrusion was around 90% in both groups. Therefore, this protein

does not seem to play a role in the regulation of asymmetric cell division in mouse oocytes, although it may be important for the regulation of membrane trafficking.

3.2.7 The accumulation of PITPβ vesicles is dependent on PtdIns3P.

The next question was why the over expression of PITPβ caused an accumulation of large EEA1 positive vesicles in oocytes. Phosphatidylinositol 3-kinase (PI3K) phosphorylates PI to make PtdIns3P, which is known to be associated with the early endosomes compartment (Lawe et al., 2000). EEA1 contains a FYVE domain and is recruited to the early endosomes partly by binding to PtdIns3P. The fact that PITPβ has been shown to stimulate the activity of PI3K (Panaretou et al., 1997) suggests that exogenous PITPβ may promote PI3P and therefore EEA1 binding. To test this possibility GV oocytes were microinjected with PITPβ WT and half the injected oocytes were treated with a PI3K inhibitor Ly294002 (10μM). The inhibitor causes oocytes to arrest at metaphase I (Hoshino et al., 2004) (Figure 3.9B), suggesting that it was active and gaining access to the cytoplasm. Both groups were fixed at the same time (8 hours after release) and labelled for PITPβ, EEA1 and Hoechst. We found that by treating the PITPβ over expressed oocytes with the PI3K inhibitor the increased accumulation of EEA1 vesicles was no longer apparent suggesting that the accumulation was dependent on PtdIns3P (Figures 3.9A-3.9B). Even though the two groups have been fixed at the same time the oocytes treated with the inhibitor have arrested at metaphase I and therefore they are at a different stage of maturation compared to the controls.

3.2.8 Over expression of PITPβ does not block Pb2 emission.

To examine whether PITPβ played a role during the second meiotic division in mouse oocytes, we over expressed the protein into MII oocytes. PITPβ WT mRNA was

microinjected in MII oocytes together with controls (microinjected with water). The oocytes were then activated with 7% ethanol to extrude the Pb2. We found that there was no significant difference in the rate of Pb2 extrusion between the over expressed oocytes and the controls (Figure 3.10A). We fixed the oocytes two hours after ethanol activation and labelled them with anti-PITPβ and anti-EEA1. We found that there was an increase in EEA1 positive vesicles compared to the controls (Figures 3.10Bii-3.10Cii) but we did not observe a significant increase in PITPβ positive vesicles (Figures 3.10Bi-3.10Ci).

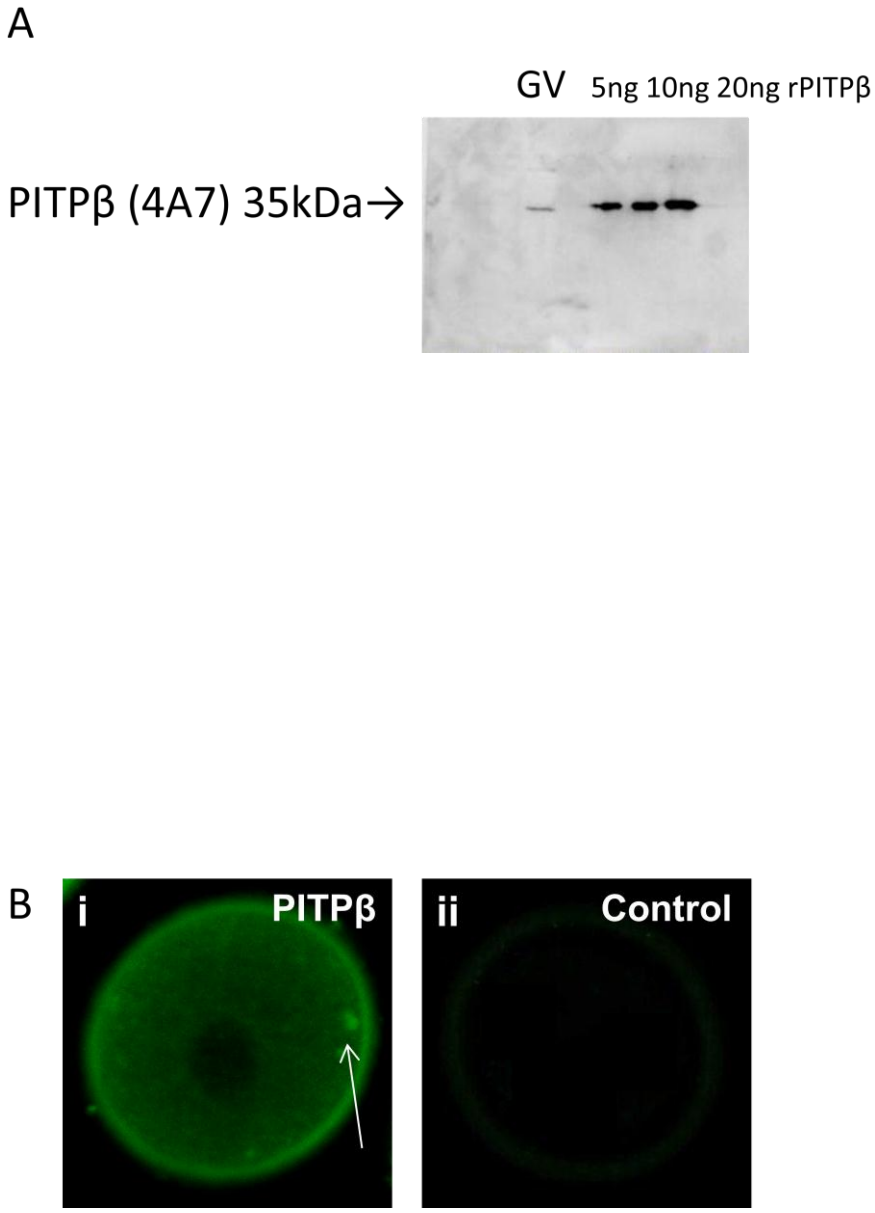


Figure 3.1. PITPβ is present in mouse oocytes

(A) Western blot analysis using 12% NuPage gel, 4.5µg sample of oocyte (130 oocytes), recombinant human PITPβ standards (from Prof. Shamshad Cockcroft), Anti-PITPβ (4A7) in a 1:1000 dilution, incubated for 1hr at RT and detected with ECL-Advanced. A 4A7-PITPβ band is displayed. (B) Oocyte at the GV stage labeled with anti-PITPβ. Control oocyte incubated without the primary antibody.

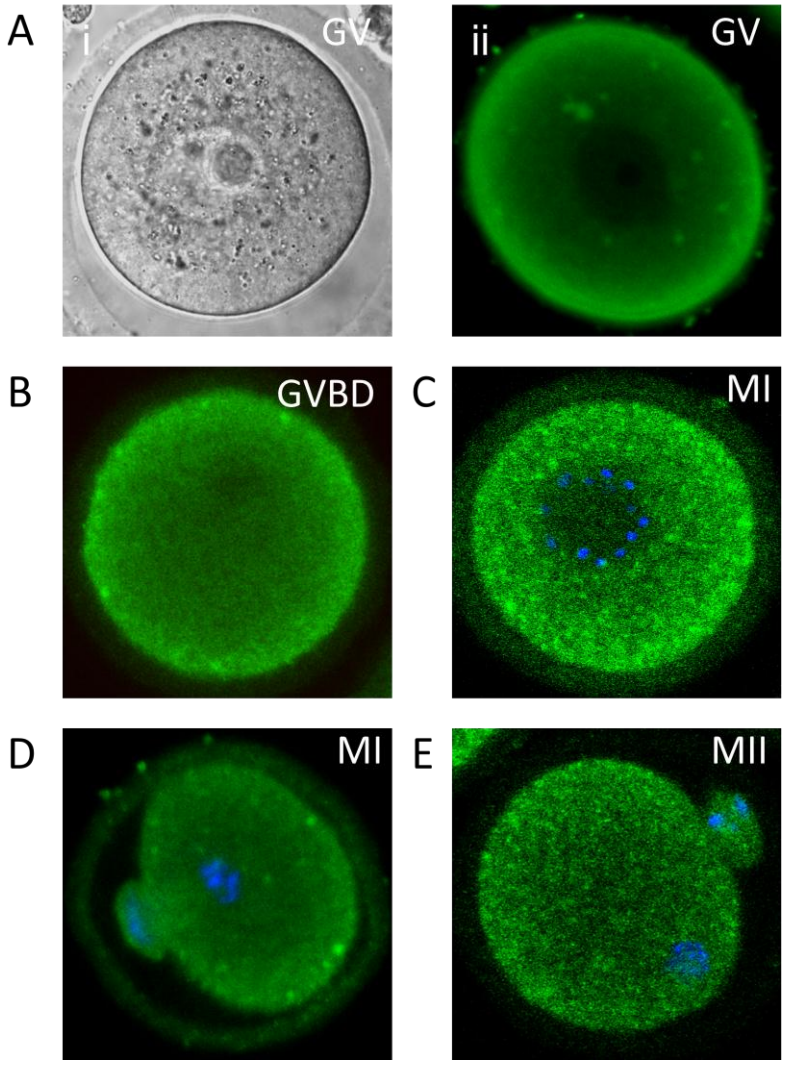


Figure 3.2. Localisation of PITPβ during oocyte maturation

(A-E) GV stage oocytes were released from prophase arrest and fixed for immunocytochemistry at different times during oocyte maturation and labelled with anti-PITPβ (green) and Hoechst 33342 (blue) to label the chromosomes. (Ai) Bright field of an oocyte at the GV stage. (Aii) GV oocyte (fixed during arrest) labelled with anti-PITPβ. (B) Localisation of PITPβ at the GVBD stage (2h after release). (C) PITPβ at Metaphase I (5h after release). (D) PITPβ at telophase I (8h after release). (E) PITPβ at Metaphase II (15 hr after release). Each image is representative of 15 oocytes per group (average).

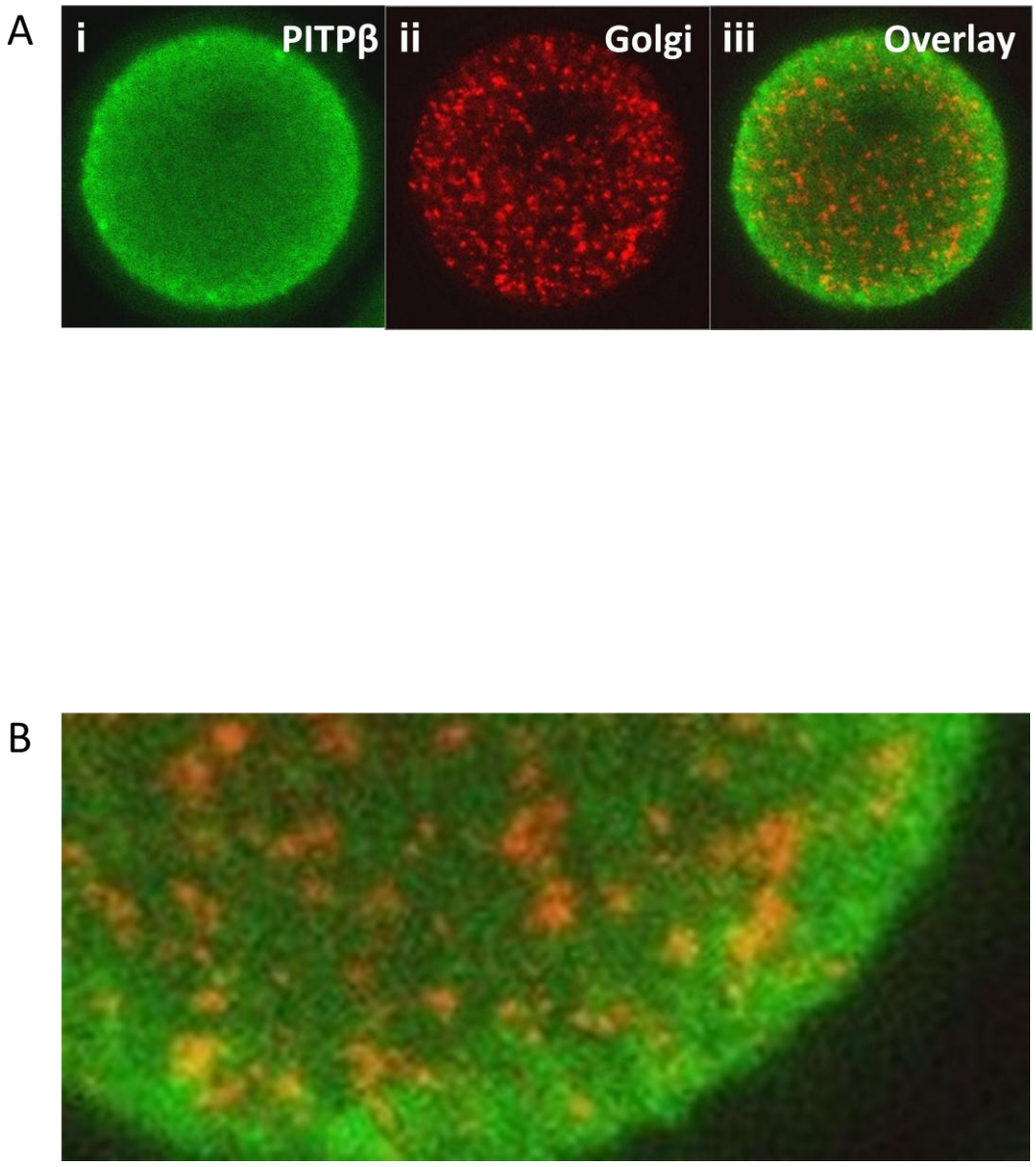


Figure 3.3. PITPβ does not localise at the Golgi compartment in mouse oocytes

(A) Oocyte at Metaphase I (fixed 5h after release from GV arrest. (Ai) Oocyte labelled with anti-PITPβ (green) and Hoechst (blue). (Aii) Oocyte labeled with anti-GM130 (red) and Hoechst (blue). (Aiii) Overlay. (B) Higher magnification of the overlay in Fig. A. In somatic cells PITPβ localises to the Golgi compartment but this is not valid for mouse oocytes. The image is representative of 14 oocytes per group (average).

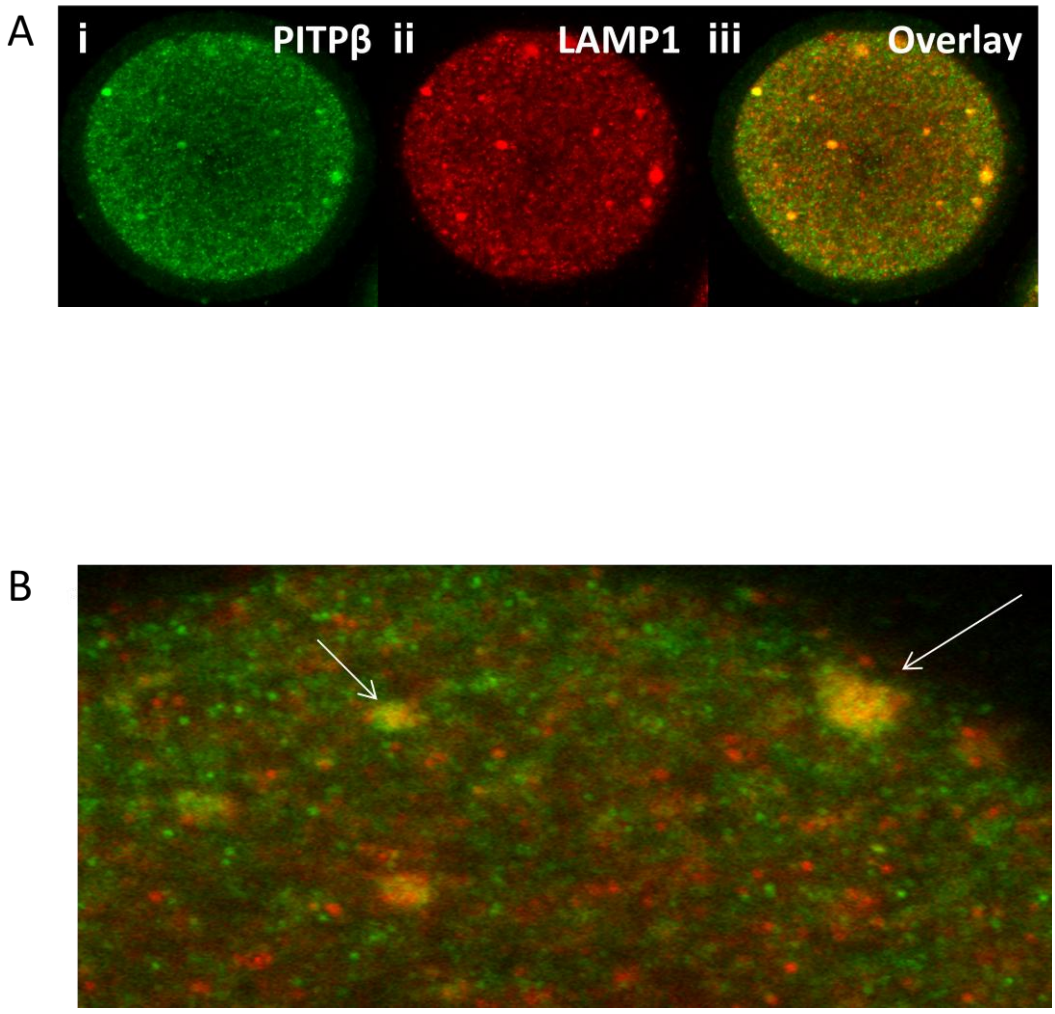


Figure 3.4. PITPβ localisation to lysosomes in mouse oocytes

(A) Oocyte fixed at the GV stage. (Ai) Oocyte labelled with anti-PITPβ. (Aii) Oocyte labeled with anti-LAMP1. (Aiii) Overlay. (B) Higher magnification of the overlay in Fig. A. The image is representative of 12 oocytes per group (average).

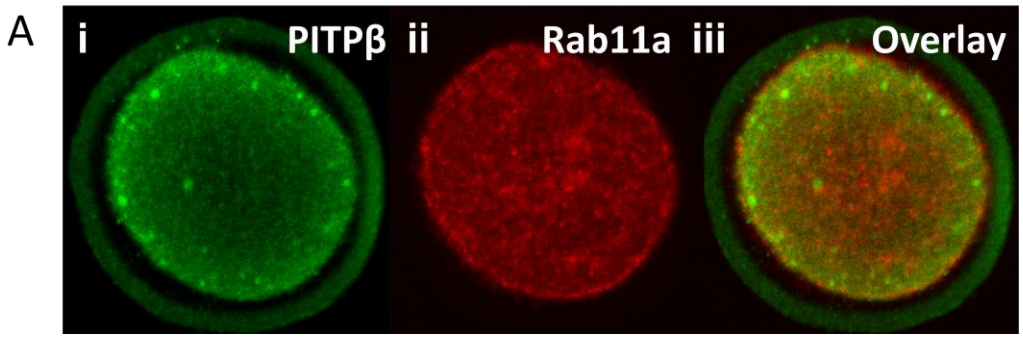


Figure 3.5. PITPβ does not localise to the recycling endosome compartment in mouse oocytes

(A) Oocyte fixed at the GVBD stage. (Ai) Oocyte labelled with anti-PITPβ. (Aii) Oocyte labelled with anti-Rab11a. (Aiii) Overlay. The image is representative of 10 oocytes per group (average).

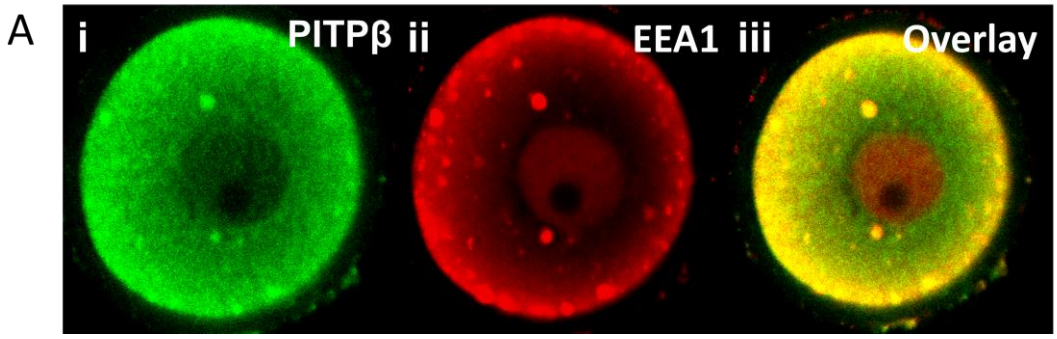
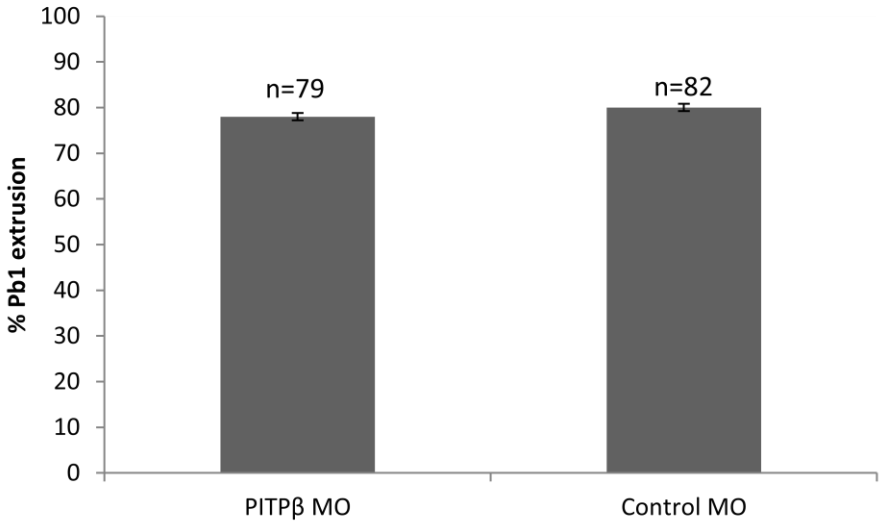


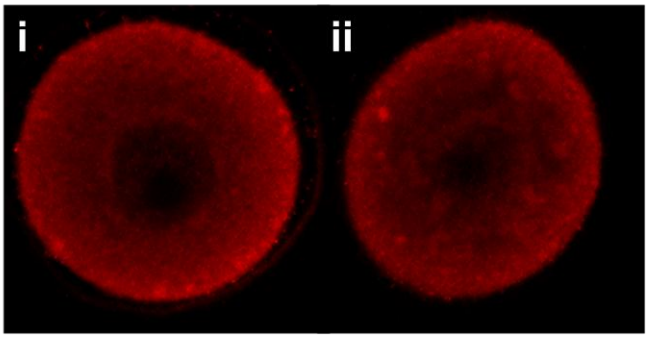
Figure 3.6. PITPβ localises to the early endosome compartment in mouse oocytes

(A) Oocyte at the GV stage (fixed during arrest). (Ai) Oocyte labelled with anti-PITPβ. (Aii) Oocyte labelled with anti-EEA1. (Aiii) Overlay. Each image is representative of 12 oocytes per group (average).

