Mitochondrial Dynamics During Mouse Oocyte Maturation

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

Abstract

Mitochondria provide the primary source of ATP for the oocyte and pre-implantation embryo and undergo a number of redistributions during oocyte maturation which may be related to developmental competence. The experiments presented in this thesis aim to examine the changes in distribution and function of mitochondria during the transition from the germinal vesicle stage to the mature metaphase II arrested egg.

Mitochondrial distribution was monitored throughout oocyte maturation and accumulation of mitochondria around the first meiotic spindle was observed. This was dependent on the activities of microtubules and their associated motor proteins dynein and kinesin. Migration of the spindle to the oocyte cortex was accompanied by mitochondria but at polar body extrusion a dramatic reorganisation of mitochondria away from the cortical domain occurred, suggesting that a mechanism exists for retention of these important organelles in the oocyte during this asymmetric cell division. The role of the mitochondrial adapter proteins Trak and Miro in establishing redistribution of mitochondria was also addressed.

Finally, a novel recombinant FRET probe for measuring ATP was validated for use in oocytes. Use of this probe revealed alterations to both ATP levels and ATP consumption at different stages of oocyte maturation. Furthermore, whilst the first meiotic spindle was found to be dependent on mitochondrial activity to retain its structure and function, attempts to identify subcellular heterogeneity in ATP supply and demand related to the distribution of mitochondria around the spindle did not reveal any differences. However, the presence of cumulus cells surrounding the oocyte as part of a cumulus-oocyte complex was found to influence ATP levels in the oocyte; oocytes matured as part of a cumulus-oocyte complex had higher ATP levels than those observed in oocytes which had been denuded of cumulus cells. This was found to be dependent on the presence of gap junctional communication between the somatic and germ cell compartments, since inhibition of gap junctions abolished the higher ATP levels observed in cumulus enclosed oocytes.

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Introduction

Mitochondria have long been recognised as the ‘powerhouse’ of the cell, providing adenosine-5'-triphosphate (ATP) to meet cellular energy demands. Of particular interest with regard to oocytes, mitochondria provide the primary source of ATP during oocyte maturation and throughout pre-implantation development as glycolysis is limited during these early developmental stages. Moreover, mitochondrial replication is inactivated in the fully grown oocyte and does not resume until after implantation. Thus, the mitochondrial complement contained within the oocyte is of critical importance for sustaining the earliest stages of life. Furthermore, the distribution of mitochondria within the oocyte and early embryo has been suggested to play a role in the establishment of developmental competence.

Experiments presented in this thesis therefore examine the changes to mitochondrial distribution and activity during maturation of the mouse oocyte. This introduction will cover a brief overview of early mammalian development, followed by a description of basic aspects of mitochondrial biology. Notable features of mitochondria in the oocyte and early embryo will be addressed and finally, the role of mitochondria in establishing development competence will be reviewed.

1.1 Early mammalian development: from oocyte to embryo

1.1.1 Oogenesis

Mammalian oocytes derive from primordial germ cells, which proliferate and migrate to the prospective ovaries during foetal development. Having reached the ovaries, the primordial germ cells cease proliferation and enter meiosis before

becoming arrested in prophase of the first meiotic division (Wolpert *et al.* 2010). Thus, in mammalian species, all of the oocytes which will be available throughout reproductive life are already present in the ovary at the time of birth, contained within primordial follicles. They are arrested at the diplotene stage of prophase of the first meiosis and will remain so until the onset of puberty, a period of a few months in the mouse, but many years in the human (Wolpert *et al.* 2010).

Throughout oocyte development, the oocyte is coupled to surrounding follicle cells by gap junctions (Anderson & Albertini 1976; Eppig 1991; Makabe *et al.* 2006). These allow the transfer of regulatory molecules and metabolites between the two cell types, which participate in the growth and development of both follicle and oocyte (Brower & Schultz 1982; Kidder & Vanderhyden 2010; Buccione *et al.* 1990). During the growth phase, the primary oocyte in the mouse expands from less than 20µm to around 80µm in diameter. This is accompanied by follicular development with the result that a fully grown oocyte is contained within an antral or Graafian follicle, surrounded by many thousands of follicle cells. It is during this period of growth that nuclear expansion to form the germinal vesicle also occurs (Chouinard 1975), and that the zona pellucida, a thick glycoprotein layer synthesised by the oocyte and which surrounds it, is formed (Bleil & Wassarman 1980a; Bleil & Wassarman 1980b).

At any given time, the ovary contains follicles in many stages of growth and development. During the reproductive cycle and in response to follicle stimulating hormone (FSH) which is synthesised and released by the anterior pituitary gland, early antral follicles are rescued from an apoptotic fate and complete the final stages

of growth and development to become antral follicles (Wolpert *et al.* 2010). In response to a surge of luteinising hormone (LH) the fully grown oocytes within the follicles can enter the next stage of development, oocyte maturation.

1.1.2 Oocyte maturation

Oocyte maturation refers to a series of nuclear and cytoplasmic changes which result in the formation of an oocyte which is competent to undergo fertilisation. The first meiotic division resumes in response to LH and the oocyte progresses from germinal vesicle (GV) stage through the first meiosis (MI), begins the second meiosis (MII) and then arrests at metaphase of MII awaiting fertilisation.

Germinal vesicle breakdown (GVBD) occurs around two hours after the initiation of oocyte maturation and is followed by the formation of a barrel-shaped metaphase I spindle some hours later (Donahue 1968; Combelles & Albertini 2001). Centrioles are absent in the mouse oocyte (Manandhar *et al.* 2005), and the meiotic spindle forms instead via the action of acentriolar MTOCs (Messinger & Albertini 1991; Schuh & Ellenberg 2007; Maro *et al.* 1985). A number of MTOCs dispersed in the cytoplasm of the oocyte are recruited to the centre of the oocyte shortly after GVBD (Maro *et al.* 1985; Messinger & Albertini 1991; Combelles & Albertini 2001; Schuh & Ellenberg 2007). Concomitant with their relocation, a dramatic increase in the number of microtubules occurs (Schuh & Ellenberg 2007; Brunet *et al.* 1998). MTOCs subsequently cluster to form the two opposing poles of the spindle and organisation of microtubules into a bipolar spindle takes place (Brunet & Maro 2005; Schuh & Ellenberg 2007).

The spindle then migrates along its axis to the oocyte cortex, a process which is dependent on a dynamic network of actin filaments (Azoury *et al.* 2008; Schuh & Ellenberg 2008; Azoury *et al.* 2011). The actin nucleator Formin-2, which nucleates the formation of straight actin filaments, is crucial to this process (Leader *et al.* 2002; Dumont *et al.* 2007). Following migration of the spindle, anaphase is initiated and homologous chromosomes begin to separate. They move towards opposing spindle poles and half of the chromosomes are then extruded in the first polar body during cytokinesis. This occurs some 10-12 hours after the LH surge. The position of the cytokinetic furrow may be determined in part by polarisation of the cortex, in that migration of the spindle induces changes to the cortex immediately overlying the position of the spindle, causing it to become rich in microfilaments and devoid of microvilli and cortical granules (Maro *et al.* 1986; Longo & CHEN 1985; Deng *et al.* 2003).

The cell division which occurs at cytokinesis is remarkably asymmetric, with the resulting polar body many times smaller than the oocyte, allowing retention of maternal stores accumulated during the growth phase (Brunet & Verlhac 2011). The polar body degenerates shortly after extrusion whilst the oocyte immediately enters meiosis II, bypassing interphase (Austin & Bishop 1957). The remaining chromosomes align on the second meiotic spindle. The egg, as it may now be referred to, is ovulated and remains arrested at metaphase of meiosis II until fertilisation occurs.

The nuclear progression of oocyte maturation is accompanied by structural and metabolic changes to the cytosol of the oocyte which include down-regulation of

transcription, up regulation of protein synthesis, alterations to cell signalling pathways and the reorganisation of a number of organelles (Eppig *et al.* 1994).

The process of oocyte maturation can be studied *in vitro* by rupture of the follicle and release of the fully grown GV stage oocyte into suitable culture media (Schroeder *et al.* 1988). The oocyte will spontaneously resume maturation, can be fertilised, undergo normal pre-implantation development and result in the birth of live offspring (Schroeder & Eppig 1984). These offspring have largely normal long term health (Eppig *et al.* 2009). Nonetheless, the rate of live births resulting from oocytes matured *in vitro* is relatively low (Moor *et al.* 1998). It has been proposed that this may be due in part to the composition of culture media which allows nuclear maturation but does not adequately support cytoplasmic maturation (Combelles *et al.* 2002; Trounson *et al.* 2001).