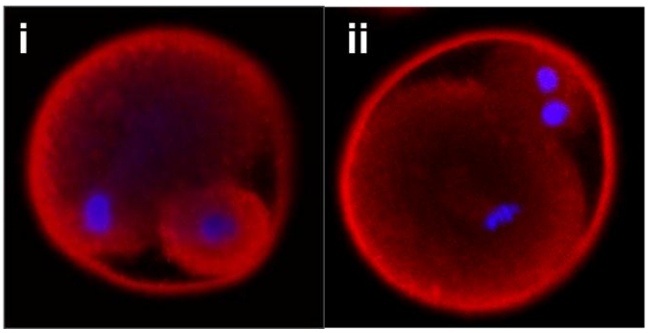
A


100
90
80
70
60
50
40
30
20
10
0
% Pb1 extrusion
n=79
n=82
PITPβ MO
Control MO

B


i
ii

C


i
ii

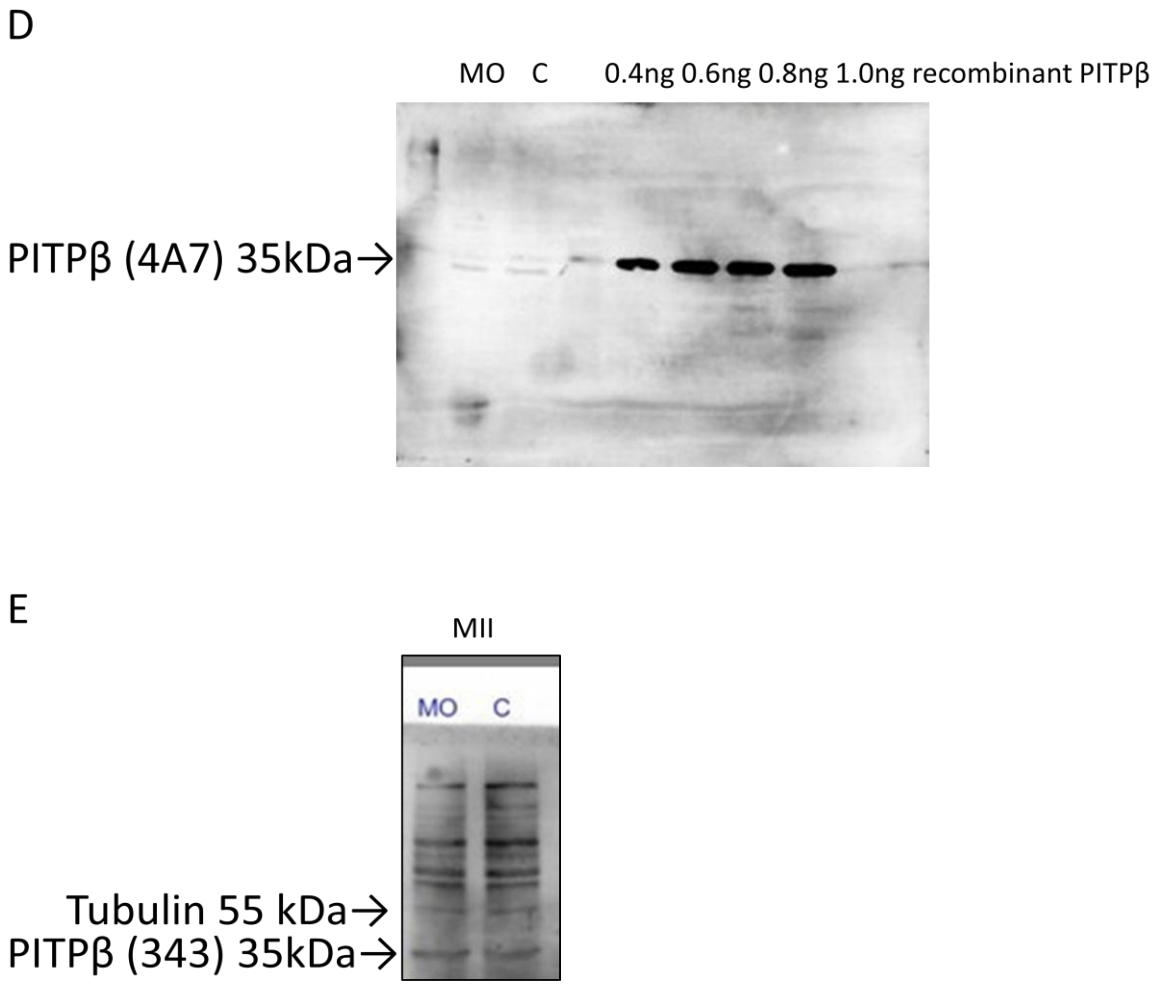
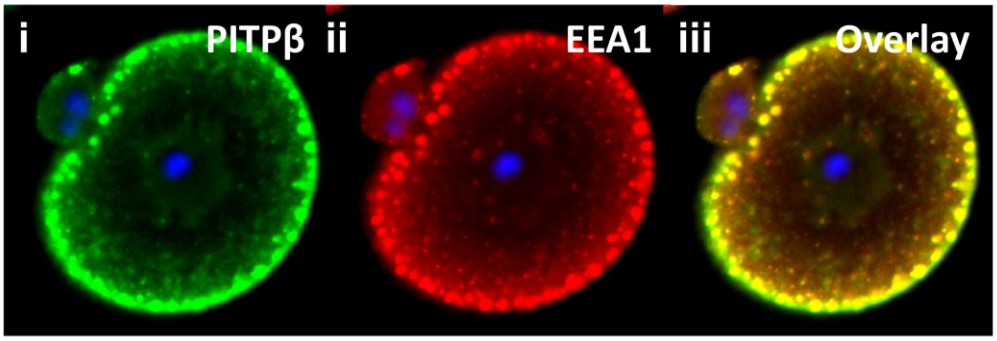


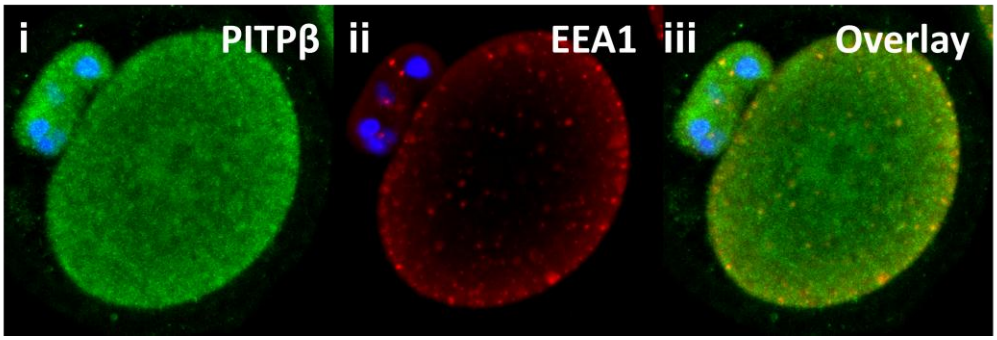
Figure 3.7. PITPβ cannot be depleted with morpholino oligonucleotides

(A-B) Oocytes injected at the GV stage, incubated O/N to deplete endogenous PITPβ and fixed at the GV stage (Bi-Bii) or sixteen hours after release from GV arrest (Ci-Cii), labelled with anti-PITPβ and Hoechst and monitored with confocal microscopy. (Ai) GV stage oocyte injected with the PITPβ MO. (Aii) GV stage oocyte injected with a control MO. (Bi) Oocyte injected with PITPβ MO. (Bii) Oocyte injected with a control MO. There is no decrease in fluorescence between the PITPβ MO oocytes and the controls suggesting that the protein could not be depleted.(C) There is no significant difference in the rate of Pb1 extrusion between the oocytes injected with the PITPβ MO and the control oocytes injected with a control MO ($P>0.05$). (D-E) Western blots showing that the protein could not be depleted with MO oligonucleotides in GV oocytes (D) and MII oocytes (E).

A


i
PITPβ
ii
EEA1
iii
Overlay

B


i
PITPβ
ii
EEA1
iii
Overlay

C

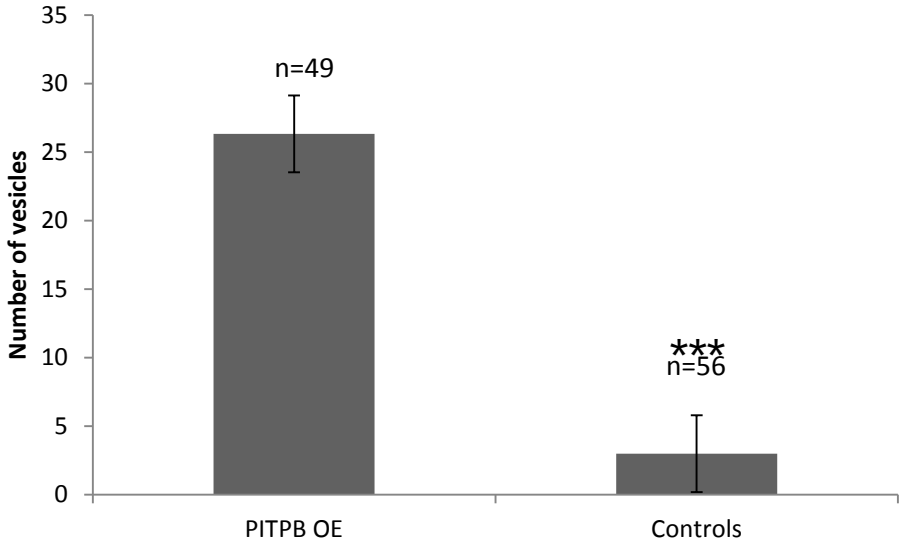
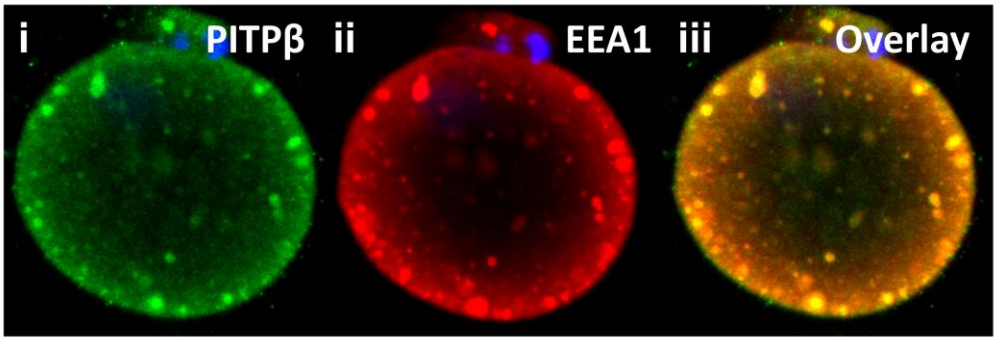

35
30
25
20
15
10
5
0
Number of vesicles
n=49
***
n=56
PITPB OE
Controls

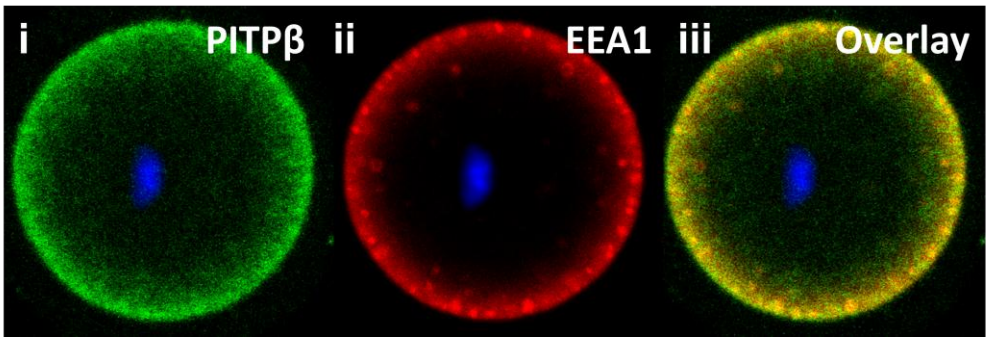
Figure 3.8. PITPβ over expression causes an accumulation of PITPβ vesicles and has no effect on polar body formation

(A) Oocytes at telophase I (fixed 8h after release) microinjected at the GV stage with mRNA PITPβ WT. (B) Control oocytes microinjected at the GV stage with water and fixed at the same stage. (Ai) Oocyte labelled with anti-PITPβ and Hoechst. (Aii) Oocyte labelled with anti-EEA1 and Hoechst. (Aiii) Overlay of anti-PITPβ, anti-EEA1 and Hoechst. (Bi) Control oocyte labelled with anti-PITPβ and Hoechst. (Bii) Control oocyte labelled with anti-EEA1 and Hoechst. (Biii) Overlay of anti-PITPβ, anti-EEA1 and Hoechst. Over expression of PITPβ causes no change in the rate of Pb1 formation compared to control oocytes. Each image is representative of approximately 20 oocytes per group. (C) There is a significant difference in the number of PITPβ positive vesicles present in oocytes over expressed with PITPβ WT compared to control oocytes. ***$P<0.001$

A


i
PITPβ
ii
EEA1
iii
Overlay

B


i
PITPβ
ii
EEA1
iii
Overlay

C

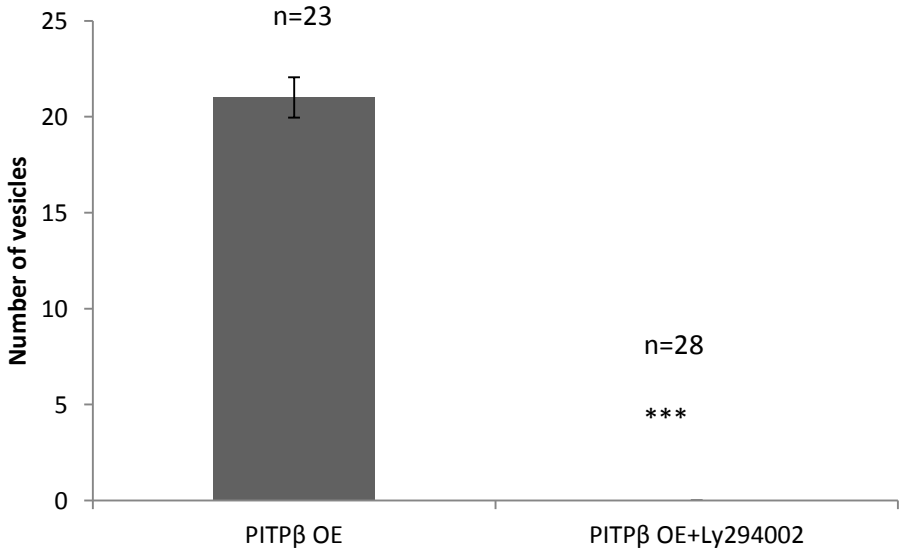
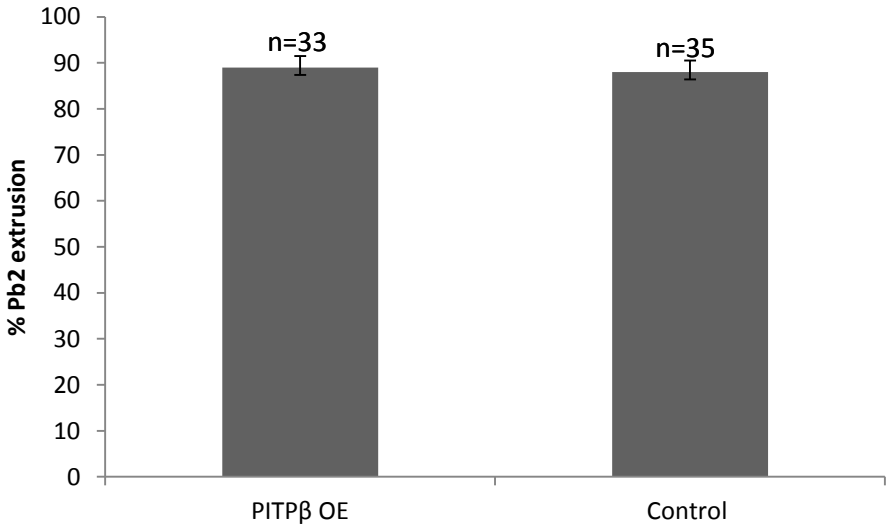

25
20
15
10
5
0
Number of vesicles
n=23
n=28
***
PITPβ OE
PITPβ OE+Ly294002

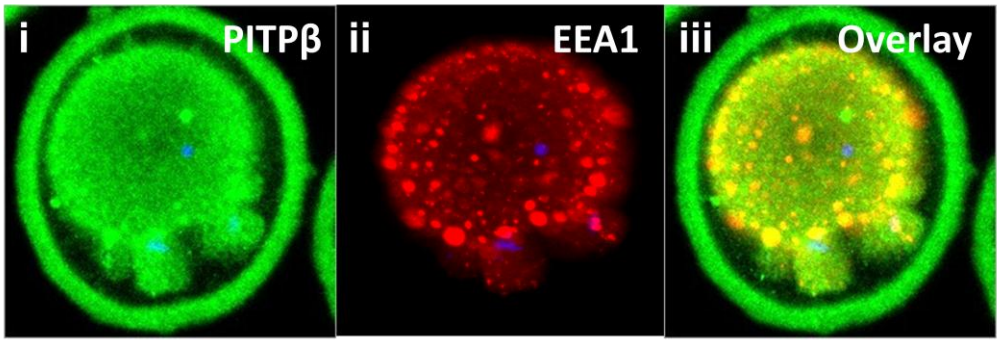
Figure 3.9. The accumulation of PITPβ vesicles is dependent on PI3P

(A) Oocytes at telophase I (fixed 8h after release) microinjected at the GV stage with mRNA PITPβ WT. (B) Oocytes microinjected at the GV stage with mRNA PITPβ WT, treated with Ly294002 (10μM) and fixed at the same stage. (Ai) Oocyte labelled with anti-PITPβ and Hoechst. (Aii) Oocyte labelled with anti-EEA1 and Hoechst. (Aiii) Overlay of anti-PITPβ, anti-EEA1 and Hoechst. (Bi) Oocyte labelled with anti-PITPβ and Hoechst. (Bii) Oocyte labelled with anti-EEA1 and Hoechst. (Biii) Overlay of anti-PITPβ, anti-EEA1 and Hoechst. Each image is representative of approximately 11 oocytes per group. (C) There is a significant difference in the number of PITPβ positive vesicles present in oocytes over expressed with PITPβ WT compared to oocytes over expressed with PITPβ WT and treated with Ly294002 ***$P<0.001$.

A


100
90
80
70
60
50
40
30
20
10
0
% Pb2 extrusion
n=33
n=35
PITPβ OE
Control

B


i
PITPβ
ii
EEA1
iii
Overlay

C

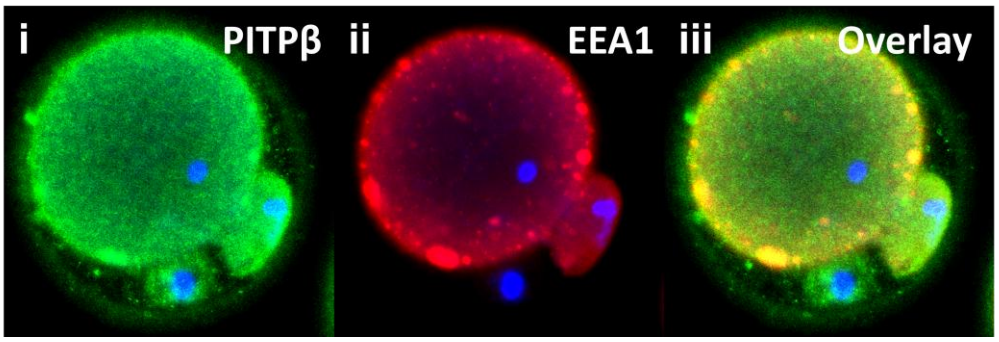

i
PITPβ
ii
EEA1
iii
Overlay

Figure 3.10. Over expression of PITPβ does not block Pb2 extrusion

(A) There is no significant difference in the rate of Pb2 extrusion in oocytes injected with PITPβ MO and control oocytes injected with water ($P>0.05$). (B-C) Oocytes at the MII stage, fixed 2 hours after ethanol activation and labelled with anti-PITPβ (green), anti-EEA1 (red) and Hoechst (blue). (B) Oocyte injected at the MII stage with PITPβ WT mRNA and activated with 7% ethanol. The green staining around the plasma membrane is non specific staining of the secondary antibody. (C) Control oocyte injected at the MII stage with water and activated with 7% ethanol. The oocytes have been cultured for 4 hours before being activated.

3.3 Discussion

The immunofluorescence experiments and the western blots presented in this chapter show that PITPβ is present in mouse oocytes throughout maturation and is localised in the cytoplasm in a punctuate/vesicular pattern from the GV stage to the MII stage. Unlike any other cell type examined thus far, my studies have revealed that PITPβ localises to the early endosomes compartment and to some extent to lysosomes in mouse oocytes.

The vesicular cytoplasmic-staining seen in mouse oocytes through meiosis with no evidence of localisation to the cleavage furrow in MI or MII is very different to that in Dm spermatocytes and to a lesser extent neuroblasts. Although there is a different PITP distribution in these two Dm cell types, there is some degree of enrichment of the protein around the spindle area and/or the contractile actin ring that is consistent with the observed effect of the *gio* mutants not being able to complete cytokinesis. The dramatically different pattern of localization seen in mouse oocytes suggests that, unlike Dm cells, PITPβ in mouse oocytes is unlikely to play a role in cytokinesis. We were not able to formally test this but the lack of localization in the cleavage furrow or spindle area in oocytes and the fact that no studies have identified early endosomes as being critical for cytokinesis, suggest that PITPβ may not be critically important for cytokinesis in mouse oocytes. The one caveat to this is that PITPβ is present at low levels throughout the cytoplasm and that this is sufficient to promote cytokinesis, that is, an accumulation of PITPβ is not necessary because the basal levels are sufficient. There is some precedent for this because, while in Dm spermatocytes there is a large accumulation in the cleavage furrow, this is not detected in neuroblasts. Since mutating *gio* in both cell types has a similar phenotype it has been suggested that PITPβ is present but that it is at levels below the limits of detection, perhaps because it is not as concentrated on the contractile ring as in spermatocytes. Further work is necessary to

determine whether PITPβ plays a similar role as PITP in spermatocytes but this will likely require deleting PITPβ from oocytes using the ZP3Cre system.

The relationship between the Golgi, ER and nuclear envelope mean that the nuclear phenotype seen in HeLa cells after PITPβ depletion is consistent with its localization. In contrast, we found no evidence to suggest that PITPβ is present on the Golgi or the ER in mouse oocyte; in the case of Golgi, no overlap in 4A7 and GM130 was examined and there was no similarity in the localisation of PITPβ and the known distribution of ER, as published by our lab and others (Mehlmann et al., 1995; FitzHarris et al., 2007). Thus we can be confident that PITPβ is playing a different role in oocytes than it appears to be in somatic cells.

Some functional roles may be attributed through understanding the localisation of PITPβ in mouse oocytes. The major site of localisation was found to be in the early endosome compartment and some limited colocalisation of PITPβ was found with LAMP1, a marker of the lysosomal compartment. This colocalization was only seen with a relatively small number of large cytoplasmic vesicles and not with the more numerous smaller LAMP1-positive vesicles.

There have been no previous reports localizing PITPβ to lysosomes. Furthermore, the identity and significance of these different size LAMP1-vesicles is not clear and thus it is not possible to attribute possible functions to PITPβ at these larger LAMP1-positive vesicles. Clearly there is much to learn about PITPβ in oocytes, in particular, its role in the compartments in which it localizes, given the localization in oocytes is on components of the endosome, it is reasonable to suggest a possible role in membrane traffic, a topic about which very little is known in oocytes.

This idea is strongly supported by the finding that expression of exogenous PITPβ caused an apparent proliferation and enlargement of vesicles in the cytoplasm of the oocyte. These vesicles were labelled by EEA1 antibody suggesting that they were also early endosomes. How the vesicles are generated is not entirely clear at this stage. The fact that they co-labelled for PITPβ and EEA1 and that such a large number of EEA1-positive vesicles were not present in the control oocytes suggests that the vesicles formed as a result of PITPβ expression. It is not clear whether the vesicles formed de novo, perhaps by increased endocytosis or whether they were generated from much smaller EEA1 positive vesicles that are not readily observed using our techniques, either by expanding in size or by fusing with other vesicles.

The C-terminal end of the EEA1 protein contains a FYVE finger domain (Gaullier et al., 2000). This protein domain, composed of Fab1p-YOPB-Vps27p-EEA1, is conserved from yeasts to humans and it binds to PtdIns3P; this binding has been proved to be necessary for the localisation and function of EEA1 (Gaullier et al., 2000). PtdIns3P is synthesised in cells by the class III phosphatidylinositol 3-kinase (PI3K), which is formed of the regulatory protein p150 and the catalytic subunit Vps34. PITPβ has been shown to stimulate the activity of PI3K, thereby increasing PtdIns3P activity (Panaretou et al., 1997). This increase in PtdIns3P would be expected to be detected by EEA1 because it has a PtdIns3P-binding FYVE domain. Therefore, we wanted to investigate if the accumulation of PITPβ vesicles in mouse oocytes was dependent on PtdIns3P. We found that by inhibiting PtdIns3P with a PI3K inhibitor (Ly294002) in oocytes over expressed with PITPβ WT, the accumulation of vesicles on the early endosome compartment disappeared, which confirmed our hypothesis.