1.1.3 Regulation of the cell cycle during oocyte maturation

Oocyte maturation is co-ordinated by the activities of the cell cycle kinases maturation promoting factor (MPF) and mitogen-activated protein kinase (MAPK). The universal cell cycle kinase MPF regulates entry into M-phase and is composed of cdk1 and cyclin B (Doree & Hunt 2002; Nurse 1990). A number of cyclin isoforms exist in mammalian cells, but cyclin B1 appears to be the isoform involved in regulation of the cell cycle in oocytes (Brandeis *et al.* 1998). Prior to resumption of meiosis, MPF is maintained in an inactive form by phosphorylation of cdk1 at Threonine-14 and Tyrosine-15 by the cdk1-specific kinase Wee1/Myt1 (Han *et al.* 2005; Gu *et al.* 1992). Activation occurs when these residues are dephosphorylated by cdc25 (Millar *et al.* 1991; Lincoln *et al.* 2002; Gautier *et al.* 1991). MPF is

maintained at a high level throughout MI before degradation of cyclin B1 is initiated at the metaphase-to-anaphase transition, as soon as the last chromosome has aligned at the metaphase plate (Clute & Pines 1999). This allows anaphase to occur and the first polar body to be extruded (Ledan *et al.* 2001). Degradation is dependent on ubiquitination by the anaphase-promoting complex/cyclosome (APC/C) triggering destruction by the 26S proteasome (Glotzer *et al.* 1991; Morgan 1999; Peters 2002). High MPF activity then resumes and is maintained during MII arrest by the activity of the cyclostatic factor (CSF) (Masui & Markert 1971; Madgwick & Jones 2007).

MAPK is also universally activated during oocyte maturation, although the timing and role of its activation differ between species. In spontaneous maturation in the mouse, activation of MAPK occurs after GVBD and is not required for MPF activation or meiotic resumption (Verlhac *et al.* 1993; Verlhac *et al.* 1994; Hashimoto *et al.* 1994; Colledge *et al.* 1994; Verlhac *et al.* 1996). Activation occurs downstream of Mos, a germ cell specific Serine/Threonine protein kinase, and unlike MPF, activity of MAPK remains high throughout maturation (Verlhac *et al.* 1993; Verlhac *et al.* 1994).

MAPK appears to be involved in the organisation of microtubules and chromatin during oocyte maturation. Indeed, MAPK has been localised to spindle poles in both the first and second meiotic spindles, as well as at cytoplasmic MTOCs (Verlhac *et al.* 1993) and MAPK activity correlates with changes to the organisation of chromatin and of the microtubule network (Verlhac *et al.* 1994). Moreover, in $Mos^{-/-}$ mice, instead of remaining in an M-phase configuration at MII, microtubules elongate to resemble those found in interphase, and chromosomes partially

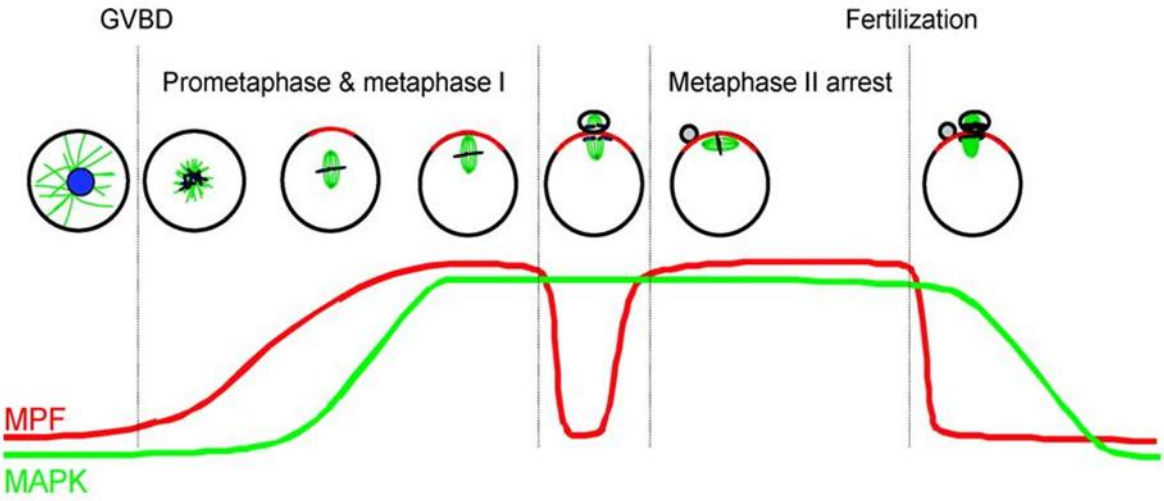

Figure 1.1 MPF and MAPK activities during meiotic maturation in mouse oocytes. MPF is activated at GVBD, and activity increases before a transient decline in MPF activity occurs during the transition between meiosis I and meiosis II. MPF is reactivated rapidly to enter meiosis II and is maintained at a high level during the metaphase II arrest. MAPK is activated later than MPF but activity remains high throughout oocyte maturation. From Brunet and Maro, Reproduction, 2005;130:801-811, © Society for Reproduction and Fertility 2005. Reproduced by permission.

decondense (Verlhac *et al.* 1996). Spindle migration is also affected in $Mos^{-/-}$ mice, resulting in the formation of abnormally large polar bodies (Tong *et al.* 2003; Choi *et al.* 1996; Verlhac *et al.* 2000). Since migration of the spindle to the cortex is controlled by microfilaments, not microtubules (Sun & Schatten 2006; Azoury *et al.* 2008), this suggests that MAPK may also play a role in modulation of the actin cytoskeleton. Furthermore, unfertilised oocytes from $Mos^{-/-}$ mice are parthenogenetically activated indicating that MAPK has a role in the maintenance of the MII arrest (Hashimoto *et al.* 1994; Colledge *et al.* 1994). An overview of MPF and MAPK activity during oocyte maturation can be found in Figure 1.1.

1.1.4 Fertilisation

Fertilisation is initiated by the fusion of the sperm to the plasma membrane of the MII arrested oocyte. In order to accomplish this feat, the sperm must first penetrate the cumulus mass surrounding the ovulated oocyte and bind to the zona pellucida glycoprotein ZP3 (Wassarman 2002; Wassarman *et al.* 2001). This triggers the acrosome reaction, releasing hydrolytic enzymes from a secretory vesicle in the head of the sperm, allowing it to penetrate the zona (Brucker & Lipford 1995). Penetration of further sperm is prevented by the exocytosis of cortical granules containing enzymes which modify the structure of the zona, making it refractory to sperm penetration (Green 1997; Horvath *et al.* 1993). The fusion of sperm and egg triggers egg activation and results in release of the egg from MII arrest and the resumption and completion of the second meiotic division (Runft *et al.* 2002). Sister chromatids are separated and extrusion of a second polar body occurs around 90 minutes after sperm penetration (Wassarman *et al.* 2001). Pronuclei become visible around 4 hours after sperm-egg fusion and are repositioned in the centre of the cell, closely apposed

but remaining separate (Howlett & Bolton 1985). The maternal and paternal chromosomes are finally united in prophase of the first embryonic division which occurs 17-20 hours after fertilisation (Austin & Bishop 1957; Howlett & Bolton 1985).

1.2 Mitochondrial structure and function

A role is emerging for mitochondria as determinants of developmental competence and many of the processes described above are likely to have high energy requirements. Before describing aspects of mitochondrial structure and function in the oocyte and early embryo which may relate to these activities, basic aspects of mitochondrial biology will be described.

1.2.1 The mitochondrial genome

Mitochondria contain their own separate genome, the presence of which is believed to indicate their bacterial origin (Andersson *et al.* 2003). Indeed the mitochondrial genome, around 16kb in size, is packaged as circular DNA, similar to that of primitive bacteria (Leblanc *et al.* 1997). The mitochondrial genome is remarkably well conserved and, in all multicellular organisms, encodes just 37 genes; 13 protein subunits required for oxidative phosphorylation, 2 rRNAs and 22tRNAs (Jansen 2000b; Bibb *et al.* 1981; Clayton 1984). A total of more than eighty proteins are required for oxidative phosphorylation, and the remainder are believed to have migrated to the nuclear genome such that co-operation between the two is required to ensure mitochondrial function (Poyton & McEwen 1996).