EEA1 is an effector of Rab5 and is involved in vesicle fusion together with SNARE proteins (Lawe et al., 2000). In fact, this protein binds to both PtdIns3P and Rab5-GTP in vitro and

the FYVE domain is essential for these bindings. The only difference is that whereas PtdIns3P binding requires only the FYVE domain, Rab5 binding requires an additional region comprised of 30 amino acids which is found adjacent to the FYVE domain (Lawe et al., 2000). This upstream region is necessary for endosomal binding. When the expression of Rab5 WT is increased, the endosomal binding of EEA1 is also increased and its dependence on PtdIns3P decreases. So, this phosphoinositide may play a role in regulating the interaction between EEA1 and Rab5 (Lawe et al., 2000). In our case PtdIns3P may regulate the interaction between EEA1 and Rab5, therefore its inhibition may have an effect on the formation of EEA1 vesicles. It has been shown that the PtdIns3P binding to EEA1 requires the FYVE domain but this domain alone is not sufficient for localisation to cellular membranes (Lawe et al., 2000). For this reason, in our experiment it is still possible to visualise some EEA1 positive vesicles even after the treatment with the PI3K inhibitor. In summary, PITPβ localises to the early endosomes compartment in mouse oocytes and the over expression of this protein causes an accumulation of this protein on this compartment.

This shows that PITPβ is involved in membrane trafficking in mouse oocytes, but it may follow a different pathway in the regulation of this mechanism. In somatic cells by localising to the Golgi or ER compartment, it regulates the exchange of lipids between these compartments and the plasma membrane, whereas in mouse oocytes it may regulate this exchange between the early endosome compartment and the other compartments involved in membrane trafficking, e.g the late endosome compartment or the lysosomal compartment. In fact, we found that some PITPβ positive vesicles are also localised to the lysosomal compartment in GV oocytes. This protein may regulate the trafficking of endocytosed material from the early endosome to the plasma membrane.

4. The role of Rab11 during asymmetric cell division in mouse oocytes.

4.1 Introduction

Rab11 has been shown to play a number of roles during cytokinesis in *Drosophila* and somatic cells.

Rab proteins constitute the largest family of monomeric small GTPases. These proteins function as molecular switches between active (GTP-bound) and inactive (GDP- bound) conformations. During their active state they cooperate with downstream 'effector' proteins, which are involved in different cellular activities such as vesicle formation, motility and fusion (Zerial and McBride 2001). Rab11 proteins also regulate the movement of vesicles and organelles along cytoskeletal elements (Zerial and McBride 2001).

More recently vesicle trafficking has been shown to be important for cytokinesis. Rab11 is thought to target recycling endosomes to the furrow during late telophase, thus regulating the abscission step of cytokinesis in HeLa cells. Rab11-containing recycling endosomes are found at the cleavage furrow of HeLa cells during telophase whereas early endosomes are not found at the furrow/midbody, suggesting that recycling endosomes play a role in cytokinesis (Wilson 2005). In addition, over-expression of Rab11-S25N (dominant negative mutant) increases considerably the number of binucleate cells (Fielding 2005; Wilson 2005). Rab11 plays a role also during cell division in *Drosophila* spermatocytes, where it accumulates at the cleavage furrow (Giansanti et al., 2007). In mutant spermatocytes the actomyosin rings form but then they fail to constrict causing a failure in cytokinesis (Giansanti et al., 2007). These mutants show also an abnormal accumulation of vesicles derived from the Golgi at the

telophase stage. Rab11 is enriched also at the acroblast of spermatids and is necessary for its formation (Giansanti et al., 2007).

The defects caused by Rab11 mutations in *Drosophila* spermatocytes resemble those observed in *gio* (PITP) and *fwd* (PI4KIIIβ) mutants (Brill et al., 2000; Giansanti et al., 2004, 2006; Gatt and Glover 2006). Mutations in these proteins cause defects in the constriction of the actomyosin rings, disorganization of the central spindle during late telophase and an abnormal accumulation of Golgi-derived vesicles at the cell equator of telophase cells (Giansanti et al., 2006; Giansanti et al., 2007). Thus these proteins appear to be essential for membrane-vesicle fusion during cytokinesis and may work in the same cytokinetic pathway. Evidence for this is shown in genetic studies where *gio* and *fwd* mutants show no Rab11 localisation at the cleavage furrow. Conversely, the *fwd* and *gio* mutants at the ana-telophase stage show a normal localisation at the cell poles and the cleavage furrow when Rab11 is mutated. This study suggests that all the proteins are involved in the pathway and that Rab11 is downstream of *gio* e *fwd* (Giansanti et al., 2007). The authors suggest that PITP (*gio*) transfers monomers of PtdIns to the cleavage furrow leading to an enrichment of PtdIns molecules at this site. The interaction of PITP with this membrane domain may help the recruitment of PI4KIIIβ encoded by *fwd*, which would then contribute to the production of PtdIns(4)P to produce PtdIns(4,5)P_2, which has also been found to be required for cytokinesis (Field et al., 2005). It has been shown that PI4KIIIβ interacts with Rab11-GTP to control the vesicular transport from the Golgi complex to the plasma membrane and it is also necessary for the localisation of Rab11 at the Golgi apparatus and not vice versa (De Graaf et al., 2004). In fact, Rab11 has been localized to both the Golgi and the recycling endosomes where it is involved in regulating vesicle transport between the Trans-Golgi network and the plasma membrane (Chen et al, 1998).

In *Drosophila* spermatocytes during prophase/prometaphase Rab11 is found in the Golgi stacks and the nuclear envelope, then it becomes concentrated at the ER compartment and at some Golgi-derived vesicles during metaphase and ana-telophase, and finally it is found at the cleavage furrow during telophase (Giansanti et al., 2007). In this Chapter, I investigate the localization and role of Rab11 in mouse oocytes.

4.2 Results

4.2.1 Localisation of Rab11 during meiosis of mouse oocytes

To investigate the distribution of Rab11 during maturation in mouse oocytes we used an anti-rabbit polyclonal antibody against Rab11a which has been already used in other cell types (e.g HeLa cells) (Wilson et al., 2005; Fielding et al., 2005). This antibody is also used as a marker for recycling endosomes (Ullrich et al., 1996). I have verified the staining pattern with two different antibodies (Rab11a rabbit polyclonal from Abcam and Rab11 rabbit polyclonal from Zymed laboratories) and the pattern results to be the same. The data shown have been obtained using the Abcam antibody.

Prophase arrested, GV stage mouse oocytes show a vesicular pattern of localisation of Rab11 distributed throughout the cytoplasm (Figure 4.1Aii). We assume that this punctuate distribution of Rab11 positive vesicles are recycling endosomes. There is also one level of cytoplasmic staining suggesting a second soluble pool of Rab11. In prometaphase, after GVBD, Rab11 remains localised in a vesicular pattern (Figure 4.1B). At metaphase I,(five hours after GVBD), when the spindle is formed and the chromosomes are aligned on the metaphase plate, Rab11 positive vesicles aggregate around the first meiotic spindle (Figure 4.1C). The spindle then migrates to the cortex of the oocyte and undergoes anaphase-telophase transition and Rab11 localises around the 'shoulders' of the first polar body and on the midzone of the spindle (Figure 4.1D) At this stage the homologous chromosomes have separated and have migrated to the spindle poles. At late telophase I, at the time of polar body formation, Rab11 localises at the cleavage furrow of the oocytes (Figure 4.1E). In MII arrested eggs, Rab11 is present in the cytoplasm but in a less punctuate and more diffuse pattern compared to the MI stage (Figure 4.1F). The distribution of Rab11 does not change markedly when the oocytes are parthenogenetically activated and allowed to progress to the pronuclear stage (Figure 4.1 G).

4.2.2 The role of microtubules in Rab11 localisation

Previous studies have shown that aggregation of ER around the mitotic spindle is mediated by microtubule-dependent processes (Fitzharris et al., 2007). In order to investigate if Rab11 positive vesicles localisation around the spindle region during metaphase I is dependent on microtubules, we have treated oocytes with nocodazole (5μM), an antimitotic agent which depolymerises microtubules by binding to β-tubulin and preventing the formation of one of the interchain disulfide linkages. This agent has been extensively used and characterised in mouse oocytes (Kubiak et al., 1993; Winston et al., 1995; Fitzharris et al., 2007). Figure 4.2A shows a control oocyte at metaphase I, exactly five hours after GVBD. The image clearly shows an aggregation of Rab11 positive staining around the spindle (transverse section through the spindle). The oocytes have been labelled with anti-Rab11a and anti-αtubulin and Hoechst to stain the chromosomes. Oocytes that have been treated with nocodazole from the GV stage up to five hours after GVBD (Figure 4.2B), the microtubules are depolymerised and Rab11 localisation fails to localise, except for a small aggregation around the chromosomes. This result shows that the localisation of spindle-associated Rab11 is dependent on microtubules.

4.2.3 Rab11 localisation does not depend on microfilaments

In order to investigate whether microfilaments play a role in localising Rab11 around the spindle region during metaphase I, we have treated oocytes with latrunculin B (0.6μM) which inhibits actin polymerization in vitro. Figure 4.3A shows a control oocyte at metaphase I, five hours after GVBD. The oocytes have been labelled with anti-Rab11a and anti-α-tubulin and Hoechst to label the chromosomes. The accumulation of Rab11 around the spindle area is intense. After the oocytes have been treated with latrunculin B ON (Figure 4.3B), Rab11 localisation does not disappear showing that its localisation is not dependent on

microfilaments. In addition, the cortical actin staining remains because it is very stable. All the oocytes treated with latrunculin B fail to extrude a polar body and therefore remain arrested at Metaphase I. This has been previously shown in mouse oocytes treated with latrunculin B (Barrett and Albertini 2010).

4.2.4 Rab11 cannot be depleted with morpholino oligonucleotides

To test the role of Rab11 during asymmetric cell division of mouse oocytes, morpholino oligonucleotide (MO) were used in an attempt to deplete Rab11 (see chapter 2). Rab11 MO-injected oocytes were cultured with IBMX for 24 hours before being released from arrest and the effects on the first meiotic division were observed. There was no significant difference in the rate of Pb1 extrusion between the Rab11 MO-injected oocytes (80%; 57/71 oocytes) and the control MO-injected oocytes (78%; 50/64 oocytes $P>0.05$) (Figure 4.4B). The metaphase II oocytes were fixed for immunocytochemistry 15-16 hours after release from arrest and labelled with anti-Rab11a and Hoechst to determine whether Rab11 had been depleted by the treatment with Rab11-MO. Figure 4.4A shows that there is no obvious decrease in the level of fluorescence in the treated oocytes compared to the control oocytes. Thus, Rab11 appears to be stable in mouse oocytes and therefore longer periods not compatible with oocyte viability would be required to deplete it.

4.2.5 Rab11 inhibition arrests the oocytes in metaphase I

In order to examine the role of Rab11 during mammalian meiosis we have used a dominant negative mutant Rab11S25N which is locked in the GDP-bound state. GV stage oocytes were injected with mRNA expressing the Rab11S25N mutant and the control oocytes were injected with water. They were then cultured in the presence of IBMX for five hours and then

released from arrest to determine the effects on the first meiotic division. A significant reduction in the extrusion of the Pb1 was observed in the oocytes injected with the mutant Rab11S25N (25%; 13/52 oocytes) compared to control oocytes injected with water (90%; 55/61 oocytes $P<0.001$) (Figure 4.5A). Figure 4.5 Bi shows a bright field of an oocyte which remained arrested at metaphase I and failed to extrude a polar body. Figure 4.5 Bii shows a bright field of a control oocyte which has extruded the Pb1. Figure 4.5 C shows an oocyte injected with Rab11S25N, arrested at metaphase I and labelled with anti-Rab11.

In order to investigate the reason for the inhibition of Pb1 extrusion oocytes were fixed for immunocytochemistry in order to examine the structure of the spindle, the chromosomes and actin. In Rab11S25N-injected oocytes that failed to extrude a polar body most were normal (Figures 4.6A and 4.6Bi), some showed evidence of misaligned chromosomes and disrupted spindles, with 20% (8/38 oocytes) having 1 or 2 misaligned chromosomes (Figure 4.6 Bii) and 15% (6/38) being scored as severe (more than three chromosomes misaligned) (Figure 4.6 Biii). In contrast only 12% (4/31) of the control MII arrested oocytes showed a slight or severe misalignment (Figure 4.6A).

Furthermore, the oocyte in figure 4.6Bi has a normal spindle and a normal actin cap. As mentioned earlier they also have a normal alignment of chromosomes on the metaphase plate. In contrast, the proportion of oocytes which show a slight misalignment or a severe misalignment of chromosomes (Figures 4.6 Bii and 4.6 Biii) do not form an actin cap due to a failure in spindle migration to the cortex and the microtubules are almost absent. Thus, inhibition of Rab11 causes oocytes to arrest at metaphase I and in a proportion of oocytes (30%) it appears to cause disruption of spindle formation and chromosome alignment.

4.2.6 Rab11 inhibition does not block polar body two emission

To investigate if Rab11 has a role during the second meiotic division of mouse oocytes, we have injected MII oocytes with Rab11 S25N mRNA and have activated them with 7% ethanol to examine the rate of Pb2 emission. We found that Rab11 inhibition in MII oocytes does not affect the rate of Pb2 emission. In fact, that there was no difference in the rate of Pb2 emission between the oocytes injected with the Rab11 mutant (90%; 23/25 oocytes) and the control oocytes injected with water (87%; 26/30 oocytes $P>0.05$) (Figure 4.7C). Furthermore, the structure of the spindle, the chromosomes and the actin ring were analysed in both groups. The oocytes injected with the Rab11 mutant S25N showed a spindle with a normal shape, the actin ring was organised and the chromosomes also showed no abnormality compared to the control oocytes (Figures 4.7A and 4.7B).

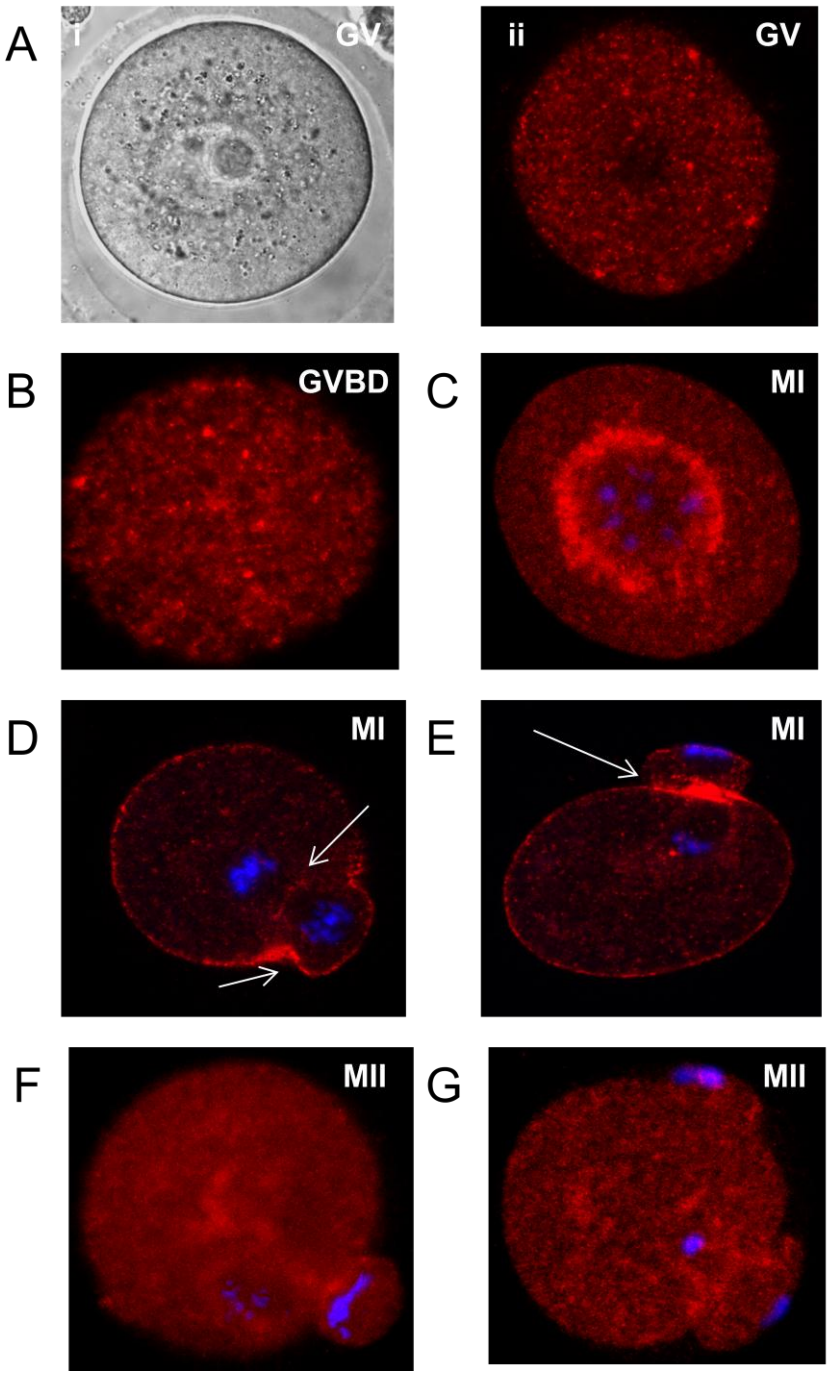


Figure 4.1 Localisation of Rab11 during meiosis

(A-G) Oocytes stained with anti-Rab11 (red) and Hoechst 33342 (blue) to label the chromosomes. (Ai) Bright field of a GV oocyte. (Aii) GV oocyte labelled with anti-Rab11. (B) Oocyte at the GVBD stage. (C) Oocyte at metaphase I. (D) Oocyte at telophase I. (E) Oocyte at late telophase I with the first polar body. (F) Oocyte arrested at Metaphase II. (G) Oocyte activated with ethanol with the second polar body. Each image is representative of approximately 15 oocytes per group.

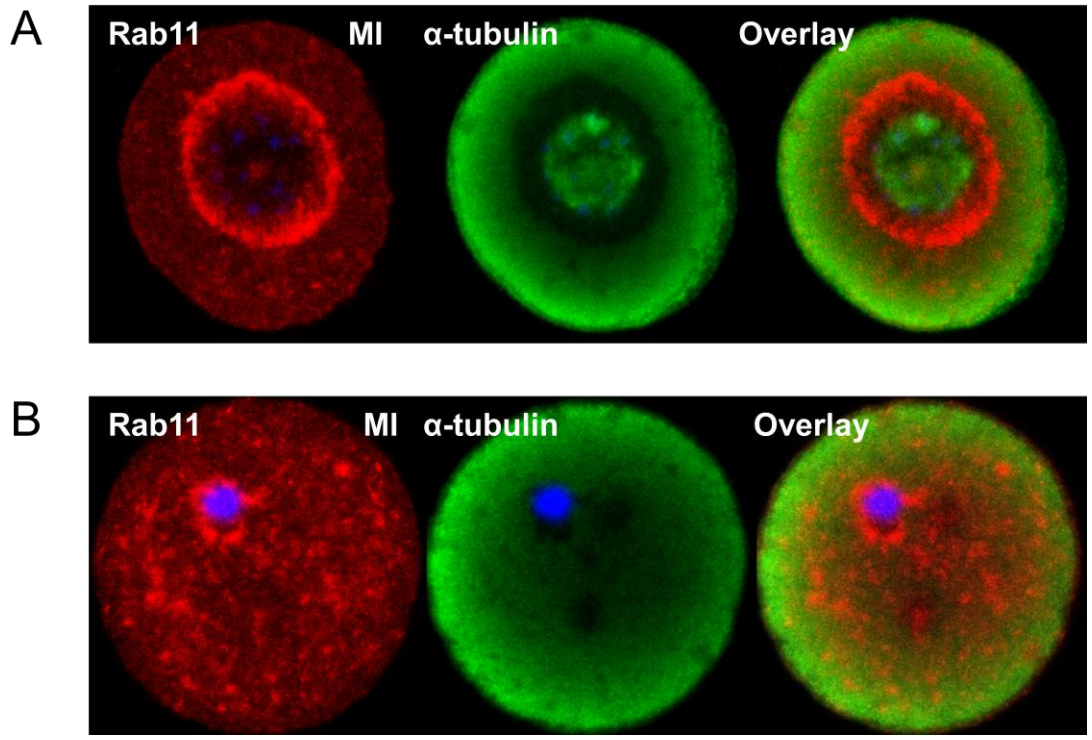


Figure 4.2 The role of microtubules in Rab11 localisation

(A-B) Oocytes fixed at Metaphase I and stained with anti-Rab11 (red), Hoechst 33342 (blue) and Anti-α-tubulin (green). (A) Oocyte fixed at Metaphase I; Rab11 is localised around the spindle. The image shows a transverse section through the spindle. (B) Oocyte treated with nocodazole (5µM) and fixed at Metaphase I. Each image is representative of 12 oocytes per group (average).

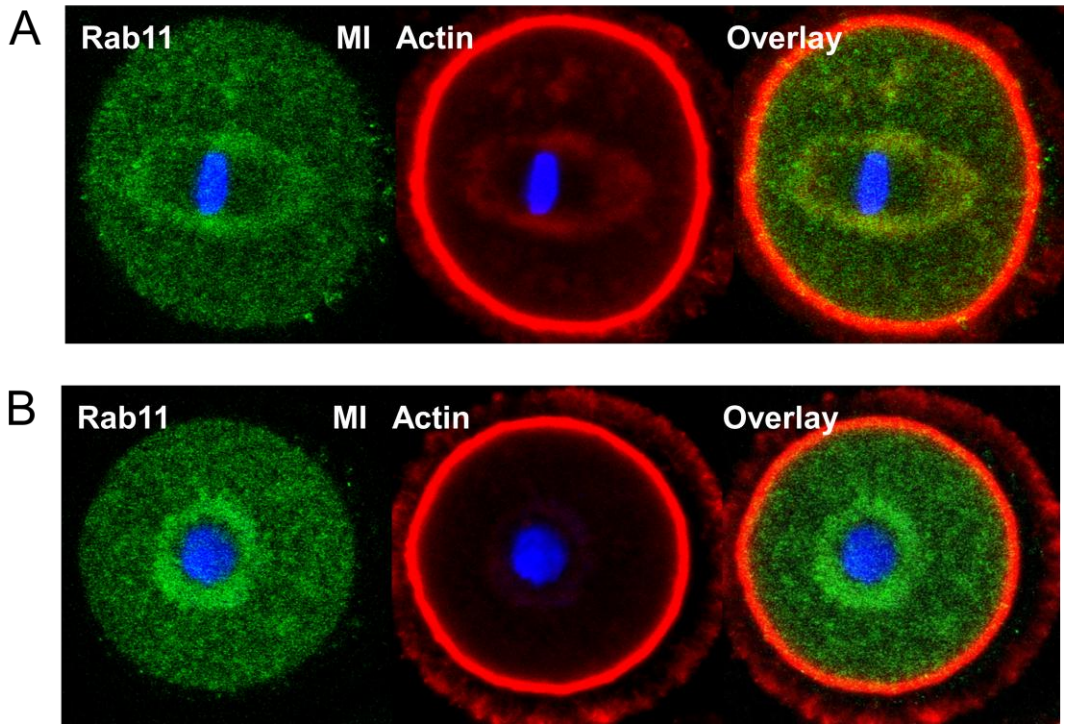


Figure 4.3 Rab11 localisation around the spindle is not dependent on microfilaments

(A-B) Oocytes fixed at Metaphase I and stained with anti-Rab11 (green), Hoechst 33342 (blue) and phalloidin (red). (A) Oocyte fixed at Metaphase I; Rab11 is localised around the spindle. (B) Oocyte treated with Latrunculin B O/N and fixed at Metaphase I. The image shows a transverse section through the spindle. Each image is representative of approximately 10-12 oocytes per group.

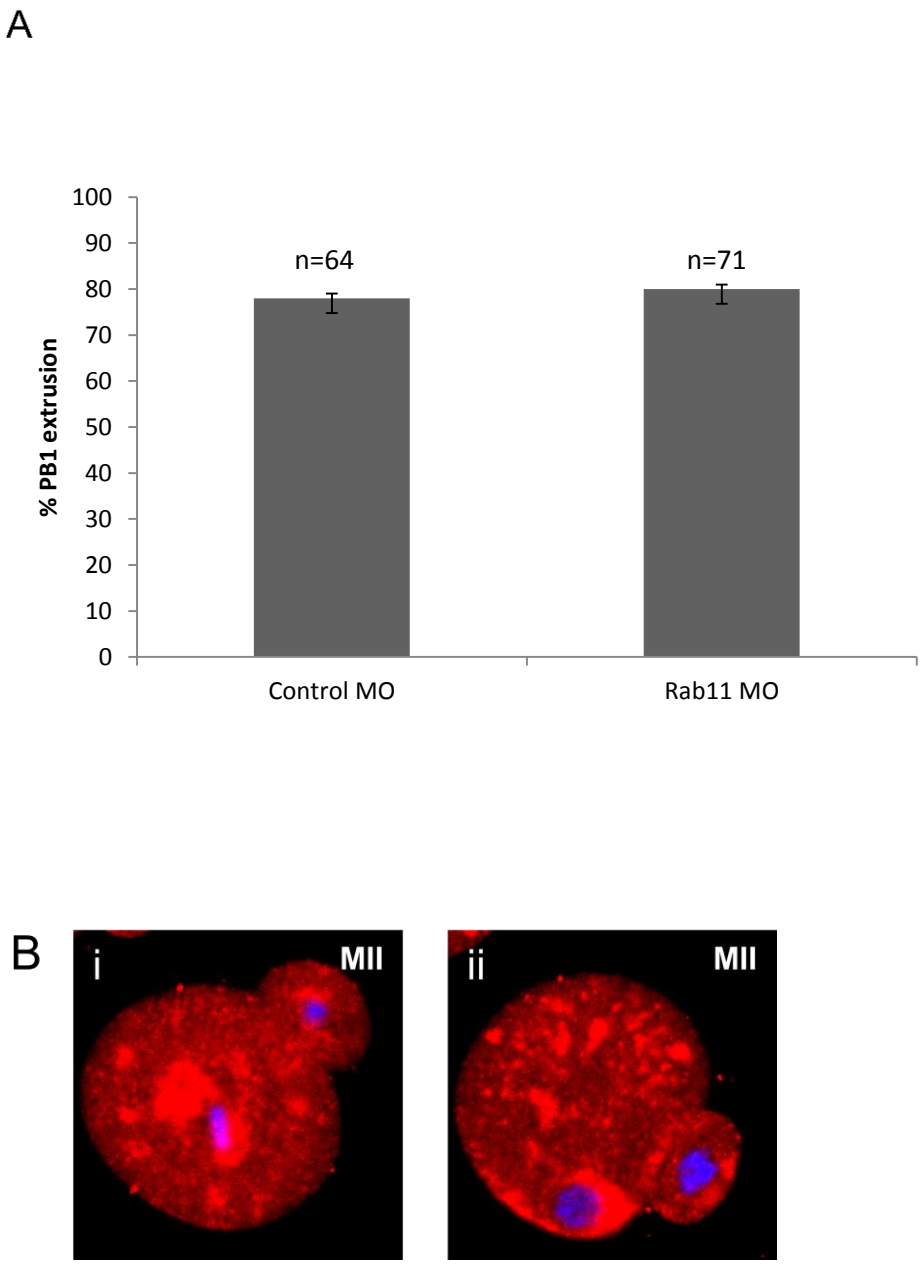


Figure 4.4 Rab11 cannot be depleted with morpholino oligonucleotides

(A) There is no significant difference in the rate of Pb1 extrusion between the oocytes injected with the Rab11 MO and the control oocytes injected with a control MO ($P>0.05$). Note the small error bars due to an all or none biological phenomenon. (Bi) MII stage oocyte injected at the GV stage with a Rab11 MO and incubated O/N in an attempt to deplete endogenous Rab11 protein. (Bii) MII stage oocyte injected with a control MO at the GV stage and incubated O/N. At sixteen hours from release from GV arrest, Rab11 MO injected (Bi) and control MO injected (Bii) were fixed, labelled for Rab11 and Hoechst and monitored with confocal microscopy. There is no decrease in fluorescence between the Rab-11 MO oocytes and the controls suggesting that the protein could not be depleted.

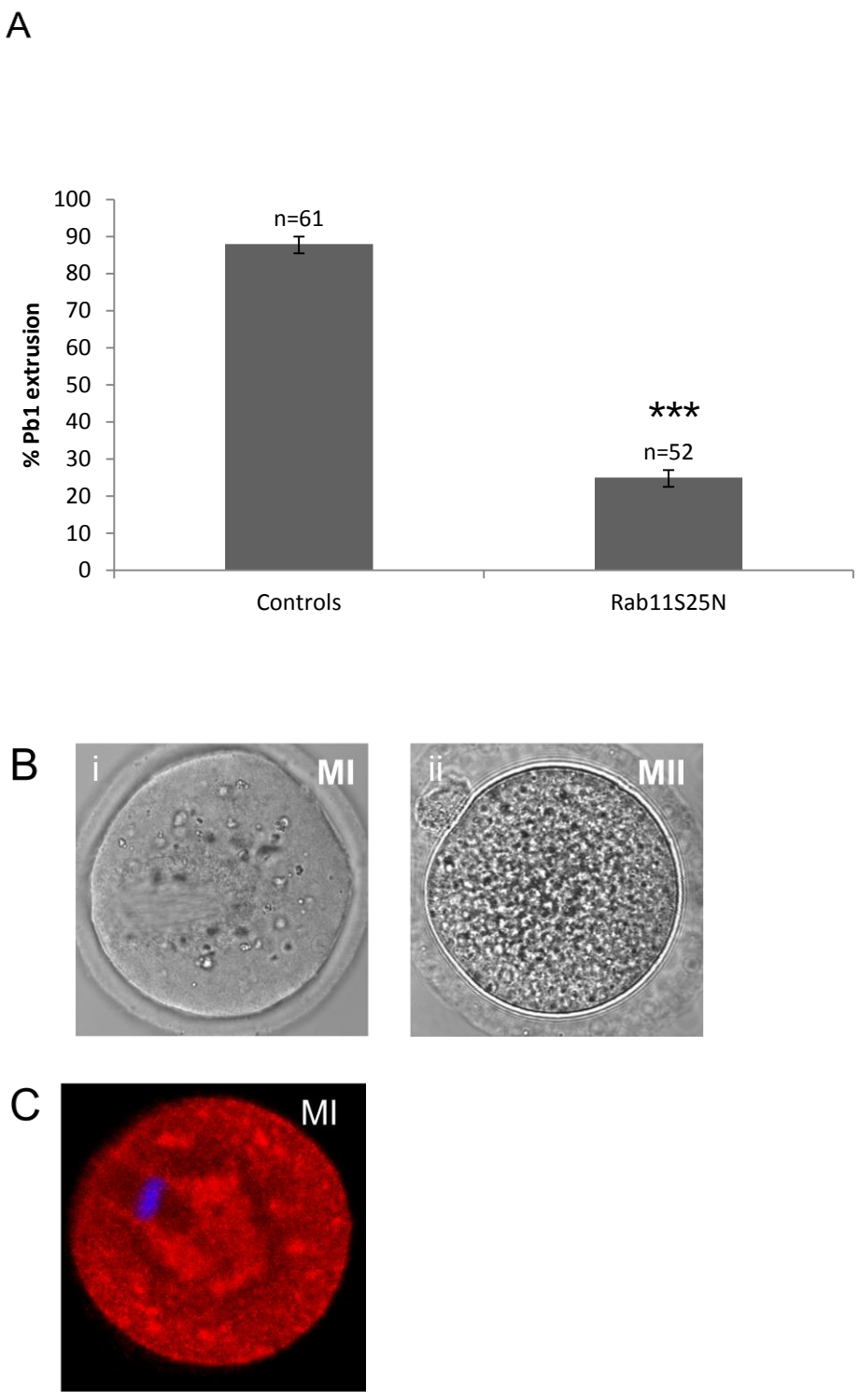


Figure 4.5 Rab11 inhibition arrests oocytes in Metaphase I

(A) There is a significant reduction in the extrusion of the first polar body in oocytes injected with a Rab-11 S25N dominant negative mutant mRNA compared to controls. P<0.001. (Bi) Bright field of oocyte injected with Rab11 S25N mRNA and arrested at Metaphase I. (Bii) Control oocyte injected with water and arrested at Metaphase II with the first polar body. (C) Oocyte injected with Rab11 S25N, arrested at MI and labelled with anti-Rab11. The data are from three experiments.

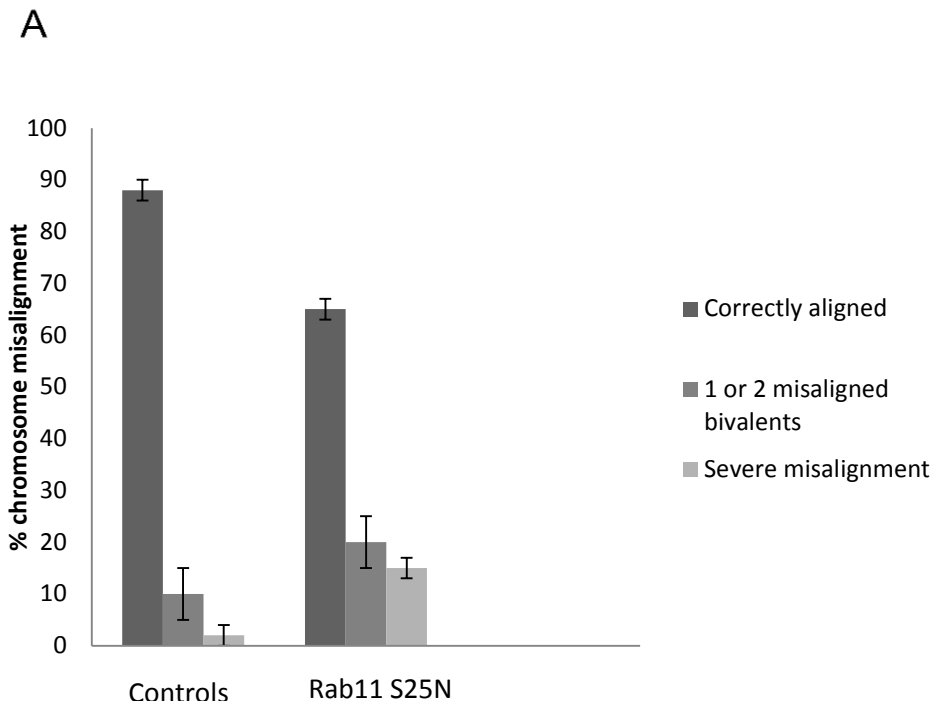


Figure 4.6 Rab11 inhibition causes chromosome misalignment and spindle disruption

(A) Analysis of chromosome alignment in oocytes injected with a Rab11 mutant (S25N) compared to controls. Data are from three different experiments with approximately 10-13 oocytes per group ($P>0.01$). (B) Oocytes injected with Rab11 S25N mRNA, arrested at metaphase I and labelled with anti-α-tubulin (green), phalloidin (red) and Hoechst (blue). (Bi) Oocyte with a normal alignment of chromosomes. (Bii) Oocyte showing a slight misalignment of chromosomes and no spindle and actin cap. (Biii) Oocyte showing a severe misalignment of chromosomes and spindle disruption and no actin cap.

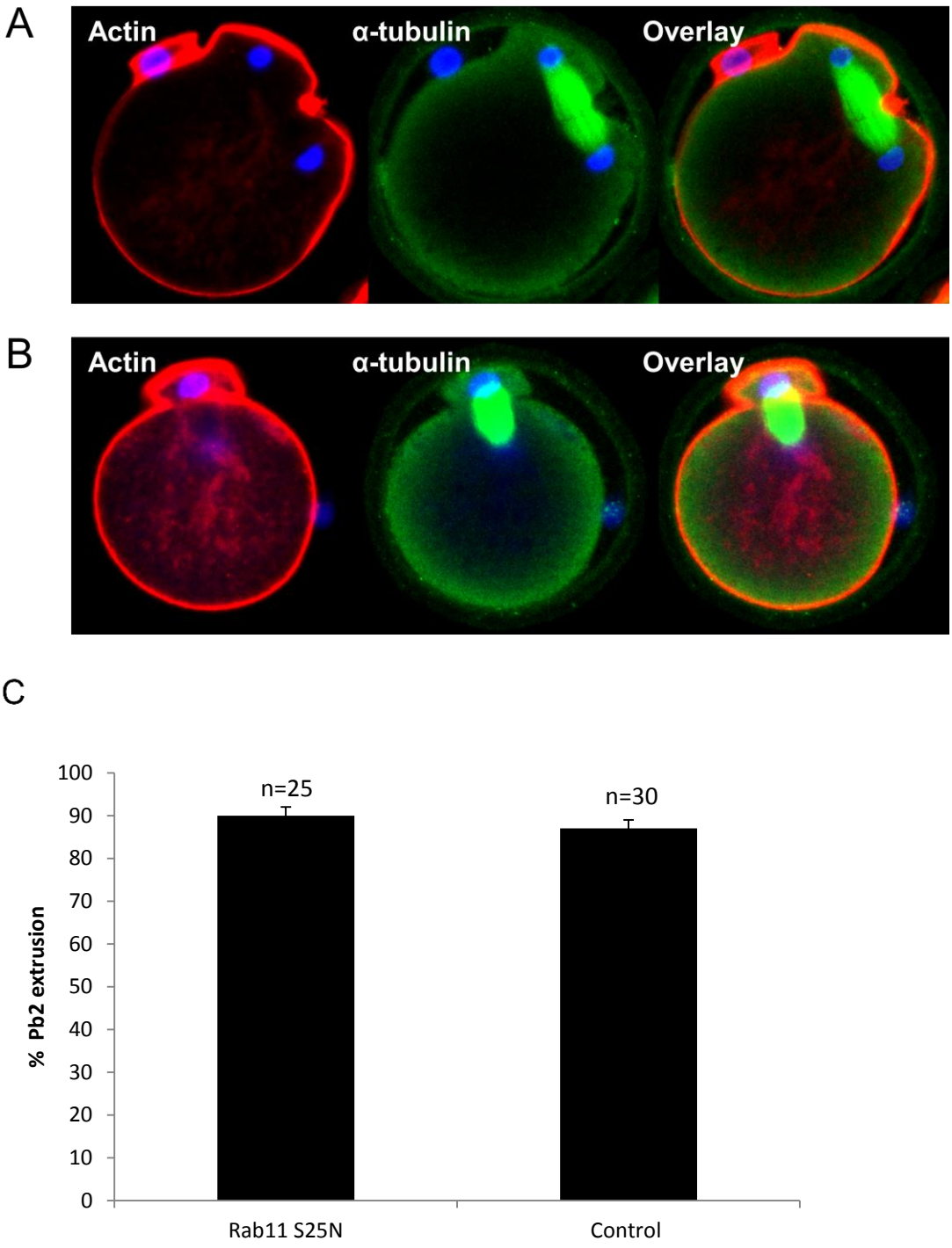


Figure 4.7 Rab11 inhibition does not block polar body two emission

(A-B) Oocytes at the MII stage, fixed 2 hours after ethanol activation and labelled with phalloidin (red), anti-α-tubulin (green) and Hoechst (blue). (A) Oocyte injected with Rab11 S25N mRNA and activated with 7% ethanol. (B) Control oocyte injected with water and activated with 7% ethanol. The oocytes have been cultured for 4 hours before being activated. (C) There is no significant difference in the rate of Pb2 extrusion in oocytes injected with Rab11 S25N and control oocytes injected with water ($P>0.05$).

4.3 Discussion

4.3.1 Rab11 localisation during meiotic maturation in mouse oocytes.

The experiments presented in this chapter show that Rab11 is present in mouse oocytes throughout maturation and is strongly localised at the cleavage furrow during telophase I suggesting a role for this protein during asymmetric cell division in mammalian meiosis. Rab11 localisation has been shown in other cells but this is the first report to show its presence in oocytes.

Our experiments indicate that in GV-stage oocytes the protein is also localised in a punctuate pattern in the cytoplasm. But in metaphase I it starts to localise strongly around the spindle region. This has not been described in somatic cells indicating that the protein may have different dynamics in mitosis compared to meiosis. At the end of Anaphase I we find that Rab11 redistributes from the spindle surrounding area to the spindle mid-zone and around the 'shoulders' of the forming Pb1. Finally, at telophase I it is localised at the cleavage furrow, which is consistent with the data observed in HeLa cells. After the extrusion of Pb1, at MII, the protein is still localised in the cytoplasm but it appears to be more soluble as the defined vesicular staining pattern seen in MI is less distinct. This localisation remains very similar after ethanol activation and the extrusion of Pb2, suggesting that it is not simply a result of the oocyte being in metaphase. Thus it appears that during oocyte maturation Rab11 is localised to a dynamic membrane compartment assumed to be the recycling endosome.

This localisation is similar to the distribution found in HeLa cells but differs to that found in *Drosophila* spermatocytes. In HeLa cells Rab11 is distributed throughout the cytoplasm in a punctuate pattern during metaphase and anaphase. At telophase it is distributed strongly at the cleavage furrow (Fielding et al., 2005; Wilson et al., 2005).

In *Drosophila* spermatocytes, at the start of the first meiotic division, following the disassembly of the Golgi at meiosis entry, Rab11 is located on the ER and it stays localised with this compartment throughout the meiotic division. Rab11 is also localised to the cleavage furrow of these cells, as in mouse oocytes and HeLa cells (Giansanti et al., 2007). We found no evidence for Rab11 localising to ER in mouse oocytes, as the pattern of distribution of the ER is different to that known for ER in mouse oocytes (Fitzharris et al., 2007).

In oocytes the vesicular nature of the staining was evident throughout much of MI and MII, with more diffuse staining appearing in MII-arrested oocytes. The vesicles showed dramatic reorganization during meiosis I; an initial aggregation around the forming MI spindle followed by localization to the shoulders of the Pb1, spindle midzone. The Pb1 shoulders are analogous to the cytokinetic furrow in other cell types. As cytokinesis progresses and the shoulders constrict, this brings the rab11 into concentrated mass at the point of polar body extrusion. It is not clear from our studies whether the Rab11 staining in the shoulders remains vesicular or whether the vesicles may be incorporated into the plasma membrane. Similarly, although it appears in many images that Rab11 positive vesicles are accumulated at the spindle-midzone, further high resolution studies are necessary to understand the nature of the Rab11 localisation in these regions.

The mechanism determining the movement and localization of Rab11 during meiosis is in part explained by a microtubule-dependent localisation to the developing spindle. Interestingly, the MI spindle appears to act as a major focal point for organising the cytoplasm. As the spindle forms it becomes enveloped in a sheath of ER and mitochondria (FitzHarris et al., 2007; Van Blerkom and Runner 1984). Studies presented in this thesis add the Rab11-positive recycling endosome compartment as another spindle-associated organelle. Furthermore, the mechanism of aggregation of all of these organelles is driven by

microtubules. The spindle-localization in all cases, including recycling endosomes, requires microtubules and in the case of ER and mitochondria, dynein is the microtubule motor that drives the movement to the spindle region.

4.3.2 Rab11 is required for the first meiotic division

In order to investigate the role of Rab11 during oocyte maturation we used a morpholino oligonucleotide designed to deplete endogenous Rab11 mRNA. This had no effect on the formation of Pb1. In fact, the rate of Pb1 extrusion was the same in the oocytes microinjected with Rab11 MO compared to the Control MO oocytes. Furthermore, immunofluorescence experiments confirmed that the protein was not depleted as the fluorescence intensity was the same. This result shows that the protein is very stable in mouse oocytes and therefore this was not the best approach to use. In fact, after the microinjection GV oocytes were left in IBMX for 24 hours to deplete endogenous Rab11 but in our case we needed longer periods of incubation which was not possible due to the low survival rate of the oocytes. For example, this approach works very well with proteins involved in the regulation of the cell cycle in mouse oocytes (e.g. cyclin B) as the turnover rate of these proteins is very fast and therefore 24 hours are enough to deplete them.

Considering the stability of Rab11 we had to try a more efficient approach and therefore we used a dominant negative mutant Rab11 mRNA (S25N) which was also microinjected into GV stage oocytes. It has been shown that this mutant inhibits the transport of transferrin to the recycling endosomes in BHK cells (Ullrich et al., 1996). Also, in HeLa cells it was used to study the function of Rab11 during mammalian cytokinesis and it caused an increase in the number of binucleate cells (Fielding et al., 2005; Wilson et al., 2005). Our experiments show that this mutant causes the oocytes to arrest in metaphase I and therefore they fail to extrude

the Pb1. Most of the oocytes arrested in metaphase did not have a normal spindle and had a slight or severe misalignment of chromosomes. This means that the spindle remained in the middle of the oocyte and could not migrate to the cortex and as a consequence no actin cap formed leading to a failure in Pb1 extrusion.

This result is different to that found in *Drosophila* spermatocytes. In fact, in these cells the actin ring formed and the spermatocytes tried to divide but abscission, the last step of cytokinesis, did not take place because the actin ring failed to constrict and looked discontinuous compared to controls (Giansanti et al. 2007). This is not surprising as spermatogenesis is a mechanism very different from oogenesis. The first crucial difference is the symmetry of division, which is highly asymmetric in oogenesis and symmetric in spermatogenesis. In fact, during MI in oogenesis the spindle migrates to the cortex, the microvilli disassemble and filaments start to accumulate under the plasma membrane forming the actin cap (Long and Chen 1984; Tremoleda et al., 2001). This could contribute to the formation of a small polar body. But in spermatogenesis the division is symmetric and therefore the spindle does not migrate but it elongates remaining in the centre of the cell (Glotzer 2005). Therefore, Rab11 may play different roles in oogenesis compared to spermatogenesis. In mouse oocytes Rab11 may be involved in the regulation of spindle migration, whereas in *Drosophila* spermatocyte it is involved in the regulation of the constriction of the actomyosin ring and in abscission, the late stage of cytokinesis (Giansanti et al., 2007). In conclusion, in spermatocytes it regulates mechanisms involved in the late stages of development, whereas in oogenesis it is involved in earlier stages of development (e.g. spindle migration).

4.3.3 Rab11 is not required for the second meiotic division

After studying the role of Rab11 during the first meiotic division, we wanted to investigate its role during the second meiotic division. MII oocytes were microinjected with a dominant negative Rab11 mRNA and after four hours activated with ethanol to examine the rate of Pb2 extrusion. Compared to control oocytes, the rate of Pb2 extrusion was normal. The spindle and the actin ring were analysed and showed no abnormality compared to control oocytes. This result suggests that Rab11 plays a role only during the first meiotic division where it may be necessary for the delivery of recycling endosomes to the cleavage furrow. In fact, at MI it localises to the spindle midzone and may contribute to the addition of new membrane at the cleavage furrow needed for cell division.

The lack of any effect of dominant-negative Rab11 at Pb2 extrusion is consistent with differences in localization seen during cytokinesis at MI and MII. At MII, we found no evidence of Rab11 localising to the shoulders of Pb2, the spindle mid-zone or the cleavage furrow. The lack of specific localization of Rab11 is consistent with the finding that dominant-negative Rab11 failed to suppress Pb2 formation, while dramatically inhibiting Pb1 formation. This finding indicates distinct differences in regulation of the two meiotic divisions. A major difference in MI and MII is that the spindle becomes surrounded by Rab11 vesicles in MI and at MII there was no evidence for a spindle-accumulation. It may be that Rab11 has already primed the cortex during MI in order to allow cortical dynamics at MII that are necessary for cytokinesis. Alternatively, it remains possible that cytokinesis itself is different in MI and MII. Pb1 is known to degenerate within a few hour of emission while Pb2 remains, in many cases through entire pre-implantation development. It has been suggested that Pb2 remains attached to the oocyte via the mid-body and it may be that abscission is much delayed compared to MI, but no direct studies have been undertaken to test this possibility (Zernicka-Goetz et al., 2009). These differences in behaviours of the Polar bodies

may underlie events of cytokinesis that are different between MI and MII. The difference in distribution and dynamics of Rab11 provide a molecular difference between the two divisions and further work is necessary to determine whether there is a functional relationship.

5. The mechanisms regulating Rab11 in mouse oocytes

5.1 Introduction

In the previous chapter we have shown that disrupting Rab11 leads to a failure in polar body extrusion in mouse oocytes. A similar phenotype in cytokinesis failure is observed in *Drosophila* spermatocytes (Giansanti et al., 2007). The mechanism of action of Rab11 and how it is regulated in order to play a role in cytokinesis is not yet understood, particularly in mammalian cells. Rab11 is an endocytic regulator and is essential for cytokinesis in various cell types and organisms by regulating various membrane trafficking events (Prekeris and Gould, 2008).