1.2.2 Maternal inheritance of mitochondria

In almost all animals, it is now established that mitochondria are inherited down the maternal line (Hutchinson *et al.* 1974; Giles *et al.* 1980). Although sperm mitochondria enter the oocyte at fertilisation, they are eliminated within a few days (Ankel-Simons & Cummins 1996; Sutovsky *et al.* 2004). In the mouse, paternal mitochondria are eliminated at the 2-cell stage (Kaneda *et al.* 1995; Shitara *et al.* 2000) but the stage of development at which sperm mitochondria are eliminated varies between species studied, from 2-cell to 8-cell stage. Notably it always seems to occur prior to embryonic genome activation suggesting that destruction is not dependent on new transcription (St.John *et al.* 2010).

Destruction of paternal mitochondria appears to be carried out via a ubiquitin-dependent mechanism (Sutovsky *et al.* 2000; Sutovsky *et al.* 1999). Sperm mitochondria are initially ubiquitinated during spermatogenesis, following which the ubiquitin signal is masked by disulphide bond cross-linking during the passage of the sperm through the epididymus. Shortly after fertilisation, the signal is restored and indeed increased, dependent on the activity of the proteolytic marker ubiquitin, and paternal mitochondria are destroyed (Sutovsky *et al.* 2000).

Elimination of paternal mitochondria appears to be subspecies-specific, such that sperm mitochondria from inter-specific crosses are not eliminated (Sutovsky *et al.* 2000; Kaneda *et al.* 1995; Shitara *et al.* 1998; Sutovsky *et al.* 1999). This subspecies-specificity has led to some confusion over whether mitochondrial inheritance is strictly maternal. Indeed, paternal inheritance of mitochondria has been demonstrated in a number of inter-specific crosses (Gyllensten *et al.* 1991; Shitara *et*

al. 1998; Zhao *et al.* 2004). However, paternal mitochondria which persisted after crossing of two mouse strains were not transmitted beyond the first generation (Shitara *et al.* 1998) and this, together with the finding that mitochondria are eliminated in intra-specific crosses indicates that paternal inheritance of mitochondria does not normally occur in most animal species. Notable exceptions include Mussels and Drosophila (Zouros *et al.* 1992; Kondo *et al.* 1992).

However, it still remains unclear why, in most animal species at least, sperm mitochondria are eliminated in the developing embryo. It has been postulated that exposure to high levels of reactive oxygen species (ROS), produced either by oxidative phosphorylation to provide sperm motility (Allen 1996), or by the environment in which the sperm develops and travels en route to the oocyte, (Sutovsky *et al.* 2004) cause damage to sperm mtDNA. Thus, the sperm mtDNA may be sacrificed, whilst mtDNA from the non-motile oocyte is protected and transmitted to subsequent generations (Allen 1996). This hypothesis supposes that oocyte mitochondria are bioenergetically repressed and do not participate in oxidative phosphorylation, which is now understood not to be the case. However, oocyte and pre-implantation embryo mitochondria are thought to have a "quiet" metabolism (Baumann *et al.* 2007; Leese *et al.* 2008), which will be described in further detail below, compatible with a theory of mtDNA protection. Alternatively, it has also been suggested that elimination of paternal mtDNA may serve to avoid potentially lethal genome conflict (Hurst 1995).

1.2.3 Mitochondrial structure and morphology

Mitochondria have two distinct cellular membranes, the inner membrane and the outer membrane. The inner membrane encircles the protein rich mitochondria matrix and is the site of the enzymes of the electron transport chain. The outer membrane encloses the inner membrane and intermembrane space and allows the passage of most small molecules and ions, as well as containing pores and channels for the passage of larger molecules. The inner membrane contains a series of invaginations termed cristae which can undergo changes in morphology to modulate mitochondrial function (Mannella 2005). For example, cristae remodelling and fusion during apoptosis has been shown to participate in the release of cytochrome c from the intermembrane space, whilst cristae dilation and increased interconnectivity has been linked to conditions of oxidative stress (Mannella 2008; Scorrano *et al.* 2002).

1.2.4 Mitochondrial organisation and distribution

Within a cell, mitochondria may exist as complex interconnected networks, as discrete individual structures, or as a dynamic, interchangeable combination of the two (Kuznetsov *et al.* 2009). Indeed, the structure of the mitochondrial network has been found to differ significantly between cell types, and even within individual cells (Bereiter-Hahn *et al.* 2008). The extent of mitochondrial interconnectivity is regulated cytoskeletal elements (Anesti & Scorrano 2005) and by the action of specific proteins, including Mfn1, Mfn2 and Opa1 which are involved in the process of fusion, and Drp1 and Fis1 which have a role in mitochondrial fission (Chen & Chan 2010; Scott & Youle 2010; Chang & Blackstone 2010). These variations in dynamics appear to be necessary to ensure proper function and distribution of

mitochondria and defects in these processes have been linked to disease (Westermann 2010).

1.2.5 Mitochondrial ATP production

ATP is produced in the mitochondria by the activities of the tricarboxylic acid (TCA) cycle in the mitochondrial matrix, and the electron transport chain located on the cristae of the inner membrane. The TCA cycle is fed by the breakdown of pyruvate, fatty acids and amino acids which are converted to CO_2 via a series of steps, with the accompanying reduction of NAD^+ to NADH and FAD^{2+} to $FADH_2$. NADH and $FADH_2$ then serve as electron donors for the electron transport chain. This is composed of a series of multi-subunit enzymes spanning the inner mitochondrial membrane termed Complex I (NADH dehydrogenase), Complex II (succinate dehydrogenase), Complex III (ubiquinol cytochrome c reductase), and Complex IV (cytochrome c oxidase). NADH enters at Complex I, whilst $FADH_2$ enters at Complex II (Nicholls & Ferguson 2003a).

Transfer of electrons along the electron transport chain is associated with a transition from a reduced to an oxidised state, and with the movement of protons across the inner mitochondrial membrane from the matrix to the inter-membrane space at Complexes I, III and IV. This generates an electrochemical gradient and results in a mitochondrial membrane potential which is 150mV to 180mV more negative in the mitochondrial matrix, and which is important for a number of mitochondrial functions including Ca^{2+} sequestration, ROS production and protein import, as well as ATP production. This electrochemical gradient is composed of both an electrical or proton gradient caused by a charge difference across the inner mitochondrial

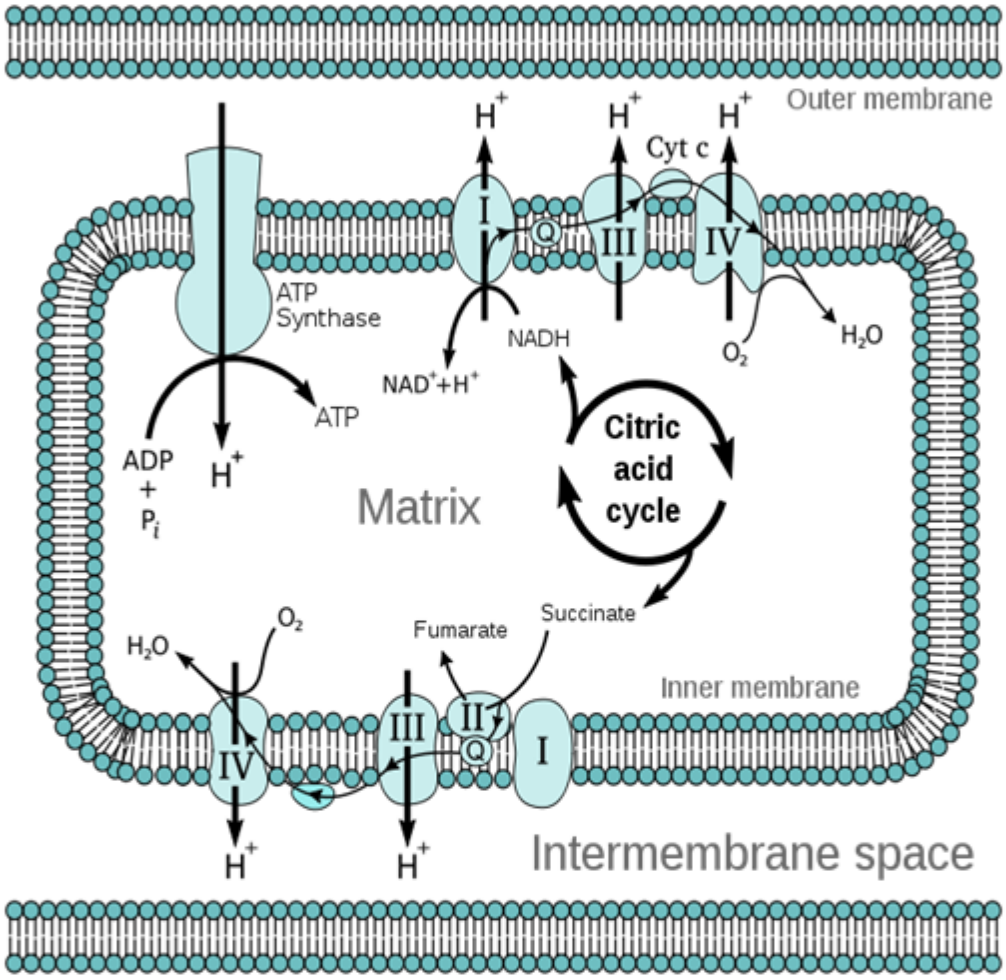

Figure 1.2. The electron transport chain. Electrons derived from the oxidation of NADH and $FADH_2$ are transferred along the electron transport chain. This is accompanied by the movement of protons across the inner mitochondrial membrane from the matrix to the inter-membrane space at Complexes I, III and IV, resulting in the establishment of an electrochemical gradient across the inner mitochondrial membrane. This drives the production of ATP by the mitochondrial ATP synthase. Image is from an open access resource (Wikimedia Commons).

membrane (the mitochondrial membrane potential, $\Delta\psi_m$), and a chemical gradient resulting from the difference in ion concentrations on either side of the membrane (the mitochondrial pH gradient, ΔpH_m). The electrochemical gradient drives the production of ATP from ADP and P_i by the final enzyme of the electron transport chain, F_1F_o-ATP synthase, or Complex V (Nicholls & Ferguson 2003b). This is a large, multi-subunit complex composed of a channel which acts as a proton-driven motor, the F_o domain, and the F_1 domain which catalyses ATP synthesis (Stock *et al.* 1999). Movement of protons through the channel as a result of the protein motive force generated by the electrochemical gradient causes rotation which drives motor activity in order synthesise ATP (Stock *et al.* 2000). Transport of ATP out of the mitochondria and into the cytosol is achieved by the adenine nucleotide transporter (ANT) which exchanges ADP for ATP. A summary can be found in Figure 1.2.

1.2.6 Measuring mitochondrial function

The mitochondrial membrane potential ($\Delta\psi_m$) can be measured using fluorescent dyes which partition to the mitochondria due to the presence of an electrochemical gradient. These dyes are usually lipophilic cations which accumulate in inverse proportion to $\Delta\psi_m$ and include tetramethylrhodamine methyl ester (TMRM), tetramethylrhodamine ethyl ester (TMRE), Rhodamine 123, 3,3′-dihexyloxacarbocyanine iodide ($DiOC_6$) and 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) (Perry *et al.* 2011). There are two main methods for use of these dyes. They can either be used at nanomolar concentrations in 'non-quenching' mode, where higher dye accumulation and fluorescent signal indicates hyperpolarisation of mitochondria, whilst low dye accumulation and a lower fluorescent signal indicates depolarisation. Alternatively,

the ‘quench/dequench’ method, whereby cells are loaded with micromolar concentrations of the dye such that collisions between dye molecule occur, which, together with the formation of dye aggregates in the mitochondria, quench the fluorescence signal, can be used. Depolarisation of the mitochondria results in dissipation of the dye from the mitochondria, unquenching the signal and resulting in an increase in fluorescence. Conversely, hyperpolarisation will cause the accumulation of more dye, resulting in increased quenching and a decrease in fluorescence (Perry *et al.* 2011). JC-1 is slightly different in that aggregation causes a shift in fluorescence from green to red, with the ratio between the two signals giving a measurement of $\Delta\psi_m$.

Caution is however required when using fluorescent dyes to measure mitochondrial membrane potential, since measurements can be influenced by the dye loading protocol, dye concentration, and export of dye from the mitochondria (Perry *et al.* 2011; Duchen 2004). Furthermore, one must also consider the concentrating effect of the plasma membrane potential and whether this is being altered by experimental manipulations, and how re-equilibration of the dye across the plasma membrane may impact upon measurements (Duchen 2004).

Particular care must be taken when interpreting results derived from JC-1 measurements as the red aggregated signal has been reported to be influenced by factors other than $\Delta\psi_m$ (Chinopoulos *et al.* 1999; Scanlon & Reynolds 1998). Moreover, the formation of aggregates is highly sensitive to dye concentration and loading times and, whilst the green monomer form of the dye equilibrates in a similar time-frame to TMRM, equilibration of the red aggregate form can take up to

90 minutes (Mathur *et al.* 2000). Indeed, since equilibration time is linked to surface-to-volume ratio, in cells where there is heterogeneity in surface to volume ratio between different subcellular regions, JC-1 may report heterogeneity in $\Delta\psi_m$ where no such difference exists (Perry *et al.* 2011). This is particularly relevant in a large cell such as the oocyte where equilibration of the probe throughout the cell may take some time and thus lead to misinterpretation of results.

Mitochondrial function can also be monitored by measuring autofluorescence of NADH and flavoproteins, which give an indication of the redox state of mitochondria (Chance *et al.* 1967). An increase in NADH fluorescence, excited in the ultraviolet (UV) range, indicates a shift towards a reduced state and an increased ratio of NADH to NAD^+ (Chance *et al.* 1979; Eng *et al.* 1989). Conversely, an increase in flavoprotein fluorescence, which is excited at a wavelength of around 450nm, reflects a shift towards a more oxidised state (Reinert *et al.* 2007).

Finally, targeting of Ca^{2+} probes (Rizzuto *et al.* 1992; Demaurex & Frieden 2003) and ATP probes (Imamura *et al.* 2009; Dumollard *et al.* 2008) to the mitochondrial compartment has allowed measurement mitochondrial Ca^{2+} uptake and ATP production.

1.2.7 Mitochondria and Ca^{2+}

Measurement of mitochondrial Ca^{2+} has shown that mitochondria are able to sequester Ca^{2+} (Rizzuto *et al.* 1992; Duchen 2000). Accumulation of Ca^{2+} into the mitochondria is driven by the electrochemical gradient and occurs through a Ca^{2+} uniporter in the inner mitochondrial membrane. Although some properties of

mitochondrial Ca^{2+} uptake have previously been characterised, it is only very recently that the molecular identity of the mitochondrial calcium uniporter (MCU) has been elucidated (De Stefani *et al.* 2011; Baughman *et al.* 2011). The MCU has low affinity for Ca^{2+} but close apposition of mitochondria and endoplasmic reticulum (ER) can create a favourable microenvironment of high Ca^{2+}, allowing efficient transmission of ER Ca^{2+} signals to mitochondria (Rizzuto *et al.* 1998; Rizzuto *et al.* 1993; Csordas *et al.* 1999; Csordas *et al.* 2010). Indeed, these microdomains or 'hotspots' can expose the mitochondria to Ca^{2+} levels 5 to 10 times higher than in the bulk cytosol (Giacomello *et al.* 2010).

Mitochondrial uptake of Ca^{2+} has a number of physiological consequences (Duchen 2000). Firstly, mitochondria provide a Ca^{2+} buffering capacity that can modulate both the spatial and temporal pattern of Ca^{2+} signals (Jouaville *et al.* 1998). In hepatocytes, mitochondrial Ca^{2+} uptake supresses positive feedback of Ca^{2+} on the IP_3R resulting in greater sensitivity to IP_3 in subcellular regions containing fewer mitochondria (Hajnoczky *et al.* 1999). Propagation of Ca^{2+} waves is enhanced in the *Xenopus* oocyte by oxidisable substrates which energise mitochondria (Jouaville *et al.* 1995), and in cortical astrocytes and pancreatic acinar cells, mitochondria act as spatial buffers, restricting the propagation of agonist-evoked Ca^{2+} (Tinel *et al.* 1999; Boitier *et al.* 1999). Moreover, proximity between ER and mitochondria may also participate in shaping Ca^{2+} oscillations by maximising ATP provision for the sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump, allowing reuptake of Ca^{2+} into the ER (Malli *et al.* 2005; Landolfi *et al.* 1998). Furthermore, Ca^{2+} uptake by the mitochondria stimulates mitochondrial oxidative phosphorylation by activating dehydrogenases of the TCA cycle (McCormack *et al.* 1990), providing a

mechanism to allow cells to up regulate mitochondrial metabolism and ATP production at times of high demand (Hansford 1994; Hajnoczky *et al.* 1995; Jouaville *et al.* 1999; Duchen 1992). Finally, transfer of Ca^{2+} from the ER to the mitochondria plays a role in regulation of cell death pathways (Pinton *et al.* 2008).