Cytokinesis in animal cells requires the addition of new membrane at the cleavage furrow. At telophase their surface area enlarges around 25% and this can occur by the storage of membrane in microvilli or other compartments or by the trafficking of new membrane through the secretory or endocytic pathways. The secretory pathway involves the transport of vesicles from the Endoplasmic Reticulum to the Golgi apparatus and then to the plasma membrane. The endocytic pathway involves the formation of vesicles derived from the plasma membrane which migrate to the early endosome and the recycling endosome, which takes them back to the plasma membrane or to lysosome for degradation (Strickland and Burgess 2004; Albertson et al., 2005).

It has been shown that Rab11 regulates the transport of transferrin to the recycling endosomes in BHK cells (Ullrich et al., 1996). The precise role of Rab11 is not clear, but it is known to be involved in several signalling pathways that regulate membrane traffic and cytokinesis.

Rab11 is known to interact with other proteins involved in trafficking and cytokinesis such as PI4KIIIβ, Arf6, Cdc42 and separase, which I am going to explore in the following chapter.

Rab11 and PI4KIIIβ

Vesicle fusion at the cleavage furrow of cells undergoing cytokinesis may also be regulated by the lipid composition of the cleavage furrow membrane. In fact, it has been shown that in different species phosphatidylinositol 4,5 bisphosphate PtdIns(4,5)P_2 is enriched at the furrow membrane and is necessary for cytokinesis together with the kinases which synthesise its production. Phosphatidylinositol (PtdIns) molecules get phosphorylated by different kinases such as PtdIns-4-kinases and (PI4K) and PtdIns(4)P-5Kinases to produce PtdIns(4,5)P_2 (Emoto et al., 2005; Field et al., 2005). It has been shown that the class I PITP (Gio), Rab11 and PI4KIIIβ (*fwd*) are required for cytokinesis in *Drosophila* spermatocytes (Brill et al., 2000; Giansanti et al., 2006, 2007). The *Drosophila* gene Four wheel drive (Fwd) is the homolog of the mammalian PI4KIIIβ (Brill et al., 2000). It has been shown that this protein is a regulator of Rab11 during cytokinesis in *Drosophila* spermatocytes. Furthermore, it is necessary for the synthesis of PI 4-phosphate (PI4P) on the Golgi membranes and for the production of secretory organelles containing PI4P which localise to the midzone. Fwd binds to Rab11 and colocalises with this protein on the Golgi membranes. It is also necessary for the localisation of Rab11 in *Drosophila* spermatocytes undergoing cell division (Polevoy et al., 2009). In *Drosophila* spermatocytes Rab11, PI4KIIIβ and PITP function in the same cytokinetic pathway, possibly with PITP found upstream, followed by PI4KIIIβ and Rab11. For this reason we wanted to investigate the relationship between Rab11, PITPβ and PI4KIIIβ in mouse oocytes.

In mammals there are four PI4K enzymes, which are divided into two groups. Type III PI4K enzymes are comprised of PI4KIIIα and PI4KIIIβ, they are conserved from yeast to men and

are relatives of PI3K enzymes (Audhya et al., 2000; Flanagan et al., 1993). Type II PI4K enzymes (which exist also in an α and β form) are a completely different family of kinases (Barilko et al 2001; Minogue et al., 2001). PI4KIIIβ and its yeast orthologue Pik1p, are localised to the peripheral membrane of the Golgi compartment and play a role in Golgi to plasma membrane secretion in yeast and mammalian cells (Walch-Solimena and Novick 1999; Godi et al., 1999).

Interactions between Rab11, FIP3 and Arf6

The Rab11 interacting protein called FIP3, is required for the enrichment of Arf6 at the midbody during cytokinesis in somatic cells (Schonteich et al., 2007). The ADP-ribosylation factors (Arfs) are GTP-binding proteins that function in the regulation of intracellular vesicular transport along secretory and endocytic pathways. This family comprises six members but Arf6 and Arf1 are the best characterized proteins. Arf6 has been shown to be involved in actin rearrangements at the cell periphery and in endocytosis (Chavrier and Goud, 1999). In this study we have investigated the relationship between Rab11 and Arf6 in mouse oocytes.

Rab11 and Cdc42

To investigate other possible mechanisms involved in the regulation of Rab11 in mouse oocytes we also studied the relationship between this protein and Cdc42, which has been shown to regulate the asymmetric positioning of the meiotic spindle in mouse oocytes (Na and Zernicka-Goetz 2006). An initial study on the relationship between Cdc42 and Rab11 has been carried out in HeLa cells by Landry et al. (2009). Cell death was shown to be induced by the **e**arly region **4** **o**pen **r**eading **f**rame **4** (E4orf4) of human adenoviruses partly by regulated changes in actin dynamics. The organization of the endocytic recycling compartment was disturbed by Cdc42 and actin during the early stages of the E4orf4 protein

expression. In addition, during these early stages of expression, the transport of recycling endosomes to the Golgi compartment was triggered, whilst recycling of protein cargos to the plasma membrane was inhibited. These changes in Golgi membrane dynamics were also dependent on Rab11a which caused the scattering of Golgi membranes leading to cell death. In response to staurosporine, a similar phenotype occurs in the mobilization of recycling endosomes traffic transported by Cdc42 and Rab11a, which also caused fragmentation of the Golgi compartment and contributed to cell death progression. Cdc42 and Rab11a were both depleted by siRNA in HeLa and MCF7 cells and this protected the cells from the toxicity of the E4orf4 protein (Landry et al., 2009). This link raises the possibility that Cdc42 may interact with Rab11.

The Rab11-separase connection

Cytokinesis is also characterised by a correct segregation of chromosomes and in *C.elegans* embryos Rab11 has been found to interact with separase, a protein known to promote sister chromosome separation (Bembenek et al., 2009). Separase is a protease which opens the cohesin ring at the onset of anaphase causing its dissociation from chromosomes (Uhlmann et al., 1999). During cytokinesis, separase localises to the furrow and midbody of *C.elegans* embryos. Depletion of separase during the early mitotic divisions causes a failure in cytokinesis and an accumulation of Rab11 positive-vesicles at the cleavage furrow and midbody which is not due to chromosome non-disjunction. Therefore, separase is necessary for cytokinesis and regulates the integration of Rab11 positive-vesicles in the plasma membrane at the cleavage furrow and midbody of *C.elegans* embryos (Bembenek et al., 2009).

In embryonic fibroblasts depletion of separase prevents sister chromatid separation but does not have any effect on other aspects of mitosis, chromosome replication or cytokinesis (Wirth

et al., 2006). Mouse oocytes that lack separase fail to segregate chromosomes and to extrude polar bodies and therefore remain permanently in meiosis I (Kudo et al., 2006). In this chapter we have also investigated the relationship between separase and Rab11 and whether Rab11 distribution is disrupted in separase knockout mouse oocytes.

5.2 Results

5.2.1 Localisation of PI4KIIIβ during meiosis of mouse oocytes

To examine the distribution of PI4KIIIβ during maturation in mouse oocytes we used an anti-rabbit polyclonal antibody against PI4KIIIβ which has been well characterised in other cell types (Toth et al., 2006). Control oocytes were incubated without the primary antibody and no labelling was present, confirming the absence of non specific binding of the secondary antibody.

Prophase arrested GV stage mouse oocytes show PI4KIIIβ distributed throughout the cytoplasm and around the nucleus (Figure 5.1Aii). In prometaphase, after GVBD, the protein remains distributed throughout the cytoplasm with a stronger localisation around the developing spindle (Figure 5.1B). At metaphase I (five hours after GVBD) the spindle is formed and the chromosomes are aligning on the metaphase plate, PI4KIIIβ distributes around the first meiotic spindle and in the cytoplasm in a vesicular pattern (Figure 5.1C). At telophase the spindle has migrated to the cortex of the oocyte, the homologous chromosomes have separated and the first polar body is being extruded; at this stage PI4KIIIβ remains distributed around the back of the meiotic spindle but does not seem to migrate to the Pb1 (Figure 5.1D). In MII arrested oocytes, the protein is present throughout the cytoplasm and around the second meiotic spindle (Figure 5.1E).

5.2.2 PIK93 does not block polar body one extrusion

To investigate the role of PI4KIIIβ in mouse oocytes and determine its relationship with Rab11, we treated oocytes with PIK93, a PI3-kinase inhibitor which also specifically inhibits type III PI4Kβ (Toth et al 2006, Balla et al 2008). In COS-7 cells PIK93 has been used to

inhibit type III PI4Kβ successfully showing that this protein is an essential enzyme in the control of sphingomyelin synthesis by regulating the flow of ceramide from the ER to the Golgi compartment (Toth et al., 2006). The protein was inhibited by 90% in COS-7 cells after 5 min of treatment at a concentration of 250nM PIK93. Treatment with PIK93 for 10 min caused the loss of Golgi localisation of the ceramide transfer protein (CERT)-PH domain (Toth et al., 2006). The ceramide transfer protein (CERT) is responsible for the quantity of ceramide transport from the endoplasmic reticulum to the Golgi. CERT has a C-terminal START domain for the binding of ceramide and an N-terminal pleck-strin homology domain that binds phosphatidylinositol 4-phosphate indicating that phosphatidylinositol (PI) 4-kinases are involved in the regulation of CERT-mediated ceramide transport.

In mouse oocytes, since we did not know the concentration necessary for a potential effect, we carried out experiments using a range of concentrations of PIK93 from 250nM up to 10μM and found no difference in the localisation of PI4KIIIβ between the treated oocytes and the controls. The oocytes were left in PIK93 overnight and fixed the day after at 15-16 hours after GVBD and labelled with anti-PI4KIIIβ and Hoechst (Figures 5.2Ai-5.2Aii). The localisation of PI4KIIIβ did not change in the treated oocytes and remained around the meiotic spindle as in the control group. In addition, there was no difference in the rate of polar body extrusion between the two groups (Figure 5.2B).

5.2.3 Cdc42 dominant negative mutant does not change Rab11 localisation at the midzone

Previous experiments in HeLa cells have demonstrated that there is a link between Rab11 and Cdc42 (Landry et al., 2009). Unpublished work (Halet et al.,) has shown that in mouse oocytes Cdc42 localises to the spindle mid-zone around 7-8 hours after GVBD, similar to

Rab11. This raises the possibility that Cdc42 is important for localising Rab11 positive vesicles in mouse oocytes. Microinjection of Cdc42 dominant negative mutant mRNA (N17S) in oocytes at the GV stage prevents the accumulation of Cdc42 at the spindle mid-zone but does not change Rab11 localisation at the mid-zone compared to controls (Figures 5.3A-5.3B). The Rab11 positive vesicles are still present at the mid-zone in oocytes microinjected with Cdc42 N17S mRNA (Figure 5.3Bii). In addition, only half of the oocytes microinjected with the dominant negative mutant of Cdc42 extruded the Pb1 (Na and Zernicka-Goetz 2006). Although we have not shown Cdc42 localisation in the same experiment, this is very reliable and as expected, we got the same percentage of polar body extrusion (50%) as in previous experiments where the same mutant was used (Halet et al., unpublished data).

5.2.4 Rab11 inhibition does not change Arf6 localisation

It has been shown in HeLa cells that Rab11 and its interacting proteins FIP3 and FIP4 interact with Arf6 and the exocyst complex to regulate membrane traffic in cytokinesis (Fielding et al., 2005). In these cells Arf6 is localised to the plasma membrane and intracellular vesicles during interphase and at the furrow and midbody during telophase. Furthermore, the binding of Arf6 to Rab11/FIP3 regulates the delivery of recycling endosomes during cytokinesis in HeLa cells (Schonteich et al., 2007). When we microinject GV oocytes with a dominant negative mutant form of Rab11 (S25N) most of the oocytes block at metaphase I (see chapter 4) and Arf6 localisation does not change and remains the same as in control oocytes (Figure 5.4A). At this stage Arf6 is present in a vesicular pattern throughout the oocyte and on the plasma membrane. In HeLa cells this distribution is observed only during interphase, whereas

at metaphase Arf6 is distributed continuously throughout the cells and is less vesicular (Fielding et al., 2005).

5.2.5 Separase does not change Rab11 localisation.

In *C.elegans* embryos depletion of separase causes a failure in early mitotic divisions and an accumulation of Rab11 vesicles at the furrow and midbody (Bembenek et al., 2009). The knockout of separase in mouse oocytes causes a failure in cytokinesis. Half of the oocytes start to form a polar body but then they retract (Kudo et al., 2006).

In our experiment knock out of separase in mouse oocytes does not cause a significant change in Rab11 localisation (Figures 5.5A-5.5B). In control oocytes which have extruded the Pb1, Rab11 localises to the cleavage furrow (Figure 5.5Ai) but it is not possible to see if the same occurs in separase knockout oocytes. Separase knock out oocytes do not extrude polar bodies or undergo metaphase to anaphase transition but they still get Rab11 localising at the back of the spindle. This suggests that the cells are trying to reorganize Rab11 at cytokinesis but it is different to *C.elegans* in that cleavage furrow does not apparently form in oocytes.

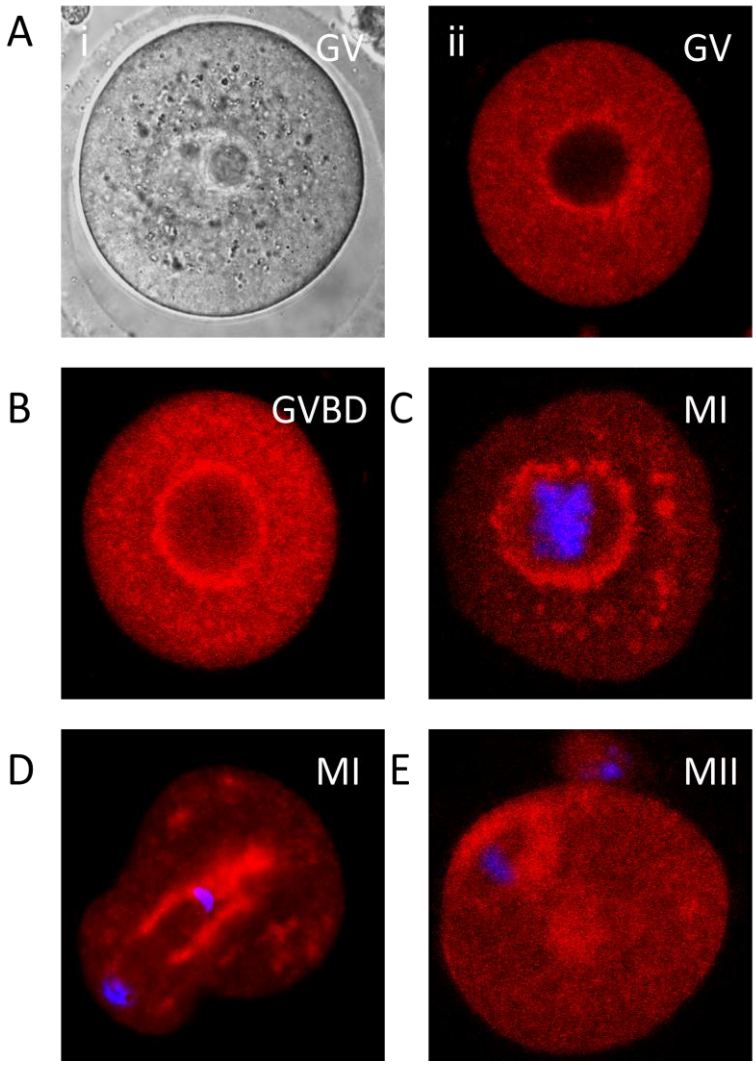
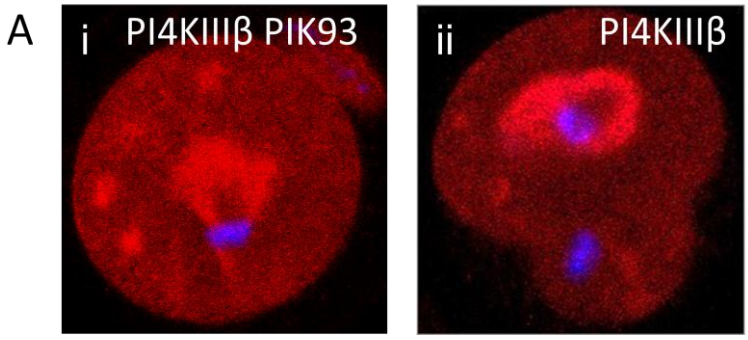


Figure 5.1. The localisation of PI4KIIIβ in mouse oocytes

(A-E) GV stage oocytes were released from prophase arrest and fixed for immunocytochemistry at different times during oocyte maturation and labelled with anti-PI4KIIIβ (red) and Hoechst 33342 (blue) to label the chromosomes. (Ai) Bright field of an oocyte at the GV stage. (Aii) GV oocyte (fixed during arrest) labelled with anti-PI4KIIIβ. (B) Localisation of PI4KIIIβ at the GVBD stage. (C) PI4KIIIβ at Metaphase I (5h after release). (D) PI4KIIIβ at telophase I (7h after release). (E) PI4KIIIβ at Metaphase II (16 hr after release). Each oocyte is representative of 13 oocytes per group (average).



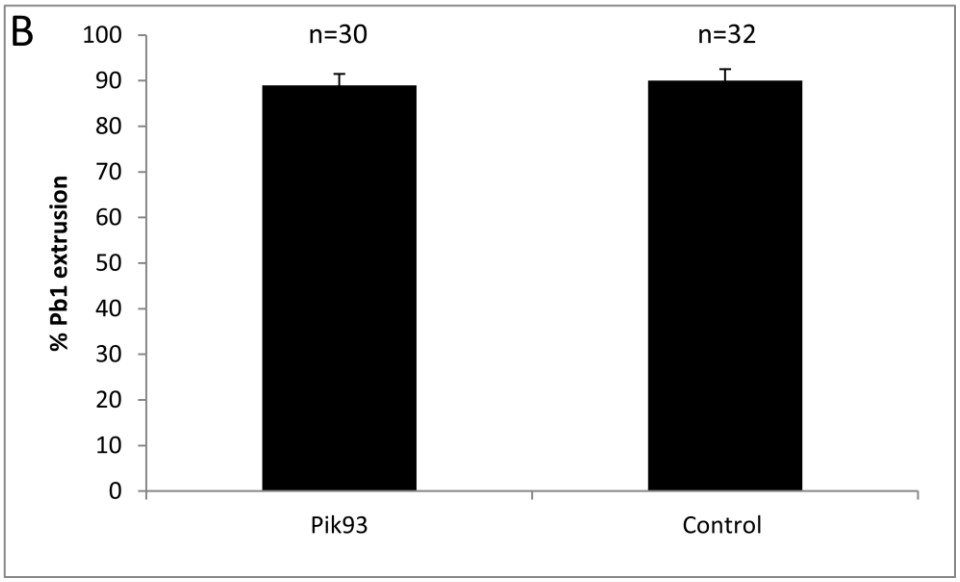


Figure 5.2. Pik93 does not block Pb1 extrusion

(A) Oocytes fixed 15 hours after GVBD. (Ai) Oocyte treated with Pik93 over night and labelled with anti-PI4KIIIβ (red) and Hoechst (blue). (Aii) Control oocyte labelled with PI4KIIIβ (red) and Hoechst (blue). The image is representative of 12 oocytes per group (average). The subtle differences are negligible and not reproducible. (B) There is no significant difference in the rate of Pb1 extrusion between the controls and the oocytes treated with Pik93 ($P>0.05$).

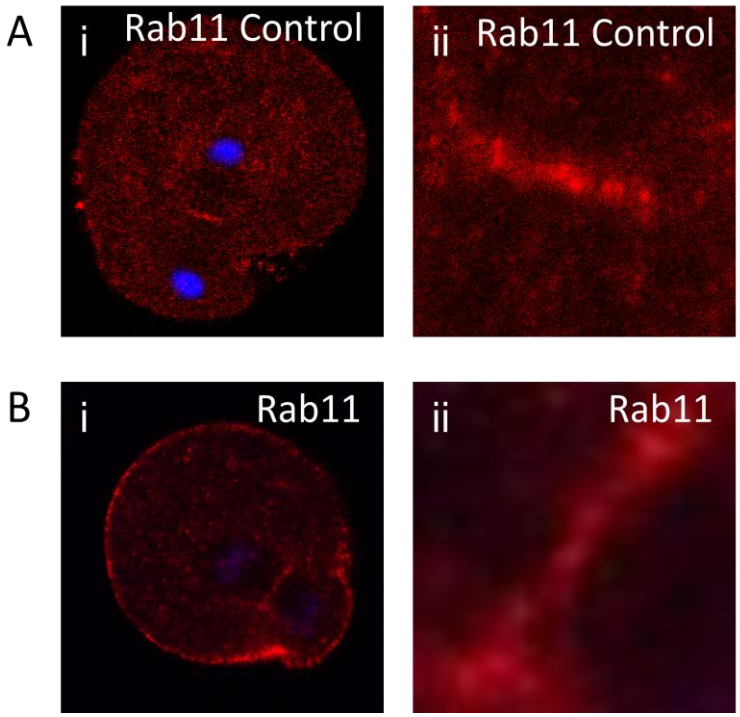


Figure 5.3. Cdc42 dominant negative mutant does not change Rab11 localisation at the midzone

(A-B) Oocytes microinjected at the GV stage, fixed 7-8 hours after GVBD and labelled with anti-Rab11 (red) and Hoechst (blue). (Ai) Control oocyte. (Aii) Higher magnification of the spindle midzone with Rab11 positive vesicles. (Bi) Oocyte microinjected with Cdc42 N17S mRNA. (Bii) Higher magnification of the spindle midzone with Rab11-positive vesicles. The image is representative of 10 oocytes per group (average).

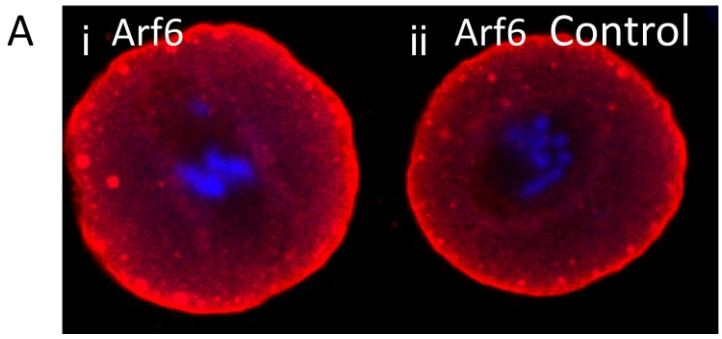


Figure 5.4. Rab11 inhibition does not change Arf6 localisation

(Ai) Oocyte microinjected with Rab11S25N mRNA at the GV stage, fixed 16hours after GVBD and labelled with anti-Arf6 (red) and Hoechst (blue). (Aii) Control oocyte fixed 16 hours after GVBD, labelled with anti-Arf6 (red) and Hoechst (blue). Each oocyte is representative of 10 oocytes per group (average) with a variable staining.

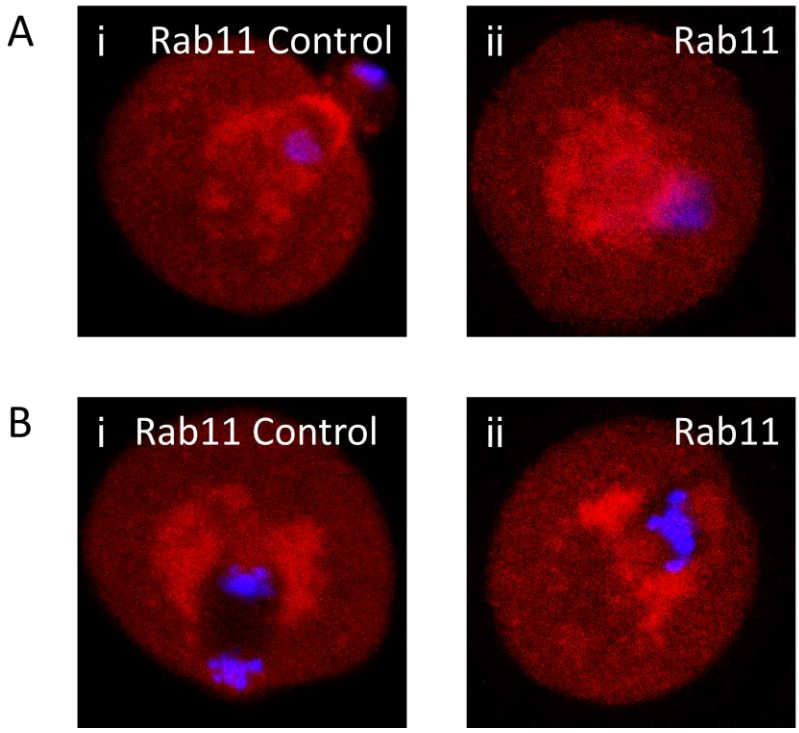


Figure 5.5. Separase does not change Rab11 localisation

(A-B) Oocytes fixed at GVBD+8 hours and labelled with anti-Rab11 and Hoechst (blue). (Ai) Control oocyte which has extruded the Pb1. (Aii) Oocyte from a KO separase mouse. (Bi) Control oocyte which is extruding the Pb1. (Bii) Oocyte from a KO separase mouse. The image is representative of 25 oocytes per group (average).