1.2.8 Mitochondria as sources and targets of ROS

Mitochondrial ROS generation occurs as an inevitable consequence of the reactions of the electron transport chain, when incomplete transfer of electrons to molecular oxygen occurs, causing superoxide anions to be produced (Adam-Vizi & Chinopoulos 2006). Whilst not themselves highly reactive, superoxide anions are readily converted to other free radical species such as hydroxyl ions and hydrogen peroxide (Turrens 2003). These can be involved in normal processes of cell signalling (Hamanaka & Chandel 2010) and antioxidant defence mechanisms can prevent their deleterious accumulation (Cadenas 1997). However, under conditions of oxidative stress, that is when there is an imbalance between ROS generation and the capacity of antioxidant mechanisms, oxidative damage to a number of cellular components including proteins and DNA can occur (Richter *et al.* 1988; Stadtman & Levine 2000).

The proximity of mtDNA to the site of ROS generation, together with the inefficiency of mtDNA repair mechanisms, may render mtDNA particularly susceptible to ROS-induced DNA damage and this has been implicated in the progression of several diseases as well as in organismal aging (Chan 2006). Indeed, the mitochondrial theory of aging proposes that dysfunction of the electron transport chain caused by oxidative damage further increases ROS production, and disrupts

ATP production (Harman 1956; Harman 1983). However, despite evidence for accumulation of mtDNA damage and decreased respiratory function associated with aging, it remains unclear whether ROS production has a causative effect (Chan 2006).

1.3 Mitochondria in the oocyte and early embryo

Mitochondria in the oocyte and embryo exhibit some unique features, and undergo a number of changes during oogenesis, oocyte maturation, fertilisation and pre-implantation development. These changes involve alterations to mitochondrial structure, numbers, and activity and will be described in relation to energy provision during early development.

1.3.1 Mitochondrial transcription and replication

Mitochondria undergo a dramatic increase in numbers during oogenesis, from a few hundred in the primordial germ cell (PGC) to around 10,000 in the primary oocyte, and eventually number in the hundreds of thousands in the mature oocyte, making them one of the most abundant cellular organelles (Piko & Matsumoto 1976; Sathananthan & Trounson 2000; Piko & Taylor 1987). Mitochondrial replication is then inactivated and resumes only after implantation (Piko & Taylor 1987; Ebert *et al.* 1988). Each mitochondrion in the oocyte is thought to contain only 1-2 copies of the mitochondrial genome, which becomes transcriptionally active at the late 2-cell stage (Piko & Taylor 1987; Telford *et al.* 1990).

Mitochondrial transcription and replication involves the co-ordination of the nuclear and mitochondrial genomes. Mitochondrial RNA polymerase and the mitochondrial

transcription factor TFAM are required (Larsson *et al.* 1998; Ekstrand *et al.* 2004) and appear to be co-ordinated by PGC-1α in a signalling pathway involving the mitochondrial transcription specificity factors TFB1M and TFB2M, and the nuclear respiratory factors NRF-1 and NRF-2 (Gleyzer *et al.* 2005; Virbasius & Scarpulla 1994). Replication is also dependent on the mitochondrial DNA polymerase γ (POLG) (Hubscher *et al.* 1979), and mitochondrial single-stranded DNA-binding protein (mtSSBP) and TWINKLE which are responsible for helicase destabilisation (Clay Montier *et al.* 2009). Interestingly, in mouse pre-implantation embryos, although transcripts of replication factors are present in the morula, mtDNA replication does not occur until the blastocyst stage, suggesting that regulation of mtDNA replication occurs at the post-transcriptional level (Thundathil *et al.* 2005).

Nonetheless, despite increasing knowledge of the molecular mechanisms governing mitochondrial transcription and replication, the pathways regulating these activities in response to physiological stimuli are not well understood. Thus, how mitochondrial transcription is activated at the 2-cell stage in the mouse, whilst replication of mDNA does not occur until after implantation is not known. It is however clear that the mitochondrial complement contained within the mature oocyte must be capable of meeting the energy requirements of fertilisation and early embryonic development until mitochondrial replication is initiated.

1.3.2 Mitochondrial DNA bottleneck

The observation that mtDNA sequence variants segregate rapidly between generations has led to the proposal that a mitochondrial bottleneck exists during oogenesis, deriving from the amplification of a limited number of mtDNA templates

in order to populate the pre-ovulatory oocyte (Shoubridge 2000). The bottleneck appears to occur in PGCs although exactly how it is achieved is not entirely clear (Cao *et al.* 2007; Cree *et al.* 2008; Khrapko 2008; Wai *et al.* 2008; Cao *et al.* 2009).

Studies in the mouse have suggested that in order to achieve the observed genetic drift in mtDNA, PGCs should contain around 200 copies of mtDNA (Jenuth *et al.* 1996). However, it has been estimated that mtDNA copy number is around 1500-3000 in PGCs, with no reduction of mtDNA occuring (Cao *et al.* 2007; Cao *et al.* 2009). These authors thus concluded that the mitochondrial bottleneck results from a limited number of effective segregation units, either due to the aggregation of mtDNA into nucleoids, or the preferential amplification of a subgroup of mtDNA. However, another group found that when PGCs were first detectable, at 7.5 days *post coitum* (d.p.c), the median mtDNA copy number was around 200, rising to around 1500 at 14.5 d.p.c (Cree *et al.* 2008). Using a mathematical model, they predicted that approximately 70% of the heteroplasmic variance detected was due to the reduction of mtDNA to around 200 copies per cell in early PGCs, with the remaining 30% accounted for by the rapid expansion of mtDNA in the expanding PGC population.

Yet another study has proposed an alternative timing for the mitochondrial bottleneck, based on measurement of mtDNA copy number and levels of genotypic variance. Indeed it was found that despite a reduction in mtDNA similar to that reported by Cree and colleagues, this is not the source of genotypic variance (Wai *et al.* 2008). The genetic bottleneck was instead proposed to occur due to the selective replication of a subset of mtDNA during the growth and maturation of oocytes in

their follicles, as shown by the incorporation of a label for mtDNA replication into only a small proportion of mitochondria in primordial and primary follicles. These authors proposed that a physical bottleneck occurs in the PGCs which serves to eliminate severely deleterious mtDNA mutations, whilst the genetic bottleneck during folliculogenesis segregates neutral and less deleterious mDNA mutations by replication of a subpopulation of mtDNA. How this preferential replication occurs has not been elucidated. Thus it remains unclear exactly when and how the mitochondrial genetic bottleneck occurs.

1.3.3 Mitochondrial structure

In contrast to the elongated, tubular mitochondria observed in somatic cells, mitochondria in growing oocytes, maturing oocytes and early embryos are generally small and spherical or oval in shape, display a dense matrix, and do not contain large numbers of cristae (Fig. 1.3A). This has led to the suggestion that oocyte and early embryonic mitochondria may exhibit low activity (Sathananthan & Trounson 2000; Motta *et al.* 2000). Although experimental evidence suggests that mitochondrial oxidative phosphorylation is crucial to ATP production in the oocyte (see section 1.3.4) the abundance of mitochondria in the oocyte (Piko & Matsumoto 1976; Sathananthan & Trounson 2000) may allow limited activity of individual mitochondria, potentially as a way of minimising harmful ROS production during the long period of arrest in the ovary.