5.3 Discussion

5.3.1 The role of PI4KIIIβ in mouse oocytes

In mammalian cells, PI4KIIIβ localises to the Golgi compartment (Godi et al., 1999). In mouse oocytes its distribution throughout meiosis I and II does not seem to resemble the distribution of the Golgi (Wang et al., 2008). Therefore, the protein seems to behave in a different manner compared to somatic cells. Also, it does not seem to colocalise with Rab11 (recycling endosomes) throughout the major stages of maturation. In fact, whereas Rab11 is found in a punctuate pattern at the GV stage and GVBD stage, PI4KIIIβ localises around the nuclear envelope during these stages. Only at metaphase I these products appear to have a similar distribution around the spindle and the organelles which surround this structure at MI (e.g mitochondria, ER). At telophase I, PI4KIIIβ is found around the back of the spindle but does not migrate to the polar body. Rab11 at this stage is found on the spindle mid-zone and at the cleavage furrow later on, but this is not the case for PI4KIIIβ. At MII PI4KIIIβ seems to distribute around the second meiotic spindle.

In *Drosophila* spermatocytes Fwd, the protein encoding PI4KIIIβ, binds to Rab11 and colocalises with this protein on the Golgi membranes. PI4KIIIβ is required also for the localisation of Rab11 during cell division of these cells (Polevoy et al., 2009). In *Drosophila* PI4KIIIβ is necessary for the synthesis of PI 4-phosphate (PI4P) on Golgi membranes and for the production of secretory organelles containing PI4P which localise to the mid-zone. Rab11 colocalises with PI4P at the mid-zone and this is dependent on PI4KIIIβ suggesting that this protein recruits Rab11 to Golgi membranes, where Rab11 starts to associate with organelles containing PI4P (Polevoy et al., 2009). But in mouse oocytes this protein does not colocalise with Rab11, therefore may not play an important role in Rab11 mediated traffic during

oocyte maturation. Further studies are required in order to understand where PI4KIIIβ localises to in mouse oocytes.

In an effort to investigate the role of PI4KIIIβ in mouse oocytes we used PIK93, an inhibitor of type III PI4Kβ. Our aim was to see whether the inhibition of the protein caused the same phenotype observed in Rab11 dominant negative mutants in order to establish a relationship between the two proteins and see which one acts upstream of the other. PIK93, like all known PI3K inhibitors, forms a hydrogen bond with the backbone amide nitrogen of Val 882. In addition, it also forms a second hydrogen bond with the backbone carbonyl of Val 882 and a third one between its sulphonamide moiety and the side chain of Asp 964 (Knight et al., 2006). In COS-7 cells, PIK93 has been shown to inhibit PI4KIIIβ blocking the conversion of serine-labelled endogenous ceramide to sphingomyelin. This protein has been proved to be necessary for the synthesis of sphingomyelin by controlling the flow of ceramide from the ER to the Golgi compartment (Toth et al., 2006). In mouse oocytes we observed no effects on major events of meiotic maturation such as the rate of polar body extrusion, despite using concentrations ranging from 250nM up to 10μM. PIK93 had no apparent effect on the localisation of PI4KIIIβ even though we used concentrations up to 10μM and increased the exposure time leaving the cells over night with the drug. We were unable to test whether the drug was effective at inhibiting the PI4KIIIβ activity in mouse oocytes as this was not possible with the amount of material available. We could have used Wortmannin, a drug which can also act as an inhibitor of PI4KIIIβ, but it is known to interfere with the cell cycle and therefore would anyway arrest the oocytes in metaphase I (Downing et al., 1996). Thus, it may be that PI4KIIIβ does not affect the same events in oocytes as it does in somatic cells or that its downstream effects are not necessary for oocyte maturation. Further studies confirming that the enzymatic activity of PI4KIIIβ is inhibited would help to verify these studies. The lack of colocalisation of Rab11 and PI4KIIIβ and the lack of effect of inhibitors

would suggest that its role in Rab11 localisation and downstream effects may not be important for the maturation of oocytes. However, it would be good to confirm this hypothesis with future experiments and to analyse the localisation of Rab11 in oocytes treated with an efficient PI4KIIIβ inhibitor.

5.3.2 Rab11 and its interacting proteins

It has been shown in various model systems that Rab11 interacts with different proteins in order to regulate cytokinesis and membrane traffic in cells (Wilson et al., 2005; Fielding et al., 2005).

FIP3, one of the two Rab11 effector proteins, can bind to Rab11 and Arf6-GTPase at the same time, forming a complex at the cleavage furrow of HeLa cells (Fielding et al., 2005). It has been shown that Arf6 binds to FIP3 at a distinct site, found before the Rab11-binding motif. In HeLa cells knockdown of FIP3 by siRNA inhibits the recruitment of Arf6 to the midbody. Also, the knock down of Arf6 has an effect on the delivery of FIP3 to the cleavage furrow. The Arf6-FIP3 complex forms in recycling endosomes before they are transported to the cleavage furrow (Schonteich et al., 2007). Knowing that FIP3 is transported to the endosomes by binding to Rab11 (Wilson et al., 2005) it has been suggested that the formation of these endocytic complexes (Rab11-FIP3-Arf6) may be implicated in the targeting of recycling endosomes to the cleavage furrow.

Arf6 cycles between the plasma membrane (GTP form) and the recycling compartment (GDP form) (Macia et al., 2004). In mouse oocytes, its localisation at the plasma membrane remains the same, even when the oocytes are microinjected with a dominant negative form of Rab11. This shows that Rab11 does not seem to regulate Arf6 in this cell type. Further studies are

required to understand the interaction of this protein with Arf6 and FIP3 in mouse oocytes. It would be interesting to investigate whether Arf6 inhibition influences Rab11 and to investigate whether Rab11 inhibition disrupts FIP3 localisation.

Cdc42 is another protein which has been implicated in many aspects of membrane traffic, including endosomes. Rab11 and Cdc42 are both found at the spindle mid-zone at telophase I in mouse oocytes (Halet et al., unpublished data), suggesting a potential role for Cdc42 in localising Rab11 positive vesicles to the mid-zone. We attempted to investigate this possibility by using a Cdc42 dominant negative mutant but no disruption was seen. We believe Cdc42 was inhibited because we got the same percentage of polar body extrusion as shown in previous experiments in which they used the same mutant (Na and Zernicka-Goetz 2006). But we do not know whether Rab11 is still localised at the cleavage furrow during telophase I or if its distribution in earlier stages of meiosis changes.

Finally we tried to investigate the relationship between Rab11 and separase in mouse oocytes. In *C.elegans* embryos, depletion of separase causes an accumulation of Rab11 at the cleavage furrow and midbody and a failure in early mitotic divisions (Bembenek et al., 2009). In WT embryos, Rab11-GFP is observed in patches on the ingressing furrow and at the midbody, whereas embryos treated with RNAi separase show a stable and persistent accumulation of Rab11-GFP at the furrow and midbody. Thus, it was postulated that separase may regulate the endocytic recycling of Rab11 from the plasma membrane after vesicle fusion during cell division, which could explain why depletion of separase leads to cytokinesis failure (Bembenek et al., 2009). Mouse oocytes lacking separase fail to extrude a polar body and remain permanently in meiosis I (Kudo et al., 2006). Half of the oocytes attempt to extrude a polar body but then protrusion retracts prior to polar body formation. In order to look at the distribution of Rab11 in these oocytes it would be interesting to use a Rab11-GFP to monitor any change occurring. We utilised a Rab11 antibody on oocytes and fixed them at various

stages of maturation. From these experiments there were no obvious changes in Rab11 distribution in oocytes lacking separase compared to controls. In separase KO oocytes Rab11 was still localised around the meiotic spindle as in control oocytes. We were not able to investigate if the protein was still localised at the shoulders of the oocyte and on the mid-zone at the beginning of anaphase I or at the cleavage furrow during telophase I due to the failure in polar body extrusion or the retraction of the polar body in these oocytes. For this reason, further studies are necessary to better understand the dynamics of Rab11 during polar body formation. Separase may be important for establishing the components of the cleavage furrow such as actin and myosin II which are necessary for the completion of cytokinesis. But from these preliminary experiments it appears that there is no interaction between Rab11 and separase in mouse oocytes.

In summary, we have attempted to investigate potential mechanisms of regulation of Rab11 so as to gain insight into its function during oocyte maturation. Remarkably, during meiosis none of the established regulators appear to play a major role in localising Rab11 to the 'shoulders' of the polar body, the spindle mid-zone or the cleavage furrow. Clearly more work is needed to understand how this protein becomes localised and what role it plays in mouse oocytes.

6. Conclusions

The main topic of this work is the understanding of the mechanisms regulating asymmetric cell division in mouse oocytes. In females gametogenesis gives rise to one functional gamete, unlike males that yield four gametes. This means that the oocyte retains as much maternal resources as possible. The mechanism of achieving this is to undergo highly asymmetric cell divisions. Recent studies have implicated membrane traffic in cytokinesis. We have investigated the localisation and function of a number of proteins. To investigate these mechanisms in fixed oocytes we used specific antibodies to localise the proteins implicated and we analysed them by immunocytochemistry and imaging techniques. The two asymmetric cell divisions which occur in mouse oocytes are needed to abolish extra sets of maternal chromosomes, maintain a high supply of maternal cytoplasm for future embryo development and prepare the oocyte for the binding of the sperm and its DNA. In meiosis I homologous chromosomes segregate and recombination occurs, while sister chromatids separate during the second meiotic division (meiosis II). It is important that the segregation happens correctly as this is crucial for normal development of the embryo and the majority of the chromosomal aneuploidies in humans are caused by non-disjunctions during the first meiotic division (Hassold and Hunt 2001).

6.1 The presence of PITPβ, Rab11 and PI4KIIIβ in mouse oocytes

Our studies show that Rab11, PITPβ and PI4KIIIβ are present in mouse oocytes throughout meiosis, from the GV to the MII stage (chapters 3-4-5). Previous experiments have shown that these proteins play a role in the regulation of cytokinesis in *Drosophila* spermatocytes

(Giansanti et al., 2006; 2007) and therefore these proteins may be involved in mouse oogenesis. The distribution of Rab11 in mouse oocytes changes throughout meiosis and suggests that the protein localises to the recycling endosomes as in somatic cells. In addition, we found that Rab11 localisation depends on microtubules but not on microfilaments (chapter 4). Surprisingly, PITPβ localises to the early endosome compartment in mouse oocytes (chapter 3). This differs from the observations in somatic cells, where the protein localises to the Golgi and the ER (Morgan et al., 2006; Shadan et al., 2008). PI4KIIIβ in mouse oocytes does not seem to localise to the Golgi compartment as in *Drosophila* spermatocytes (chapter 5) (Polevoy et al., 2009). Further studies are needed in order to investigate its localisation.

6.2 PITPβ

To investigate the potential role of PITPβ in mouse oocytes we tried to knockdown the protein using a specific morpholino for PITPβ-MO but this method did not show any positive result as the protein seems to be very stable in these cells. This is not surprising considering that in somatic cells the knockdown of the protein was effective only after 72hrs (Carvou et al., 2010). It is also known that PITPβ deficiency in murine embryonic stem cells leads to an early failure in embryonic development (Alb et al., 2002). Moreover, the single class I PITP was shown to be required for cytokinesis in *Drosophila* spermatocytes (Giansanti et al., 2006). Giotto, a gene that encodes the class I PITP, is necessary for mitotic and meiotic cytokinesis in Neuroblasts and spermatocytes. These cells form normal actomyosin rings, but these rings fail to constrict to completion leading to a failure in cytokinesis.

Here, we used an alternative approach to investigate the role of PITPβ in mouse oocytes, which was to over-express the protein. As described in chapter 3, over expression of PITPβ causes an abnormal accumulation of PITPβ-positive vesicles in the oocyte. These vesicles

were identified to be early endosomes. In contrast, in somatic cells the protein localises to the ER and the Golgi compartment. It is not clear why this protein localises to a different compartment in mouse oocytes compared to that in somatic cells, however this difference probably reflects the highly specialised mechanisms involved in oogenesis. As seen in chapter 3, PITPβ may be involved in membrane trafficking in mouse oocytes but this does not affect the rate of polar body extrusion and therefore the regulation of cytokinesis. Unfortunately little is known about membrane trafficking in mouse oocytes.

The early endosome compartment (EE) is the first endocytic compartment to receive incoming cargo derived from the plasma membrane (Gruenberg and Howell 1989). Phospholipids play an significant role in membrane trafficking pathways. The EE compartment is enriched in PtdIns(3)P (Gillooly et al., 2000). One of the proteins that is recruited at the EE by PtdIns(3)P is PIKFyve, an enzyme which synthesizes PtdIns(3,5)P_2 from PtdIns(3)P in mammalian cells (Cabezas et al., 2006). This enzyme is sensitive to Wortmannin treatment and is recruited to the EE via its FYVE domain (Sbrissa et al., 2002). Knockdown of this protein induces an enlargement of the EE compartment and cytoplasmic vacuolation (Rutherford et al., 2006). This may be caused by a lack of PtdIns(3,5)P_2 synthesis which causes endosomal fission and an increase in PtdIns(3)P which leads to EE fusion (Shisheva 2008). The same phenotype is caused when PITPβ is over-expressed in mouse oocytes (chapter 3). The over-expression of PITPβ causes an accumulation of PITPβ-positive vesicles that colocalise with the EE compartment. The EE vesicles are already present in the control oocytes but when PITPβ is over-expressed these vesicles enlarge. This may be caused by the same mechanism described previously. In addition, PITPβ has been shown to stimulate the activity of PI3K (Panaretou et al., 1997) and therefore we raise the possibility that PITPβ may regulate the production of PtdIns3P on the early endosomes.

6.3 Rab11

It appears that Rab11 in mouse oocytes is also localised to the recycling compartment as found in other cell types. It would have been useful to use as a control another specific antibody known to localise to this compartment, unfortunately this was not possible. As a first approach to investigate the role of Rab11 in mouse oocytes we have used a specific Rab11-MO to knockdown the protein but the result was negative probably due to the high stability of this protein in oocytes. Since Rab11 accumulates at the cleavage furrow of oocytes undergoing cytokinesis it is potentially involved in this process (Chapter 4). A similar accumulation occurs in HeLa cells and *Drosophila* spermatocytes (Fielding et al., 2005; Giansanti et al., 2007). However, the most successful approach was to inject mouse oocytes with a dominant negative Rab11 mutant (mRNA). This caused chromosome misalignment and spindle disruption during the first meiotic division causing a failure in the extrusion of the Pb1 (chapter 4). The spindle remains in the centre of the oocyte and fails to migrate to the cortex preventing the formation of the actin cap and as a consequence the polarization of the oocyte which is necessary for the extrusion of the polar body. The meiotic spindle and the chromatin are known to provide the information to polarize the oocyte (Brunet and Maro 2005). As the spindle reaches the cortex cortical polarity is established. The region overlying the spindle starts to accumulate actin, myosin and other proteins like Rho, Rac and Cdc42, which are essential for polarity in various systems (Brunet and Maro 2005; Sun and Schatten 2006). At anaphase I the chromosomes have divided into two sets; the set found toward the cortex gets cleaved from the oocyte together with the actin cap by the constriction of the myosin ring. This leads to the formation of the Pb1.

In HeLa cells Rab11 mediates the transport of the recycling endosomes to the cleavage furrow during late telophase (Wilson et al., 2005). This result indicates that recycling

endosomes are important for cytokinesis in HeLa cells and are required for the completion of abscission. It is also known that cytokinesis requires the addition of new membrane at the cleavage furrow in order for the cell to divide (Glotzer 2005). Therefore Rab11 may be required for the delivery of vesicles (recycling endosomes) at the cleavage furrow of mouse oocytes undergoing cell division. Vesicles which derive from recycling endosomes have been identified by the existence of Rab11/FIP3 and Rab11/FIP4 protein complexes migrating along microtubules via a motor protein to reach the furrow and the midbody during cytokinesis. The recruitment of these proteins to the furrow is controlled by active Arf6 and by the binding of FIP3/4 to Arf6-GTP, possibly together with Rab11 (Fielding et al., 2005). We looked at the distribution of Arf6 in Rab11 mutants in mouse oocytes but further work is needed to determine if there is an obvious interplay between these two proteins. The recruitment of the proteins to the furrow happens when Rab11 is found in the active form (Rab11-GTP); Rab11-GTP recruits FIP3 to vesicles originated from the recycling endosomes in the area of the centrosomes. When the Rab11-GEF is not activated, Rab11 remains in the closed conformation (Rab11-GDP) and therefore cannot recruit FIP3 to these vesicles. When we inject mouse oocytes with a dominant negative Rab11 the oocytes remain arrested at metaphase I and Rab11 accumulates at the back of the spindle (chapter 4). The vesicles fail to migrate along microtubules and cannot get delivered to the cleavage furrow where they are required for cytokinesis. The metaphase arrest may be caused by a failure of the activation of the spindle assembly checkpoint. In fact, this block after spindle migration resembles the metaphase I arrest which has been identified in oocytes that fail to satisfy the spindle assembly checkpoint. This suggests that the block may be due to the misalignment of chromosomes and to a possible defect in the assembly of kinetochore fibers (Brunet et al., 1999; Wassmann et al., 2003; Homer et al., 2005). There is no evidence in the literature showing that mutation of Rab11 causes an arrest of the cells in metaphase. In fact, in other

systems the cells attempt to undergo cytokinesis but then they fail to divide properly. Also, the inhibition of spindle formation together with chromosome misalignment does not occur in all the oocytes and this may be the result of different levels of Rab11 inhibition throughout precise stages of spindle formation. A similar phenotype has been observed in mouse oocytes after inhibition of Cdc42 (Na and Zernicka-Goetz 2006). These oocytes were blocked in metaphase I but in addition they showed an elongated spindle. This Rho GTPase protein has been found to regulate microtubule attachment to kinetochores in somatic cells (Yasuda et al., 2004). Hence, Rab11 and Cdc42 may belong to the same signalling cascade which regulates spindle stability through the regulation of microtubule-kinetochore attachment. Furthermore, a study done in HeLa cells showed that Cdc42 perturbed the organization of the recycling endosomes compartment and favoured the transport of recycling endosomes to the Golgi compartment whilst inhibiting the recycling of protein cargos to the plasma membrane (Landry et al., 2009). For this reason we started to investigate the relationship between Rab11 and Cdc42 in mouse oocytes but found no obvious change in the distribution of Rab11 in oocytes injected with a dominant negative Cdc42 mutant (chapter 5).

The majority of the oocytes injected with a Rab11 dominant negative mutant, which arrested at metaphase I, migrated to the oocyte cortex and failed to extrude a polar body but still formed an actin cap showing that Rab11 may contribute to the regulation of pre-existing actin filaments to stimulate polar body extrusion. In fact, the block in metaphase I may be due to a failure in the polymerization or reorganization of a subset of actin filaments which cause a failure in spindle migration. In mouse oocytes spindle migration depends on actin filaments. Oocytes treated with cytochalasin D, an agent which inhibits actin filament polymerization, have chromosomes which remain located in the centre of the oocytes, whereas oocytes treated with nocodazole, a drug which depolymerises microtubules, have chromosomes still able to migrate to the cortex (Longo and Chen 1985; Verlhac et al., 2000). It is also known

that kinases are involved in the control of spindle migration and asymmetry. Two kinases have been identified until now which regulate spindle migration in meiosis I: the Mos/MAPK (mitogen-activated preotein kinase) pathway and the Pyk2 (proline-rich tyrosine kinase-2) pathway (Azoury et al., 2009). The control of spindle migration is dependent on microfilaments, therefore targets of the Mos/MAPK pathway which should control the microfilament cytoskeleton still need to be discovered. A possible target may be the protein formin-2; in yeast and somatic cells it has been discovered a connection between formins and MAPK already (Matheos et al., 2004).

Our results show that Rab11 is required for the first asymmetric division of mouse oocytes by controlling the formation of the spindle and the alignment of chromosomes (chapter 4). Polarity is an essential mechanism regulating asymmetric cell division in mouse oocytes. It is known that disruption of the follicular architecture in vivo may disturb the asymmetry of the oocyte and lead to abnormalities in embryos (Sanfins et al., 2003). It has been shown that the animal pole of the mammalian oocyte involves the anchoring of the GV via an MTOC to make sure that the asymmetry remains during the extrusion of the polar body (Sanfins et al., 2003). In mouse oocytes the meiotic spindle and chromatin provide the information required to polarise the oocyte (Brunet and Maro 2005; Sun and Schatten 2006). The position of the spindle determines the location of the contractile ring, which divides the cytoplasm. The orientation of the spindle needs to be accurate relative to the cell as this is required for the asymmetric division. How does the spindle move to the right location? It has been shown that pulling forces act on the two spindle poles and another force acts on the posterior pole leading to a displacement of the spindle in *C.elegans* embryo (Grill et al., 2001). These pulling forces need dynamic astral microtubules and cytoplasmic dynein, a motor protein regulated by cortical polarity factors (Gonczy 2008). In mouse oocytes meiotic spindles lack centrosomes and have less astral microtubules compared to other model systems, suggesting

that there may be a diverse mechanism responsible for the position of the spindle (Longo and Chen 1985). Recently it has been shown that actin and the actin polymerizing factor formin2 (Fmn2) are necessary for the position of the spindle during meiosis of mammalian oocytes. In oocytes a cloud of F-actin surrounds the spindle; the breaking of the symmetry causes an asymmetric cloud of F-actin which pushes the spindle towards the cortex. At the same time myosin, which is found at the spindle poles, pulls on actin filaments (Schuh and Ellenberg 2008). The small GTP-ase Ran is necessary for the establishment of chromatin-mediated polarity in mouse oocytes (Clarke and Zhang 2001). Cortical polarity is required for the formation of the polar body in mouse oocytes; in fact, disruption of actin, myosin, Rac, Rho and Cdc42 prevents the formation of the Pb1 (Halet and Carroll 2007; Na and Zernicka-Goetz 2006).

6.4 Meiosis I differs from meiosis II

Since we found that the injection of a Rab11 dominant negative mutant in mouse oocytes blocks the formation of the Pb1 but does not have any effect on the formation of the Pb2, (chapter 4) it appears that there are differences in regulation of MI and MII of the meiotic cell division. During MI the spindle assembly checkpoint (SAC) does not start the progression to anaphase and degradation of securin and cyclin B until all the chromosomes are aligned on the metaphase plate. Once the chromosomes are aligned, the APC binds to its activator Cdc20. This event occurs spontaneously after the alignment (Brunet et al., 1999). This mechanism does not occur in MII. At this stage the oocyte remains arrested at MII while all the chromosomes are aligned on the metaphase plate. This arrest is due to the activity of CSF, which is present only in MII oocytes and it appears around the time of Pb1 formation (Ciemerych and Kubiak 1998). Various factors establish the presence of CSF activity and the

arrest at metaphase in these oocytes. One of these is the Mos/MAPK pathway (Sagata et al., 1989). In MII oocytes the APC remains inactive until fertilization. MAPK and other proteins which regulate the activity of CSF in MII oocytes are already present during MI but they seem to have a different role here as they do not cause an arrest at metaphase after chromosome alignment, as they do in MII. One possibility could be that in MI some components of the CSF activity may not be present. The other main difference between the two stages is that the Pb2 is released after an increase in intracellular $Ca^{2+,}$ whereas the Pb1 not**.**

6.5 Advantages and limitations of immunocytochemistry

As a first approach to study the membrane trafficking proteins Rab11 and PITPβ we fixed oocytes at different stages of maturation and labelled them with specific antibodies for these proteins which have been extensively used in other model systems. This is the first study investigating the location and role of these proteins in this model system. The specificity of the antibodies for these proteins was proven by the use of western blots and by imaging control oocytes without the primary antibody. The advantage of performing immunofluorescence experiments is that they don't interfere with the maturation of the oocyte and they do not cause possible artefacts or autofluorescence problems which may be caused by the use of fluorescent probes. In fact, it would have been useful to use a Rab11 or PITPβ-GFP protein and microinject it into oocytes to monitor the distribution of these proteins in living oocytes. But this approach is not always the best as the microinjection can cause artefacts and the amount of mRNA injected needs to be monitored constantly as too much protein or too low could interfere with the maturation of the oocyte (e.g. the rate of polar body extrusion). The use of fluorescent probes is more useful when we want to monitor

the level of a protein at different stages of oocyte maturation, for example, for a protein involved in the control of cell cycle like cyclin B1. In this case, it is possible to study the events occurring and compare them to the developmental processes in which the proteins examined are involved in. One disadvantage of doing immunofluorescence experiments is that the process of fixation could cause possible artefacts. In certain experiments we performed, the use of a fluorescent probe would have been more appropriate. For example, in the study of the distribution of Rab11 in separase knock out oocytes or in Cdc42 dominant negative mutant oocytes. The limitation of using antibodies is that we can always get non-specific interactions with the primary antibody. A further control in this case would be to deplete the protein and check if the staining is abolished completely.

6.6 Future approaches

The most common way to study the role of a protein in mouse oocytes is by specific mRNA degradation provided by double-stranded RNA (dsRNA), which is named RNA interference (RNAi) (Svoboda et al., 2000; Wianny and Zernicka-Goetz 2000). In our experiments we have tried to knockdown proteins by using Morpholino antisense oligos (Gene Tools, USA). They have been proved to be non toxic and very effective in mouse oocytes (Lefebvre et al., 2002). However, the proteins we studied appear to be very stable in mouse oocytes therefore this method was not efficient.

Another useful approach to study the action of a specific protein is the use of a dominant negative mutant, as we used for the small GTPase protein Rab11 (chapter 4). A mutation is called ‘dominant’ because its phenotype is observed in the presence of the endogenous protein. The mutation inactivates the function of the endogenous protein by the sequestration of the upstream activators, in this case the Rab-GEF. This method has been proved to be very

efficient in mouse oocytes for the study of various GTPase proteins such as Cdc42 and Rac (Na and Zernicka-Goetz 2006; Halet and Carroll 2007). Future experiments are necessary to investigate the role of PITPβ in mouse oocytes by using alternative approaches; one may be the injection of a dominant negative mutant of PITPβ, but this has not been identified. Also, the involvement of the protein in membrane trafficking of mouse oocytes needs to be confirmed by the study of other proteins known to be associated and involved in this mechanism. The best approach to study the role of a protein in a specific model system is to get a knockdown of the protein but this was not possible for PITPβ or Rab11. Also, to further investigate the relationship between Rab11 and its interacting proteins (Arf6, FIP3 etc) the best approach would be the injection of a dominant negative mutant of these proteins attached to a fluorescent probe (e.g GFP) to analyse the changes occurring during oocyte maturation. The same is valid to investigate the role of PI4KIIIβ in mouse oocytes.

Finally, in order to investigate whether the oocytes injected with a Rab11 dominant negative mutant and arrested at metaphase I, attempt to undergo anaphase, or not, we need to perform a chromosome spread or do a series of live imaging experiments to see if they try to divide and then retract or they simply block at metaphase I.

Reference list

Ahringer, J. "Control of cell polarity and mitotic spindle positioning in animal cells." Curr.Opin.Cell Biol. 15.1 (2003): 73-81.

Alb,J.G.,Jr; Phillips,S.E; Rostand,K; Cui,X; Pinxteren,J; Cotlin,L; Manning,T; Guo,S; York,J.D; Sontheimer,H; Collawan,J.F; Bankaitis,V.A. "Genetic ablation of phosphatidylinositol transfer protein function in murine embryonicstem cells." Mol.Biol.Cell 13.3 (2002): 739-54.

Alberts, B; Johnson A; Lewis J; Raff M; Roberts K; Walter P. Molecular Biology of the cell (Fifth edition) (2010).

Albertson, R., B. Riggs, and W. Sullivan. "Membrane traffic: a driving force in cytokinesis." Trends Cell Biol. 15.2 (2005): 92-101.

Allen-Baume, V., B. Segui, and S. Cockcroft. "Current thoughts on the phosphatidylinositol transfer protein family." FEBS Lett. 531.1 (2002): 74-80.

Altan-Bonnet,N.; Phair,R.D.; Polishchuk,R.S.; Weigert,R.; Lippincott-Schwartz,J.
"A role for Arf1 in mitotic Golgi disassembly, chromosome segregation, and cytokinesis." Proc.Natl.Acad.Sci.U.S.A 100.23 (2003): 13314-19.

Anderson, E. and D. F. Albertini. "Gap junctions between the oocyte and companion follicle cells in the mammalian ovary." J.Cell Biol. 71.2 (1976): 680-86.

Andreassen,P.R.; Palmer,D.K.; Wener,M.H.; Margolis,R.L.
"Telophase disc: a new mammalian mitotic organelle that bisects telophase cells with a possible function in cytokinesis." J.Cell Sci. 99 (Pt 3) (1991): 523-34.

Audhya, A., M. Foti, and S. D. Emr. "Distinct roles for the yeast phosphatidylinositol 4-kinases, Stt4p and Pik1p, in secretion, cell growth, and organelle membrane dynamics." Mol.Biol.Cell 11.8 (2000): 2673-89.

Azoury,J.; Lee,K.W.; Georget,V.; Rassinier,P.; Leader,B.; Verlhac,M.H.
"Spindle positioning in mouse oocytes relies on a dynamic meshwork of actin filaments." Curr.Biol. 18.19 (2008): 1514-19.

Azoury, J., M. H. Verlhac, and J. Dumont. "Actin filaments: key players in the control of asymmetric divisions in mouse oocytes." Biol.Cell 101.2 (2009): 69-76.

Balla, A; Kim, Y.J; Varnai, P; Szentpetery, Z; Knight, Z; Shokat, K.M; Balla, T. Maintenance of Hormone-sensitive Phosphoinositide Pools in the Plasma Membrane Requires Phosphatidylinositol 4-kinase IIIα. Molecular Biology of the Cell.19, 711-721 (2008)

Barrett, S. L. and D. F. Albertini. "Cumulus cell contact during oocyte maturation in mice regulates meiotic spindle positioning and enhances developmental competence." J.Assist.Reprod.Genet. 27.1 (2010): 29-39.

Barylko,B; Gerber,S.H; Binns,D.D; Grichine,N; Khvotchev,M; Sudhof,T.C; Albanesi,J.P. A novel family of phosphatidylinositol 4-kinases conserved from yeast to humans. J.Biol.Chem. 276.11 (2001); 7705-08

Bembenek, J. N., J. G. White, and Y. Zheng. "A role for separase in the regulation of RAB-11-positive vesicles at the cleavage furrow and midbody." Curr.Biol. 20.3 (2010): 259-64.

Bernards, A. "GAPs galore! A survey of putative Ras superfamily GTPase activating proteins in man and Drosophila." Biochim.Biophys.Acta 1603.2 (2003): 47-82.

Bluemink, J. G. and S. W. de Laat. "New membrane formation during cytokinesis in normal and cytochalasin B-treated eggs of Xenopus laevis. I. Electron microscope observations." J.Cell Biol. 59.1 (1973): 89-108.

Brill,J.A.; Hime,G.R.; Scharer-Schuksz,M.; Fuller,M.T. A phospholipid kinase regulates actin organization and intercellular bridge formation during germline cytokinesis." Development 127.17 (2000): 3855-64.

Bringmann, H. and A. A. Hyman. "A cytokinesis furrow is positioned by two consecutive signals." Nature 436.7051 (2005): 731-34.

Brunet,S.; Polanski,Z.; Verlhac,M.H.; Kubiak,J.Z.; Maro,B. "Bipolar meiotic spindle formation without chromatin." Curr.Biol. 8.22 (1998): 1231-34.

Brunet,S.; Maria,A.S.; Guillaud,P.; Dujardin,D.; Kubiak,J.Z.; Maro,B. "Kinetochore fibers are not involved in the formation of the first meiotic spindle in mouse oocytes, but control the exit from the first meiotic M phase." J.Cell Biol. 146.1 (1999): 1-12.

Brunet, S. and B. Maro. "Cytoskeleton and cell cycle control during meiotic maturation of the mouse oocyte: integrating time and space." Reproduction. 130.6 (2005): 801-11.

Cabezas, A., K. Pattni, and H. Stenmark. "Cloning and subcellular localization of a human phosphatidylinositol 3-phosphate 5-kinase, PIKfyve/Fab1." Gene 371.1 (2006): 34-41.

Canman, J. C., D. B. Hoffman, and E. D. Salmon. "The role of pre- and post-anaphase microtubules in the cytokinesis phase of the cell cycle." Curr.Biol. 10.10 (2000): 611-14.

Carmena,M; Riparbelli,M.G; Minsetrini,G; Tavares,A.M; Adams,R; Callaini,G; Glover,D.M. "Drosophila polo kinase is required for cytokinesis." J.Cell Biol. 143.3 (1998): 659-71

Carneiro,G.F.; Liu,I.K.; Hyde,D.; Anderson,G.B.; Lorenzo,P.L.; Ball,B.A.
"Quantification and distribution of equine oocyte cortical granules during meiotic maturation and after activation." Mol.Reprod.Dev. 63.4 (2002): 451-58.

Carvou,N.; Holic,R.; Li,M.; Futter,C.; Skippen,A.; Cockcroft,S.
"Phosphatidylinositol- and phosphatidylcholine-transfer activity of PITPbeta is essential for COPI-mediated retrograde transport from the Golgi to the endoplasmic reticulum." J.Cell Sci. 123.Pt 8 (2010): 1262-73.

Chapman, D. L. and D. J. Wolgemuth. "Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis." Development 118.1 (1993): 229-40.

Chavrier, P. and B. Goud. "The role of ARF and Rab GTPases in membrane transport." Curr.Opin.Cell Biol. 11.4 (1999): 466-75.

Chen,W.; Feng,Y.; Chen,D.; Wandinger-Ness,A.
"Rab11 is required for trans-golgi network-to-plasma membrane transport and a preferential target for GDP dissociation inhibitor." Mol.Biol.Cell 9.11 (1998): 3241-57.

Chouinard, L. A. "A light- and electron-microscope study of the oocyte nucleus during development of the antral follicle in the prepubertal mouse." J.Cell Sci. 17.3 (1975): 589-615.

Ciemerych, M. A. and J. Z. Kubiak. "Cytostatic activity develops during meiosis I in oocytes of LT/Sv mice." Dev.Biol. 200.2 (1998): 198-211.

Clarke, P. R. and C. Zhang. "Ran GTPase: a master regulator of nuclear structure and function during the eukaryotic cell division cycle?" Trends Cell Biol. 11.9 (2001): 366-71.

Clayton, C., T. Hausler, and J. Blattner. "Protein trafficking in kinetoplastid protozoa." Microbiol.Rev. 59.3 (1995): 325-44.

Clayton, L., J. M. McConnell, and M. H. Johnson. "Control of the surface expression of uvomorulin after activation of mouse oocytes." Zygote. 3.2 (1995): 177-89.

Cockcroft, S. "Trafficking of phosphatidylinositol by phosphatidylinositol transfer proteins." Biochem.Soc.Symp.74 (2007): 259-71.

Cockcroft, S. and N. Carvou. "Biochemical and biological functions of class I phosphatidylinositol transfer proteins." Biochim.Biophys.Acta 1771.6 (2007): 677-91.

Cockcroft, S. and K. Garner. "Function of the phosphatidylinositol transfer protein gene family: is phosphatidylinositol transfer the mechanism of action?" Crit Rev.Biochem.Mol.Biol. (2011).

Colanzi, A., C. Suetterlin, and V. Malhotra. "Cell-cycle-specific Golgi fragmentation: how and why?" Curr.Opin.Cell Biol. 15.4 (2003): 462-67.

Connors,S.A.; Kanatsu-Shinohara,M.; Schultz,R.M.; Kopf,G.S.
"Involvement of the cytoskeleton in the movement of cortical granules during oocyte maturation, and cortical granule anchoring in mouse eggs." Dev.Biol. 200.1 (1998): 103-15.

Cosker, K. E., Shadan, S., van Diepen, M., Morgan, C., Li, M., Allen-Baume, V., Hobbs, C., Doherty, P., Cockcroft, S. and Eickholt, B. J. (2008). Regulation of PI3K signalling by the phosphatidylinositol transfer protein PITP{alpha} during axonal extension in hippocampal neurons. *J. Cell Sci.* 121, 796-803.

Cowan, C. R. and A. A. Hyman. "Asymmetric cell division in C. elegans: cortical polarity and spindle positioning." Annu.Rev.Cell Dev.Biol. 20 (2004): 427-53.

Cox,D.; Lee,D.J.; Dale,B.M.; Calafat,J.; Greenberg,S.
"A Rab11-containing rapidly recycling compartment in macrophages that promotes phagocytosis." Proc.Natl.Acad.Sci.U.S.A 97.2 (2000): 680-85.

Danilchick, M., H. B. Peng, and B. K. Kay. "Xenopus laevis: Practical uses in cell and molecular biology. Pictorial collage of embryonic stages." Methods Cell Biol. 36 (1991): 679-81.

De Vries,K.J; Heinrichs,A.A; Cunningham,E; Brunink,F; Westerman,J; Somerharju,P.J; Cockcroft,S; Wirtz,K.W; Snoek,G.T. "An isoform of the phosphatidylinositol-transfer protein transfers sphingomyelin and is associated with the Golgi system." Biochem.J. 310 (Pt 2) (1995): 643-49.

de,Graaf P; Zwart,W.T; van Dijken,R.A; Deneka,M; Schulz,T.K; Geijsen,N; Coffer,P.J; Gadella,B.M; Verkleij,A.J; van der Sluijs,P; van Bergen en Henegouwen PM. "Phosphatidylinositol 4-kinasebeta is critical for functional association of rab11 with the Golgi complex." Mol.Biol.Cell 15.4 (2004): 2038-47.

De,Lozanne A.; Berlot,C.H.; Leinwand,L.A.; Spudich,J.A.
"Expression in Escherichia coli of a functional Dictyostelium myosin tail fragment." J.Cell Biol. 105.6 Pt 2 (1987): 2999-3005.

Dechant, R. and M. Glotzer. "Centrosome separation and central spindle assembly act in redundant pathways that regulate microtubule density and trigger cleavage furrow formation." Dev.Cell 4.3 (2003): 333-44.

Deng,M.; Suraneni,P.; Schultz,R.M.; Li,R.
"The Ran GTPase mediates chromatin signaling to control cortical polarity during polar body extrusion in mouse oocytes." Dev.Cell 12.2 (2007): 301-08.

Denis-Donini, S., B. Baccetti, and A. Monroy. "Morphological changes of the surface of the eggs of Xenopus laevis in the course of development. 2. Cytokinesis and early cleavage." J.Ultrastruct.Res. 57.1 (1976): 104-12.

Dickeson,S.K.; Lim,C.N.; Schuyler,G.T.; Dalton,T.P.; Helmkamp,G.M.,Jr.; Yarbrough,L.R.
"Isolation and sequence of cDNA clones encoding rat phosphatidylinositol transfer protein." J.Biol.Chem. 264.28 (1989): 16557-64.

Donaldson, J. G., D. Finazzi, and R. D. Klausner. "Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein." Nature 360.6402 (1992): 350-52.

Downing,G.J.; Kim,S.; Nakanishi,S.; Catt,K.J.; Balla,T.
"Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases." Biochemistry 35.11 (1996): 3587-94.

Drechsel,D.N.; Hyman,A.A.; Hall,A.; Glotzer,M.
"A requirement for Rho and Cdc42 during cytokinesis in Xenopus embryos." Curr.Biol. 7.1 (1997): 12-23.

Ducibella,T.; Anderson,E.; Albertini,D.F.; Aalberg,J.; Rangarajan,S.
"Quantitative studies of changes in cortical granule number and distribution in the mouse oocyte during meiotic maturation." Dev.Biol. 130.1 (1988): 184-97.

Dumont,J.; Million,K.; Sunderland,K.; Rassinier,P.; Lim,H.; Leader,B.; Verlhac,M.H
"Formin-2 is required for spindle migration and for the late steps of cytokinesis in mouse oocytes." Dev.Biol. 301.1 (2007): 254-65.

Echard,A.; Hickson,G.R.; Foley,E.; O'Farrell,P.H.
"Terminal cytokinesis events uncovered after an RNAi screen." Curr.Biol. 14.18 (2004): 1685-93.

Eckley,D.M.; Ainsztein,A.M.; Mackay,A.M.; Goldberg,I.G.; Earnshaw,W.C.
"Chromosomal proteins and cytokinesis: patterns of cleavage furrow formation and inner centromere protein positioning in mitotic heterokaryons and mid-anaphase cells." J.Cell Biol. 136.6 (1997): 1169-83.

Eggert, U. S., T. J. Mitchison, and C. M. Field. "Animal cytokinesis: from parts list to mechanisms." Annu.Rev.Biochem. 75 (2006): 543-66.

Emoto,K.; Inadome,H.; Kanaho,Y.; Narumiya,S.; Umeda,M.
"Local change in phospholipid composition at the cleavage furrow is essential for completion of cytokinesis." J.Biol.Chem. 280.45 (2005): 37901-07.

Feng, B., H. Schwarz, and S. Jesuthasan. "Furrow-specific endocytosis during cytokinesis of zebrafish blastomeres." Exp.Cell Res. 279.1 (2002): 14-20.

Ferreira,E.M.; Vireque,A.A.; Adona,P.R.; Meirelles,F.V.; Ferriani,R.A.; Navarro,P.A
"Cytoplasmic maturation of bovine oocytes: structural and biochemical modifications and acquisition of developmental competence." Theriogenology 71.5 (2009): 836-48.

Field,S.J.; Madson,N.; Kerr,M.L.; Galbraith,K.A.; Kennedy,C.E.; Tahiliani,M.;
Wilkins,A; Cantley,L.C.
"PtdIns(4,5)P2 functions at the cleavage furrow during cytokinesis." Curr.Biol. 15.15 (2005): 1407-12.

Fielding,A.B.; Schonteich,E.; Matheson,J.; Wilson,G.; Yu,X.; Hickson,G.R.;
Srivastava,S. Baldwin,S.A; Prekeris,R; Gould,G.W.
"Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic
in cytokinesis.'' EMBO J. 24.19 (2005): 3389-99

Fishkind, D. J. and Y. L. Wang. "Orientation and three-dimensional organization of actin filaments in dividing cultured cells." J.Cell Biol. 123.4 (1993): 837-48.

Fisk,N.M.; Ware,M.; Stanier,P.; Moore,G.; Bennett,P.
"Molecular genetic etiology of twin reversed arterial perfusion sequence." Am.J.Obstet.Gynecol. 174.3 (1996): 891-94.

FitzHarris, G., P. Marangos, and J. Carroll. "Cell cycle-dependent regulation of structure of endoplasmic reticulum and inositol 1,4,5-trisphosphate-induced Ca2+ release in mouse oocytes and embryos." Mol.Biol.Cell 14.1 (2003): 288-301.

FitzHarris, G; Marangos, P; Carroll J. "Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein." Dev.Biol. 305.1 (2007): 133-44.

Flanagan,C.A.; Schnieders,E.A.; Emerick,A.W.; Kunisawa,R.; Admon,A.; Thorner,J.
"Phosphatidylinositol 4-kinase: gene structure and requirement for yeast cell viability." Science 262.5138 (1993): 1444-48.

Frank J. Longo. Fertilization. London, New York: Chapman and Hall 1987.

Fujiwara T; Oda K; Yokota S; Takatsuki A; Ikehara Y. " Brefeldin A causes disassembly of the Golgi complex and accumulation of secretory proteins in the endoplasmic reticulum". J.Biol.Chem. (1988);263(34):18545-52.

Gatt, M. K. and D. M. Glover. "The Drosophila phosphatidylinositol transfer protein encoded by vibrator is essential to maintain cleavage-furrow ingression in cytokinesis." J.Cell Sci. 119.Pt 11 (2006): 2225-35.

Gaullier,J.M.; Ronning,E.; Gillooly,D.J.; Stenmark,H.
"Interaction of the EEA1 FYVE finger with phosphatidylinositol 3-phosphate and early endosomes. Role of conserved residues." J.Biol.Chem. 275.32 (2000): 24595-600.

Gerald,N.J.; Damer,C.K.; O'Halloran,T.J.; De,Lozanne A.
"Cytokinesis failure in clathrin-minus cells is caused by cleavage furrow instability." Cell Motil.Cytoskeleton 48.3 (2001): 213-23.

Giansanti,M.G.; Bonaccorsi,S.; Bucciarelli,E.; Gatti,M.
"Drosophila male meiosis as a model system for the study of cytokinesis in animal cells." Cell Struct.Funct. 26.6 (2001): 609-17.

Giansanti,M.G.; Farkas,R.M.; Bonaccorsi,S.; Lindsley,D.L.; Wakimoto,B.T.;
Fuller,M.T.; Gatti,M.
"Genetic dissection of meiotic cytokinesis in Drosophila males." Mol.Biol.Cell 15.5 (2004): 2509-22.

Giansanti,M.G.; Bonaccorsi,S.; Kurek,R.; Farkas,R.M.; Dimitri,P.; Fuller,M.T.;
Gatti,M.
"The class I PITP giotto is required for Drosophila cytokinesis." Curr.Biol. 16.2 (2006): 195-201.

Giansanti, M. G., G. Belloni, and M. Gatti. "Rab11 is required for membrane trafficking and actomyosin ring constriction in meiotic cytokinesis of Drosophila males." Mol.Biol.Cell 18.12 (2007): 5034-47.

Gillooly,D.J.; Morrow,I.C.; Lindsay,M.; Gould,R.; Bryant,N.J.; Gaullier,J.M.;
Parton,R.G; Stenmark,H.
"Localization of phosphatidylinositol 3-phosphate in yeast and mammalian cells." EMBO J. 19.17 (2000): 4577-88.

Glotzer, M. "Cytokinesis." Curr.Biol. 7.5 (1997): R274-R276.

Glotzer, M. "The molecular requirements for cytokinesis." Science 307.5716 (2005): 1735-39.

Glotzer, M. "The 3Ms of central spindle assembly: microtubules, motors and MAPs." Nat.Rev.Mol.Cell Biol. 10.1 (2009): 9-20.

Godi,A.; Pertile,P.; Meyers,R.; Marra,P.; Di,Tullio G.; Iurisci,C.; Luini,A.; Corda,D.;
De Matteis,M.A.
"ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex." Nat.Cell Biol. 1.5 (1999): 280-87.

Gonczy, P. "Mechanisms of asymmetric cell division: flies and worms pave the way." Nat.Rev.Mol.Cell Biol. 9.5 (2008): 355-66.

Goody, R. S., A. Rak, and K. Alexandrov. "The structural and mechanistic basis for recycling of Rab proteins between membrane compartments." Cell Mol.Life Sci. 62.15 (2005): 1657-70.

Grill,S.W.; Gonczy,P.; Stelzer,E.H.; Hyman,A.A.
"Polarity controls forces governing asymmetric spindle positioning in the Caenorhabditis elegans embryo." Nature 409.6820 (2001): 630-33.

Grosshans, B. L., D. Ortiz, and P. Novick. "Rabs and their effectors: achieving specificity in membrane traffic." Proc.Natl.Acad.Sci.U.S.A 103.32 (2006): 11821-27.

Gruenberg, J. and K. E. Howell. "Membrane traffic in endocytosis: insights from cell-free assays." Annu.Rev.Cell Biol. 5 (1989): 453-81.

Halet, G. and J. Carroll. "Rac activity is polarized and regulates meiotic spindle stability and anchoring in mammalian oocytes." Dev.Cell 12.2 (2007): 309-17.

Hassold, T. and P. Hunt. "To err (meiotically) is human: the genesis of human aneuploidy." Nat.Rev.Genet. 2.4 (2001): 280-91.

Helmkamp,G.M.,Jr.; Harvey,M.S.; Wirtz,K.W.; Van Deenen,L.L.
"Phospholipid exchange between membranes. Purification of bovine brain proteins that preferentially catalyze the transfer of phosphatidylinositol." J.Biol.Chem. 249.20 (1974): 6382-89.