Indeed, mitochondrial ultrastructure changes progressively through early development, and by the 8-cell stage the matrix begins to become progressively less dense, although the cristae remain largely unchanged (Sathananthan & Trounson

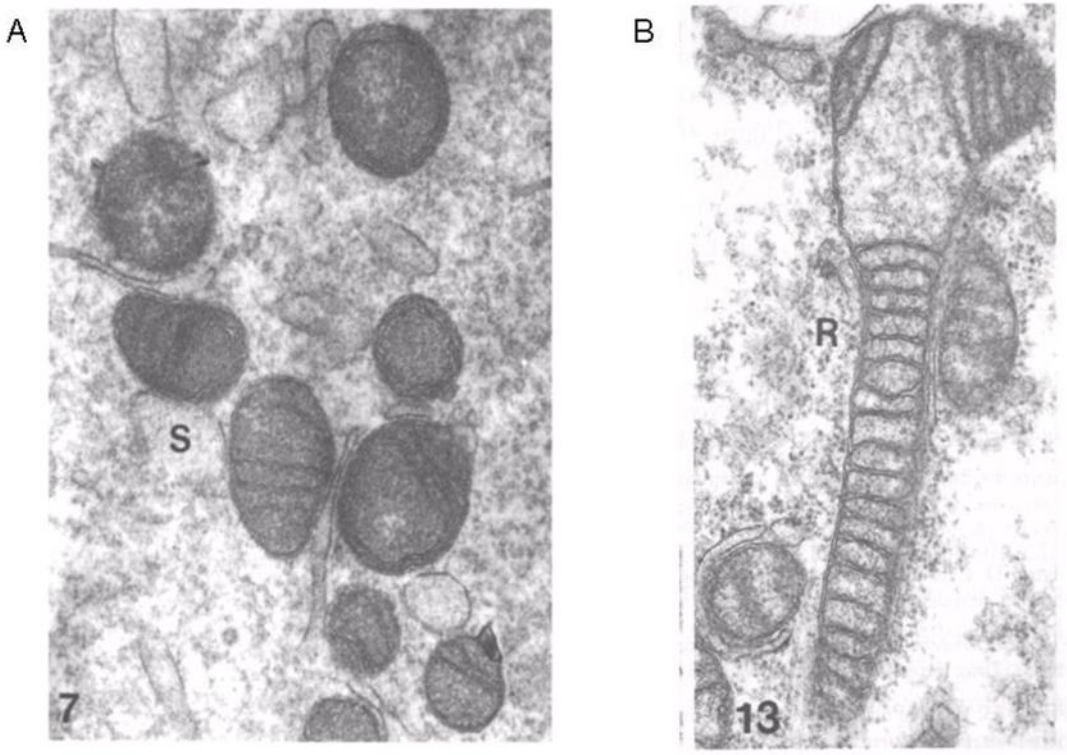

Figure 1.3 Structural changes to mitochondria during early development. EM micrographs showing mitochondrial structure. A) Mitochondria in a mature MII oocyte are round, have a dense matrix and few cristae. B) Mitochondria in the hatched blastocyst are elongated, have decreased matrix density and exhibit well developed cristae. From Sathananthan and Trounson, Hum. Reprod., 2000;15 (suppl2):148-159, by permission of Oxford University Press.

2000). At the time of differentiation, expansion and hatching of the blastocyst, and coincident with a change of substrate preference from pyruvate and lactate to glucose and an increase in metabolic activity (Brinster 1967; Leese & Barton 1984; Gardner & Leese 1986; Houghton *et al.* 1996b), mitochondria begin to take on an appearance more similar to that observed in somatic cells, displaying increasing numbers of cristae and becoming more tubular and elongated in shape (Sathananthan & Trounson 2000) (Fig. 1.3B).

1.3.4 Metabolism of the oocyte and pre-implantation embryo

Mouse oocytes and early embryos exhibit a preference for pyruvate as a metabolic substrate, and maturation and development to 2-cell stage can be supported in media supplemented with pyruvate but not glucose or lactate (Biggers *et al.* 1967). Glucose is unable to support development in the mouse until the 4-cell stage, whilst lactate can support development from the 2-cell stage (Brinster 1965). The key role of pyruvate as an energy substrate in pre-implantation embryo development is supported by studies demonstrating a preferential uptake of pyruvate until the morula to blastocyst transition, at which point there is an increase in metabolic activity accompanied by a dramatic increase in glucose uptake (Brinster 1967; Leese & Barton 1984; Gardner & Leese 1986; Houghton *et al.* 1996b). Additionally, oocytes lacking activity of the enzyme pyruvate dehydrogenase are unable to support development and exhibit compromised maturation (Johnson *et al.* 2007).

Glycolytic activity is suppressed in the mouse oocyte and early embryo and thus contributes little to ATP production (Dumollard *et al.* 2007a; Cetica *et al.* 2002; Saito *et al.* 1994). This is thought to be due to a block in a key step of the glycolytic

pathway, the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate, catalysed by the enzyme phosphofructokinase (Barbehenn *et al.* 1974; Barbehenn *et al.* 1978). Regulation of hexokinase activity (Houghton *et al.* 1996a; Ayabe *et al.* 1994), expression of glucose transporters (Pantaleon & Kaye 1998; Hogan *et al.* 1991; Morita *et al.* 1992), and the ratio of ATP/ADP (Leese *et al.* 1984) may also play a role in maintaining low glycolytic activity.

Despite the inability of lactate to support development before the 2-cell stage (Brinster 1965) it is readily taken up by 1 and 2-cell embryos, albeit in reduced amounts compared to pyruvate (Wales & Whittingham 1967). Interestingly, it has been shown that in the mouse oocyte and zygote exogenous pyruvate but not lactate-derived pyruvate is used for mitochondrial ATP production, implying firstly that the source of pyruvate is important, and secondly providing an explanation for the inability of lactate to support development until after the 2-cell stage (Dumollard *et al.* 2007a). Lactate has been proposed instead to participate in regulation of redox potential (Dumollard *et al.* 2007a; Lane & Gardner 2000). The oocyte and pre-implantation mouse embryo thus rely on oxidative phosphorylation of pyruvate to supply ATP, with a switch to a combined metabolism of both glycolysis and oxidative phosphorylation occurring at the blastocyst stage.

1.3.5 Cumulus cell derived metabolic support

Cumulus cells are specialised granulosa cells, which surround the fully grown oocyte in compact layers and are linked to the oocyte via gap junctions assembled by connexin 37, allowing bi-directional communication to occur (Anderson & Albertini 1976). Interaction between cumulus cells and the oocyte participates in numerous

processes in the oocyte, including control of meiotic induction, regulation of membrane potential, and suppression of transcriptional activity in the oocyte, whilst the oocyte also regulates aspects of the development and activity of granulosa cells (Eppig 2001; Gilchrist *et al.* 2008).

Bi-directional communication between oocyte and cumulus cells also contributes to metabolic activity of the cumulus-oocyte complex. The first indication of a role for cumulus cells in providing metabolic support to the oocyte came from studies which showed that in the absence of cumulus cells, only pyruvate was able to sustain oocyte maturation, but in the presence of cumulus cells, glucose and lactate were sufficient to allow maturation of the oocyte, suggesting that cumulus cells are able to metabolise glucose into products which can be used by the oocyte (Biggers *et al.* 1967). Indeed, expression of glycolytic enzymes and levels of glycolytic activity are high in cumulus cells but low in oocytes (Sugiura *et al.* 2005; Downs & Utecht 1999). Furthermore, cumulus cells produce pyruvate (Leese & Barton 1985; Donahue & Stern 1968), which could be supplied to the oocyte, although whether this is secreted into the media and taken up by oocytes or transmitted to the oocyte via gap junctions is unclear. Moreover, the presence of cumulus cells can partially compensate for a lack of pyruvate dehydrogenase activity in oocytes (Johnson *et al.* 2007), and cumulus cells may also contribute to oocyte metabolism by uptake of amino acids which the oocyte is unable to take up, and subsequent transfer of these amino acids to the oocyte via gap junctions (Colonna & Mangia 1983; Eppig *et al.* 2005; Haghighat & Van Winkle 1990).

Figure 1.4 Metabolic cooperation between oocytes and cumulus cells. Oocytes can obtain pyruvate, cholesterol and alanine from cumulus cells. Secretion of paracrine factors by the oocyte can regulate these processes. From Su et al., Semin. Reprod.Med., 2009;27(1):32-42.

Interestingly, the oocyte appears to be able to regulate metabolic activity in the cumulus cells, at least in the mouse. In the case of amino acid uptake, fully grown oocytes were able to promote expression of an amino acid transporter and uptake of L-alanine in cumulus cells, either as part of a cumulus-oocyte complex or when oocytectomised complexes were co-cultured with oocytes (Eppig *et al.* 2005). A similar finding was obtained for cumulus cell expression of transcripts encoding glycolytic proteins, which were up regulated in the presence of the oocyte, as were both glycolytic and TCA cycle activity in cumulus cells (Sugiura *et al.* 2005), suggesting that paracrine signalling by the oocyte is able to regulate at least two aspects of metabolic cooperation between the oocyte and the cumulus cells. BMP15 and FGFs have been implicated as paracrine factors produced by the oocyte which can regulate glycolysis in the cumulus cells (Sugiura *et al.* 2007). Thus, a functional metabolic relationship between the oocyte and its surrounding cumulus cells exists, which is regulated by the oocyte to ensure adequate metabolic support. A summary can be found in Figure 1.4.

1.4 The influence of mitochondria on developmental competence

1.4.1 Mitochondrial complement

In the human oocyte, measurements of mitochondrial content based on mtDNA copy number have found variable amounts, ranging from 190,000 to 800,000 (Reynier *et al.* 2001; Santos *et al.* 2006; May-Panloup *et al.* 2005; Steuerwald *et al.* 2000; Barritt *et al.* 2002), whilst in the mouse, numbers range between around 100,000 to 160,000 (Steuerwald *et al.* 2000; Piko & Matsumoto 1976; Piko & Taylor 1987). It would seem therefore that the mitochondrial complement in oocytes is quite variable, and

this has been shown to be the case even in oocytes from the same donor (Tamassia *et al.* 2004; Barritt *et al.* 2002; Reynier *et al.* 2001).

Nonetheless, mitochondrial complement of the oocyte has been suggested to have an impact on developmental competence. Low mitochondrial content, as measured by mtDNA copy number, has been linked to fertilisation or developmental failure (Santos *et al.* 2006; Reynier *et al.* 2001; El Shourbagy *et al.* 2006; Wai *et al.* 2010; May-Panloup *et al.* 2005), the absence of a meiotic spindle in *in vitro* matured human oocytes (Zeng *et al.* 2007) and to decreased developmental competence of *in vitro* matured rat oocytes from small antral follicles (Zeng *et al.* 2009). However, other studies have not found a correlation between mtDNA copy number and developmental outcome (Barritt *et al.* 2002; Tamassia *et al.* 2004), thus it remains unclear whether it is a factor. However, given the lack of mitochondrial replication until after implantation, and the dependence on mitochondria for ATP provision during oocyte maturation and early embryogenesis, it might follow that a minimum number of mitochondria is required in the oocyte to ensure successful development.

1.4.2 ATP content

ATP content has been reported to reflect developmental capacity in oocytes from several species. A higher rate of successful pregnancy outcome was reported in women undergoing *in vitro* fertilisation (IVF) where the average ATP content of oocytes was above 2 pmol/oocyte (Van Blerkom *et al.* 1995). Higher ATP levels were also found in human oocytes which matured successfully *in vitro* and in which a meiotic spindle was detectable, compared to those which failed to mature and in which a meiotic spindle was not detected (Zeng *et al.* 2007).In bovine oocytes, those

that were classified as good based on morphological criteria had higher ATP levels during maturation, which persisted to the expanded blastocyst stage and were accompanied by greater cell numbers per blastocyst (Stojkovic *et al.* 2001). Additionally, ATP levels were found to be higher in oocytes from donor cows with good reproductive success and this was associated with a greater proportion of development to blastocyst (Tamassia *et al.* 2004).

Conversely, reduced developmental competence of rat oocytes matured *in vitro* has been associated with a reduction in ATP content (Zeng *et al.* 2009), and inducing mitochondrial damage in mouse oocytes leads to a decreased pre- and post-implantation development, associated with lower $\Delta\Psi_m$ and lower ATP (Thouas *et al.* 2004; Thouas *et al.* 2006). Furthermore, induction of ROS leads to reduced ATP and damages MII spindle structure (Zhang *et al.* 2006). In mouse oocytes deficient in pyruvate dehydrogenase, thereby prevented from using pyruvate for oxidative phosphorylation, ATP levels in ovulated oocytes are lower and the majority fail to mature successfully and exhibit spindle and chromosomal abnormalities (Johnson *et al.* 2007).

However, whilst these studies suggest a link between ATP content and developmental outcome, no difference in ATP levels was detected between high and low competency pig oocytes (Brevini *et al.* 2005) or between *in vivo* and *in vitro* matured pig oocytes, despite a different capacity for continued development (Brad *et al.* 2003), suggesting that whilst ATP content of the oocyte may reflect developmental competency in some species, it is not necessarily correlated with developmental success in all species.

Changes to ATP levels during oocyte maturation have also been reported. In pig (Sun *et al.* 2001; Brevini *et al.* 2005) and bovine oocytes (Stojkovic *et al.* 2001), this manifests itself as an increase between GV and MII stages. In the cat oocyte however, a decrease in ATP content was observed (Freistedt *et al.* 2001). Interestingly, in the human oocyte, no change to ATP content was measured before and after maturation (Van Blerkom *et al.* 1995), although this study measured ATP content of pooled oocytes which may have masked changes in individual oocytes. It is not clear however what impact changes in ATP levels during maturation may have in terms of developmental competence.

1.4.3 Mitochondrial membrane potential

$\Delta\Psi_m$ is thought to be an indicator of mitochondrial activity and thus has also been proposed to have an effect on ATP levels and developmental competence, although it should be noted that $\Delta\Psi_m$ does not necessarily reflect the level of metabolic activity (Richter *et al.* 1996; Diaz *et al.* 1999). In human oocytes and pre-implantation embryos, $\Delta\Psi_m$ has been reported to be inversely correlated with maternal age, and reduced $\Delta\Psi_m$ has been linked to decreased developmental potential, aberrant spindle structure and chaotic mosaicism (Wilding *et al.* 2001; Wilding *et al.* 2003). Lower $\Delta\Psi_m$ has also been detected in thawed cryopreserved oocytes, accompanied by a decreased rate of development to blastocyst, although this was not associated with a decrease in ATP levels (Jones *et al.* 2004; Ahn *et al.* 2002). $\Delta\Psi_m$ has also been reported to change during pre-implantation development and these changes were proposed to be associated with developmental potential (Acton *et al.* 2004).

Additionally, heterogeneity in $\Delta\Psi_m$ at the subcellular level in oocytes and embryos has been reported. In oocyte and pre-implantation embryos, $\Delta\Psi_m$ appears to be related to cell-to-cell contact, with pericortical mitochondria exhibiting higher $\Delta\Psi_m$, (Van Blerkom *et al.* 2002; Jones *et al.* 2004) although it should be noted that this was not detected in other studies (Wilding *et al.* 2001; Dell'Aquila *et al.* 2009). At the blastocyst stage, high polarised mitochondria are evident in the mural trophectoderm whilst those of the inner cell mass exhibit low $\Delta\Psi_m$ (Van Blerkom *et al.* 2006; Barnett *et al.* 1996), although this is not the case in the pig (Sun *et al.* 2001). However, it should be noted that these studies were carried out using the indicator JC-1 which, as described above (see section 1.2.6), is not necessarily appropriate for detecting subcellular heterogeneity in $\Delta\Psi_m$. Furthermore, the functional significance of subcellular $\Delta\Psi_m$ heterogeneity in the oocyte and embryo remains unclear (Van Blerkom & Davis 2006).

1.4.4 Stage-specific redistributions

Mitochondria undergo changes to their distribution during oocyte maturation in a number of species and disruptions to the normal pattern of distribution have been linked to fertilisation and developmental failure. These redistributions were first observed in the mouse, where Van Blerkom and Runner described a mitochondrial translocation to the perinuclear region during formation of the first meiotic spindle, followed by a redistribution of mitochondria at the time of polar body extrusion (Van Blerkom & Runner 1984). These changes were only seen to occur in maturing oocytes and thus were proposed to be a necessary feature of oocyte maturation. Another study in mouse oocytes described a similar redistribution of mitochondria during oocyte maturation but also noted some mitochondrial accumulation around

the MII spindle, accompanied by a concentration of non-spindle associated mitochondria in the hemisphere of the oocyte containing the MII spindle (Calarco 1995).

Mitochondrial redistributions have also been noted in the human oocyte (Wilding *et al.* 2001), the bovine oocyte, where they were correlated with increased development to blastocyst and higher ATP levels (Stojkovic *et al.* 2001), and the pig oocyte (Sun *et al.* 2001; Brevini *et al.* 2005), where the pattern of mitochondrial distribution was found to influence mitochondrial activity, as measured MitoTracker Orange fluorescence intensity (Torner *et al.* 2004). The abnormal distribution of mitochondria in mouse oocytes at MII has been linked to reduced capacity for embryo development (Nagai *et al.* 2006).