Homer,H.A.; McDougall,A.; Levasseur,M.; Murdoch,A.P.; Herbert,M.
"Mad2 is required for inhibiting securin and cyclin B degradation following spindle depolymerisation in meiosis I mouse oocytes." Reproduction. 130.6 (2005): 829-43.

Hoshino,Y.; Yokoo,M.; Yoshida,N.; Sasada,H.; Matsumoto,H.; Sato,E.
"Phosphatidylinositol 3-kinase and Akt participate in the FSH-induced meiotic maturation of mouse oocytes." Mol.Reprod.Dev. 69.1 (2004): 77-86.

Kline, D. "Attributes and dynamics of the endoplasmic reticulum in mammalian eggs." Curr.Top.Dev.Biol. 50 (2000): 125-54.

Knight,Z.A.; Gonzalez,B.; Feldman,M.E.; Zunder,E.R.; Goldenberg,D.D.;
Williams,O; Loewith,R; Stokoe,D; Balla,A; Toth,B; Balla,T; Weiss,W.A;
Williams,R.L; Shokat,K.M.
"A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling." Cell 125.4 (2006): 733-47.

Kovar,D.R.; Kuhn,J.R.; Tichy,A.L.; Pollard,T.D.
"The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin." J.Cell Biol. 161.5 (2003): 875-87.

Krisher, R. L. "The effect of oocyte quality on development." J.Anim Sci. 82 E-Suppl (2004): E14-E23.

Kubiak,J.Z.; Weber,M.; de,Pennart H.; Winston,N.J.; Maro,B.
"The metaphase II arrest in mouse oocytes is controlled through microtubule-dependent destruction of cyclin B in the presence of CSF." EMBO J. 12.10 (1993): 3773-78.

Kudo,N.R.; Wassmann,K.; Anger,M.; Schuh,M.; Wirth,K.G.; Xu,H.; Helmhart,W.; Kudo,H.; McKay,M.; Maro,B.; Ellenberg,J.; de,Boer P.; Nasmyth,K.
"Resolution of chiasmata in oocytes requires separase-mediated proteolysis." Cell 126.1 (2006): 135-46.

Landry,M.C.; Sicotte,A.; Champagne,C.; Lavoie,J.N.
"Regulation of cell death by recycling endosomes and golgi membrane dynamics via a pathway involving Src-family kinases, Cdc42 and Rab11a." Mol.Biol.Cell 20.18 (2009): 4091-106.

Lauber,M.H.; Waizenegger,I.; Steinmann,T.; Schwarz,H.; Mayer,U.; Hwang,I.; Lukowitz,W.; Jurgens,G.
"The Arabidopsis KNOLLE protein is a cytokinesis-specific syntaxin." J.Cell Biol. 139.6 (1997): 1485-93.

Lawe,D.C.; Patki,V.; Heller-Harrison,R.; Lambright,D.; Corvera,S.
"The FYVE domain of early endosome antigen 1 is required for both phosphatidylinositol 3-phosphate and Rab5 binding. Critical role of this dual interaction for endosomal localization." J.Biol.Chem. 275.5 (2000): 3699-705.

Leader,B.; Lim,H.; Carabatsos,M.J.; Harrington,A.; Ecsedy,J.; Pellman,D.; Maas,R.; Leder,P.
"Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes." Nat.Cell Biol. 4.12 (2002): 921-28.

Leaf,D.S.; Roberts,S.J.; Gerhart,J.C.; Moore,H.P.
"The secretory pathway is blocked between the trans-Golgi and the plasma membrane during meiotic maturation in Xenopus oocytes." Dev.Biol. 141.1 (1990): 1-12.

Lefebvre,C.; Terret,M.E.; Djiane,A.; Rassinier,P.; Maro,B.; Verlhac,M.H.
"Meiotic spindle stability depends on MAPK-interacting and spindle-stabilizing protein (MISS), a new MAPK substrate." J.Cell Biol. 157.4 (2002): 603-13.

Leung,K.F.; Baron,R.; Ali,B.R.; Magee,A.I.; Seabra,M.C.
"Rab GTPases containing a CAAX motif are processed post-geranylgeranylation by proteolysis and methylation." J.Biol.Chem. 282.2 (2007): 1487-97.

Longo, F. J. and D. Y. Chen. "Development of surface polarity in mouse eggs." Scan Electron Microsc.Pt 2 (1984): 703-16.

Longo, F.J. and D.Y.Chen. "Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus." Dev.Biol. 107.2 (1985): 382-94.

Low,S.H.; Li,X.; Miura,M.; Kudo,N.; Quinones,B.; Weimbs,T.
"Syntaxin 2 and endobrevin are required for the terminal step of cytokinesis in mammalian cells." Dev.Cell 4.5 (2003): 753-59.

Mabuchi, I. and M. Okuno. "The effect of myosin antibody on the division of starfish blastomeres." J.Cell Biol. 74.1 (1977): 251-63.

Macia,E.; Luton,F.; Partisani,M.; Cherfils,J.; Chardin,P.; Franco,M.
"The GDP-bound form of Arf6 is located at the plasma membrane." J.Cell Sci. 117.Pt 11 (2004): 2389-98.

Mackay,A.M.; Ainsztein,A.M.; Eckley,D.M.; Earnshaw,W.C.
"A dominant mutant of inner centromere protein (INCENP), a chromosomal protein, disrupts prometaphase congression and cytokinesis." J.Cell Biol. 140.5 (1998): 991-1002.

Madgwick, S. and K. T. Jones. "How eggs arrest at metaphase II: MPF stabilisation plus APC/C inhibition equals Cytostatic Factor." Cell Div. 2 (2007): 4.

Maro,B.; Johnson,M.H.; Pickering,S.J.; Flach,G.
"Changes in actin distribution during fertilization of the mouse egg." J.Embryol.Exp.Morphol. 81 (1984): 211-37.

Maro, B., S. K. Howlett, and E. Houliston. "Cytoskeletal dynamics in the mouse egg." J.Cell Sci.Suppl 5 (1986): 343-59.

Maro, B. and M. H. Verlhac. "Polar body formation: new rules for asymmetric divisions." Nat.Cell Biol. 4.12 (2002): E281-E283.

Matheos,D.; Metodiev,M.; Muller,E.; Stone,D.; Rose,M.D.
"Pheromone-induced polarization is dependent on the Fus3p MAPK acting through the formin Bni1p." J.Cell Biol. 165.1 (2004): 99-109.

Mehlmann,L.M.; Terasaki,M.; Jaffe,L.A.; Kline,D.
"Reorganization of the endoplasmic reticulum during meiotic maturation of the mouse oocyte." Dev.Biol. 170.2 (1995): 607-15.

Minogue,S.; Anderson,J.S.; Waugh,M.G.; dos,Santos M.; Corless,S.; Cramer,R.;
Hsuan,J.J.
"Cloning of a human type II phosphatidylinositol 4-kinase reveals a novel lipid kinase family." J.Biol.Chem. 276.20 (2001): 16635-40.

Mishima,M.; Pavicic,V.; Gruneberg,U.; Nigg,E.A.; Glotzer,M.
"Cell cycle regulation of central spindle assembly." Nature 430.7002 (2004): 908-13.

Morgan,C.P.; Allen-Baume,V.; Radulovic,M.; Li,M.; Skippen,A.; Cockcroft,S.
"Differential expression of a C-terminal splice variant of phosphatidylinositol transfer protein beta lacking the constitutive-phosphorylated Ser262 that localizes to the Golgi compartment." Biochem.J. 398.3 (2006): 411-21.

Motosugi,N.; Dietrich,J.E.; Polanski,Z.; Solter,D.; Hiiragi,T.
"Space asymmetry directs preferential sperm entry in the absence of polarity in the mouse oocyte." PLoS.Biol. 4.5 (2006): e135.

Murray, A. W. and M. W. Kirschner. "Cyclin synthesis drives the early embryonic cell cycle." Nature 339.6222 (1989): 275-80.

Murthy, M. and T. L. Schwarz. "The exocyst component Sec5 is required for membrane traffic and polarity in the Drosophila ovary." Development 131.2 (2004): 377-88.

Na, J. and M. Zernicka-Goetz. "Asymmetric positioning and organization of the meiotic spindle of mouse oocytes requires CDC42 function." Curr.Biol. 16.12 (2006): 1249-54.

Nakanishi, S., K. J. Catt, and T. Balla. "A wortmannin-sensitive phosphatidylinositol 4-kinase that regulates hormone-sensitive pools of inositolphospholipids." Proc.Natl.Acad.Sci.U.S.A 92.12 (1995): 5317-21.

O W and T. G. Baker. "Initiation and control of meiosis in hamster gonads in vitro." J.Reprod.Fertil. 48.2 (1976): 399-401.

Ohkura, H., I. M. Hagan, and D. M. Glover. "The conserved Schizosaccharomyces pombe kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells." Genes Dev. 9.9 (1995): 1059-73.

Ou, Y. and J. B. Rattner. "The centrosome in higher organisms: structure, composition, and duplication." Int.Rev.Cytol. 238 (2004): 119-82.

Palade, G. "Intracellular aspects of the process of protein synthesis." Science 189.4200 (1975): 347-58.

Panaretou,C.; Domin,J.; Cockcroft,S.; Waterfield,M.D.
"Characterization of p150, an adaptor protein for the human phosphatidylinositol (PtdIns) 3-kinase. Substrate presentation by phosphatidylinositol transfer protein to the p150.Ptdins 3-kinase complex." J.Biol.Chem. 272.4 (1997): 2477-85.

Pasqualato,S.; Senic-Matuglia,F.; Renault,L.; Goud,B.; Salamero,J.; Cherfils,J.
"The structural GDP/GTP cycle of Rab11 reveals a novel interface involved in the dynamics of recycling endosomes." J.Biol.Chem. 279.12 (2004): 11480-88.

Payne, C. and G. Schatten. "Golgi dynamics during meiosis are distinct from mitosis and are coupled to endoplasmic reticulum dynamics until fertilization." Dev.Biol. 264.1 (2003): 50-63.

Pedersen R.A., Rossant J. Experimental approaches to mammalian embryonic development. Cambridge University press 1986.

Pelissier, A., J. P. Chauvin, and T. Lecuit. "Trafficking through Rab11 endosomes is required for cellularization during Drosophila embryogenesis." Curr.Biol. 13.21 (2003): 1848-57.

Pfeffer, S. and D. Aivazian. "Targeting Rab GTPases to distinct membrane compartments." Nat.Rev.Mol.Cell Biol. 5.11 (2004): 886-96.

Phillips,S.E.; Vincent,P.; Rizzieri,K.E.; Schaaf,G.; Bankaitis,V.A.; Gaucher,E.A.
"The diverse biological functions of phosphatidylinositol transfer proteins in eukaryotes." Crit Rev.Biochem.Mol.Biol. 41.1 (2006): 21-49.

Piekny, A., M. Werner, and M. Glotzer. "Cytokinesis: welcome to the Rho zone." Trends Cell Biol. 15.12 (2005): 651-58.

Piel,M.; Nordberg,J.; Euteneuer,U.; Bornens,M.
"Centrosome-dependent exit of cytokinesis in animal cells." Science 291.5508 (2001): 1550-53.

Polevoy,G.; Wei,H.C.; Wong,R.; Szentpetery,Z.; Kim,Y.J.; Goldbach,P.;
Steinbach,S.K.; Balla,T.; Brill,J.A.
"Dual roles for the Drosophila PI 4-kinase four wheel drive in localizing Rab11 during cytokinesis." J.Cell Biol. 187.6 (2009): 847-58.

Prekeris, R. and G. W. Gould. "Breaking up is hard to do - membrane traffic in cytokinesis." J.Cell Sci. 121.Pt 10 (2008): 1569-76.

Prigent,M.; Dubois,T.; Raposo,G.; Derrien,V.; Tenza,D.; Rosse,C.; Camonis,J.;
Chavrier,P.
"ARF6 controls post-endocytic recycling through its downstream exocyst complex effector." J.Cell Biol. 163.5 (2003): 1111-21.

Riggs,B.; Rothwell,W.; Mische,S.; Hickson,G.R.; Matheson,J.; Hays,T.S.;
Gould,G.W.; Sullivan,W.
"Actin cytoskeleton remodeling during early Drosophila furrow formation requires recycling endosomal components Nuclear-fallout and Rab11." J.Cell Biol. 163.1 (2003): 143-54.

Rutherford,A.C.; Traer,C.; Wassmer,T.; Pattni,K.; Bujny,M.V.; Carlton,J.G.;
Stenmark,H.; Cullen,P.J.
"The mammalian phosphatidylinositol 3-phosphate 5-kinase (PIKfyve) regulates endosome-to-TGN retrograde transport." J.Cell Sci. 119.Pt 19 (2006): 3944-57.

Sagata,N.; Watanabe,N.; Vande Woude,G.F.; Ikawa,Y.
"The c-mos proto-oncogene product is a cytostatic factor responsible for meiotic arrest in vertebrate eggs." Nature 342.6249 (1989): 512-18.

Sanfins,A.; Lee,G.Y.; Plancha,C.E.; Overstrom,E.W.; Albertini,D.F.
"Distinctions in meiotic spindle structure and assembly during in vitro and in vivo maturation of mouse oocytes." Biol.Reprod. 69.6 (2003): 2059-67.

Saunders,C.M.; Larman,M.G.; Parrington,J.; Cox,L.J.; Royse,J.; Blayney,L.M.;
Swann,K.; Lai,F.A.
"PLC zeta: a sperm-specific trigger of Ca(2+) oscillations in eggs and embryo development." Development 129.15 (2002): 3533-44.

Sbrissa, D., O. C. Ikonomov, and A. Shisheva. "Phosphatidylinositol 3-phosphate-interacting domains in PIKfyve. Binding specificity and role in PIKfyve. Endomenbrane localization." J.Biol.Chem. 277.8 (2002): 6073-79.

Scapin,S.M.; Carneiro,F.R.; Alves,A.C.; Medrano,F.J.; Guimaraes,B.G.; Zanchin,N.I.
"The crystal structure of the small GTPase Rab11b reveals critical differences relative to the Rab11a isoform." J.Struct.Biol. 154.3 (2006): 260-68.

Schonteich,E.; Pilli,M.; Simon,G.C.; Matern,H.T.; Junutula,J.R.; Sentz,D.;
Holmes,R.K.; Prekeris,R.
"Molecular characterization of Rab11-FIP3 binding to ARF GTPases." Eur.J.Cell Biol. 86.8 (2007): 417-31.

Schuh, M. and J. Ellenberg. "A new model for asymmetric spindle positioning in mouse oocytes." Curr.Biol. 18.24 (2008): 1986-92.

Sciaky,N.; Presley,J.; Smith,C.; Zaal,K.J.; Cole,N.; Moreira,J.E.; Terasaki,M.;
Siggia,E.; Lippincott-Schwartz,J.
"Golgi tubule traffic and the effects of brefeldin A visualized in living cells." J.Cell Biol. 139.5 (1997): 1137-55.

Segev, N. "Ypt/rab gtpases: regulators of protein trafficking." Sci.STKE. 2001.100 (2001): re11.

Shadan,S.; Holic,R.; Carvou,N.; Ee,P.; Li,M.; Murray-Rust,J.; Cockcroft,S.
"Dynamics of lipid transfer by phosphatidylinositol transfer proteins in cells." Traffic. 9.10 (2008): 1743-56.

Shisheva, A. "PIKfyve: Partners, significance, debates and paradoxes." Cell Biol.Int. 32.6 (2008): 591-604.

Shuster, C. B. and D. R. Burgess. "Parameters that specify the timing of cytokinesis." J.Cell Biol. 146.5 (1999): 981-92.

Shuster,C.B. and D.R.Burgess. "Targeted new membrane addition in the cleavage
furrow is a late, separate event in cytokinesis." Proc.Natl.Acad.Sci.U.S.A 99.6
(2002): 3633-38.

Simonsen,A.; Wurmser,A.E.; Emr,S.D.; Stenmark,H.
"The role of phosphoinositides in membrane transport." Curr.Opin.Cell Biol. 13.4 (2001): 485-92.

Skop,A.R.; Bergmann,D.; Mohler,W.A.; White,J.G.
"Completion of cytokinesis in C. elegans requires a brefeldin A-sensitive membrane accumulation at the cleavage furrow apex." Curr.Biol. 11.10 (2001): 735-46.

Skop,A.R.; Liu,H.; Yates,J.,III; Meyer,B.J.; Heald,R.
"Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms." Science 305.5680 (2004): 61-66.

Straight, A. F. and C. M. Field. "Microtubules, membranes and cytokinesis." Curr.Biol. 10.20 (2000): R760-R770.

Straight,A.F.; Cheung,A.; Limouze,J.; Chen,I.; Westwood,N.J.; Sellers,J.R.; Mitchison,T.J.
"Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor." Science 299.5613 (2003): 1743-47.

Strickland, L. I. and D. R. Burgess. "Pathways for membrane trafficking during cytokinesis." Trends Cell Biol. 14.3 (2004): 115-18.

Strickland, L. I., E. J. Donnelly, and D. R. Burgess. "Induction of cytokinesis is independent of precisely regulated microtubule dynamics." Mol.Biol.Cell 16.10 (2005): 4485-94.

Sun,Q.Y.; Lai,L.; Park,K.W.; Kuhholzer,B.; Prather,R.S.; Schatten,H.
"Dynamic events are differently mediated by microfilaments, microtubules, and mitogen-activated protein kinase during porcine oocyte maturation and fertilization in vitro." Biol.Reprod. 64.3 (2001): 879-89.

Sun, Q. Y. and H. Schatten. "Regulation of dynamic events by microfilaments during oocyte maturation and fertilization." Reproduction. 131.2 (2006): 193-205.

Svoboda,P.; Stein,P.; Hayashi,H.; Schultz,R.M.
"Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference." Development 127.19 (2000): 4147-56.

Tanaka, S. and K. Hosaka. "Cloning of a cDNA encoding a second phosphatidylinositol transfer protein of rat brain by complementation of the yeast sec14 mutation." J.Biochem. 115.5 (1994): 981-84.

Thompson,H.M.; Skop,A.R.; Euteneuer,U.; Meyer,B.J.; McNiven,M.A.
"The large GTPase dynamin associates with the spindle midzone and is required for cytokinesis." Curr.Biol. 12.24 (2002): 2111-17.

Tilley,S.J.; Skippen,A.; Murray-Rust,J.; Swigart,P.M.; Stewart,A.; Morgan,C.P.; Cockcroft,S.; McDonald,N.Q.
"Structure-function analysis of human [corrected] phosphatidylinositol transfer protein alpha bound to phosphatidylinositol." Structure. 12.2 (2004): 317-26.

Torner,H.; Brussow,K.P.; Alm,H.; Ratky,J.; Pohland,R.; Tuchscherer,A.
"Mitochondrial aggregation patterns and activity in porcine oocytes and apoptosis in surrounding cumulus cells depends on the stage of pre-ovulatory maturation." Theriogenology 61.9 (2004): 1675-89.

Toth,B.; Balla,A.; Ma,H.; Knight,Z.A.; Shokat,K.M.; Balla,T.
"Phosphatidylinositol 4-kinase IIIbeta regulates the transport of ceramide between the endoplasmic reticulum and Golgi." J.Biol.Chem. 281.47 (2006): 36369-77.

Tremoleda,J.L.; Schoevers,E.J.; Stout,T.A.; Colenbrander,B.; Bevers,M.M.
"Organisation of the cytoskeleton during in vitro maturation of horse oocytes." Mol.Reprod.Dev. 60.2 (2001): 260-69.

Tung JJ; Hansen DV; Ban KH; Loktev AV; Summers MK; Adler JR; Jackson PK. " A role for the anaphase-promoting complex inhibitor Emi2/XErp1, a homolog of early mitotic inhibitor 1, in cytostatic factor arrest of Xenopus eggs". Proc Natl Acad Sci (2005). 22;102(12):4318-23.

Uhlmann, F., F. Lottspeich, and K. Nasmyth. "Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1." Nature 400.6739 (1999): 37-42.

Ullrich,O.; Reinsch,S.; Urbe,S.; Zerial,M.; Parton,R.G.
"Rab11 regulates recycling through the pericentriolar recycling endosome." J.Cell Biol. 135.4 (1996): 913-24.

Van Blerkom, J. and Runner MN. Mitochondrial reorganization during resumption of arrested meiosis in the mouse oocyte. Am J Anat. (1984) 171(3):335-55

Van Paridon, P. A., A. J. Visser, and K. W. Wirtz. "Binding of phospholipids to the phosphatidylinositol transfer protein from bovine brain as studied by steady-state and time-resolved fluorescence spectroscopy." Biochim.Biophys.Acta 898.2 (1987): 172-80.

van Tiel,C.M.; Schenning,M.; Snoek,G.T.; Wirtz,K.W.
"Overexpression of phosphatidylinositol transfer protein beta in NIH3T3 cells has a stimulatory effect on sphingomyelin synthesis and apoptosis." Biochim.Biophys.Acta 1636.2-3 (2004): 151-58.

Verlhac,M.H.; Lefebvre,C.; Guillaud,P.; Rassinier,P.; Maro,B.
"Asymmetric division in mouse oocytes: with or without Mos." Curr.Biol. 10.20 (2000): 1303-06.

Walch-Solimena, C. and P. Novick. "The yeast phosphatidylinositol-4-OH kinase pik1 regulates secretion at the Golgi." Nat.Cell Biol. 1.8 (1999): 523-25.

Wang,L.; Wang,Z.B.; Zhang,X.; FitzHarris,G.; Baltz,J.M.; Sun,Q.Y.; Liu,X.J.
"Brefeldin A disrupts asymmetric spindle positioning in mouse oocytes." Dev.Biol. 313.1 (2008): 155-66.

Wang, Y. L. "The mechanism of cytokinesis: reconsideration and reconciliation." Cell Struct.Funct. 26.6 (2001): 633-38.

Wassmann, K., T. Niault, and B. Maro. "Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes." Curr.Biol. 13.18 (2003): 1596-608.

Wei and Seemann. “Spindle-dependent partitioning of the Golgi ribbon”. (2009). Commun Integr Biol. 2(5):406-7.

Wianny, F. and M. Zernicka-Goetz. "Specific interference with gene function by double-stranded RNA in early mouse development." Nat.Cell Biol. 2.2 (2000): 70-75.

Wilson,G.M.; Fielding,A.B.; Simon,G.C.; Yu,X.; Andrews,P.D.; Hames,R.S.; Frey,A.M.; Peden,A.A.; Gould,G.W.; Prekeris,R.
"The FIP3-Rab11 protein complex regulates recycling endosome targeting to the cleavage furrow during late cytokinesis." Mol.Biol.Cell 16.2 (2005): 849-60.

Winston,N.J.; McGuinness,O.; Johnson,M.H.; Maro,B.
"The exit of mouse oocytes from meiotic M-phase requires an intact spindle during intracellular calcium release." J.Cell Sci. 108 (Pt 1) (1995): 143-51.

Wirth,K.G.; Wutz,G.; Kudo,N.R.; Desdouets,C.; Zetterberg,A.; Taghybeeglu,S.; Seznec,J.; Ducos,G.M.; Ricci,R.; Firnberg,N.; Peters,J.M.; Nasmyth,K.
"Separase: a universal trigger for sister chromatid disjunction but not chromosome cycle progression." J.Cell Biol. 172.6 (2006): 847-60.

Wirtz, K. W., A. Schouten, and P. Gros. "Phosphatidylinositol transfer proteins: from closed for transport to open for exchange." Adv.Enzyme Regul. 46 (2006): 301-11.

Wong, K., ddR Meyers, and L. C. Cantley. "Subcellular locations of phosphatidylinositol 4-kinase isoforms." J.Biol.Chem. 272.20 (1997): 13236-41.

Yasuda,S.; Oceguera-Yanez,F.; Kato,T.; Okamoto,M.; Yonemura,S.; Terada,Y.; Ishizaki,T.; Narumiya,S.
"Cdc42 and mDia3 regulate microtubule attachment to kinetochores." Nature 428.6984 (2004): 767-71.

Zerial, M. and H. McBride. "Rab proteins as membrane organizers." Nat.Rev.Mol.Cell Biol. 2.2 (2001): 107-17.

Zernicka-Goetz M; Morris, SA; Bruce, AW. Making a firm decision: multifaceted regulation of cell fate in the early mouse embryo. Nat Rev Genet (2009). 10(7):467-77

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