The oocyte microtubule network appears to be responsible for the translocations of mitochondria observed in many species (Van Blerkom 1991; Brevini *et al.* 2005; Sun *et al.* 2001; Liu *et al.* 2010) and is likely to involve cytoplasmic rather than spindle associated microtubules (Maro *et al.* 1985; Brevini *et al.* 2005). Interestingly, *in vitro* matured oocytes, which have lower developmental capacity than those matured *in vivo*, display reduced mitochondrial transport to the central region which is associated with a reduced cytoplasmic microtubule network (Brevini *et al.* 2005; Sun *et al.* 2001).

However, microfilaments also appear to have a role in more subtle changes to mitochondrial distribution in mouse oocytes. Yu and colleagues described three phases of mitochondrial translocation, the first occurring at GVBD when

mitochondria were found to move to the perinuclear region (Yu *et al.* 2010). Subsequently, at around 3-4 hours after the onset of maturation, mitochondria dispersed throughout the cytoplasm, before aggregating again around the spindle region. This ring of mitochondria persisted until late MI when dispersal occurred a second time, followed by re-formation of the spindle-associated ring around the time of polar body extrusion. Finally, in MII oocytes, the mitochondrial ring was not observed. Mitochondrial translocations were found to be accompanied by bursts of ATP production at the time of ring formation (Yu *et al.* 2010). Surprisingly however, whilst a burst of ATP observed at polar body extrusion was found to be associated with this event and did not occur when polar body extrusion was prevented, the first two bursts of ATP production were linked to the presence of mitochondrial clusters, the formation of which was microfilament dependent. Disruption of the formation of the mitochondrial ring by inhibition of microtubules did not affect the first two bursts of ATP production, suggesting they are unrelated to the translocation of mitochondria to the perinuclear and spindle regions.

Mitochondrial redistribution also occurs during early development in embryos from several species, where they accumulate around the perinuclear region (Bavister & Squirrell 2000; Van Blerkom *et al.* 2000; Barnett *et al.* 1997; Sun *et al.* 2001; Tokura *et al.* 1993; Wilding *et al.* 2001; Muggleton-Harris & Brown 1988). Abnormal distribution of mitochondria at the pronucleus stage results in some blastomeres with reduced mitochondrial content and diminished ATP generating capacity (Van Blerkom *et al.* 2000).

Thus, mitochondrial redistributions appear to be a feature of competent oocytes and successful early development. However, the precise way in which mitochondrial translocations contribute to developmental competence has not been established. It has been proposed by a number of investigators that compartmentalisation of mitochondria in specific subcellular locations may serve to concentrate ATP supply in areas of high demand (Van Blerkom & Runner 1984; Van Blerkom 1991; Sun *et al.* 2001; Barnett *et al.* 1996; Eichenlaub-Ritter *et al.* 2004). This could act to minimise mitochondrial activity, and resultant ROS production, which would otherwise be required to sustain sufficiently high ATP levels throughout the oocyte to meet localised high demand. Such a compartmentalisation of mitochondria to meet energy demands or Ca^{2+} buffering requirements has been described in other cell types (Kuznetsov *et al.* 2009), nonetheless experimental data supporting this proposal in oocytes is lacking.

1.4.5 Mitochondria at fertilisation; interactions with ER

Mitochondria are found in close proximity to the ER in oocytes and interaction between the two organelles has been reported. Fertilisation of the oocyte triggers a series of Ca^{2+} oscillations due to IP_3-dependent release of Ca^{2+} from the ER (Miyazaki *et al.* 1993; Kline & Kline 1994). These oscillations are necessary for egg activation (Stricker 1999). Production of ATP by the mitochondria is crucial both to maintain resting Ca^{2+} levels in the mature oocyte, and to sustain the Ca^{2+} oscillations at fertilisation (Dumollard *et al.* 2004). Furthermore, sperm-triggered Ca^{2+} oscillations are transmitted to the mitochondria where they up regulate mitochondrial ATP production to meet the energy requirements of egg activation (Dumollard *et al.* 2008; Campbell & Swann 2006; Liu *et al.* 2001).

Disruptions to this tightly regulated signalling process have been shown to have detrimental effects on development. This is perhaps not surprising given that the number, amplitude, frequency, and duration of increases in cytosolic Ca^{2+} have been shown to be important for triggering specific events at fertilisation, and to have a role in determining continuing development (Ducibella *et al.* 2002; Ozil & Huneau 2001; Bos-Mikich *et al.* 1997; Ozil *et al.* 2005). Inhibition of mitochondrial function in the oocyte leads to sustained elevation of ER-released Ca^{2+} in place of Ca^{2+} oscillations (Liu *et al.* 2001) and impaired mitochondrial activity appears to contribute to disrupted Ca^{2} signalling in *in vitro* aged oocytes, possibly contributing to their reduced ability to give rise to developmentally competent embryos.

Indeed, the pattern of Ca^{2+} oscillations in post-ovulatory aged oocytes has been shown to be abnormal, and Ca^{2+} re-uptake is impaired (Igarashi *et al.* 1997; Jones & Whittingham 1996). Fertilisation of *in vitro* aged oocytes can cause induction of abnormally high frequency Ca^{2+} oscillations resulting in the triggering of apoptosis rather than activation (Gordo *et al.* 2000; Gordo *et al.* 2002). This is accompanied by the failure of *in vitro* aged oocytes to up regulate ATP production at fertilisation (Igarashi *et al.* 2005) and decreased content of ER Ca^{2+} stores, likely deriving from insufficient supply of ATP for store refilling (Takahashi *et al.* 2000), leading to a reduction in successful continued development (Takahashi *et al.* 2009).

1.4.6 Aging

Female fertility declines with age and oocytes and embryos from older women exhibit increased levels of chromosomal abnormalities. Mitochondrial activity has been found to decrease with increasing maternal age, possibly contributing to this

reproductive aging. In human metaphase II oocytes and pre-implantation embryos from older women, it has been reported that $\Delta\Psi_m$ is lower, and this was linked with the presence of a disrupted spindle and increased chaotic mosaicism, and with a decreased capacity of the embryo to develop (Wilding *et al.* 2001; Wilding *et al.* 2003). Similar findings have been reported for oocytes from older mice in which $\Delta\Psi_m$ and ATP levels were lower than in control oocytes retrieved from younger mice, and the oocytes exhibited a greater sensitivity to induced mitochondrial damage (Thouas *et al.* 2005). In post-ovulatory aged oocytes, used as a model for reproductive aging, ATP levels have been found to be lower than in freshly isolated oocytes (Chi *et al.* 1988). Furthermore, aberrations in both Ca^{2+} signalling and upregulation of ATP levels at fertilisation have been observed in post-ovulatory oocytes, as described above, providing a functional link between the deficiencies in mitochondrial activity observed in aged oocytes and important developmental events.

The mechanisms by which aging contributes to mitochondrial dysfunction and decreased fertility are not entirely clear. It has been reported that oocytes from older women have decreased numbers of mitochondria, accompanied by decreased density of the mitochondrial matrix in oocytes, and an increase in the frequency of ruptured mitochondrial membranes in granulosa cells (de Bruin *et al.* 2004; Chan *et al.* 2005). However, another study has reported increased numbers of mitochondria, possibly reflecting changes in oxidative phosphorylation capacity, although the changes were not associated with any mutations in mtDNA, or defects in the activity of respiratory chain enzymes (Muller-Hocker *et al.* 1996).

It has been proposed that mtDNA mutations may accumulate during the time the oocyte is arrested in the ovary and contribute to reduced developmental competence with increasing age; it remains unclear however whether an increase in mtDNA mutations actually occurs. Whilst some studies have reported increased incidence of mtDNA mutations (Barritt *et al.* 2000; Chan *et al.* 2005), other studies have failed to find a correlation between advanced reproductive age and rearrangements of mtDNA, although it was observed that there is a high incidence of a common mtDNA mutation in all oocytes and embryos and this is associated with failure to develop (Barritt *et al.* 1999; Brenner *et al.* 1998; Hsieh *et al.* 2002). These discrepancies may be explained by the fact that the studies examined different regions of mtDNA which may have different susceptibilities to mutation with increasing age such that some regions exhibit increasing levels of mutation with age whilst others do not.

In somatic cells, it has been postulated that mitochondria contribute to aging due to production of ROS, leading to accumulation of mtDNA mutations, resulting in a reduced capacity for oxidative phosphorylation and ATP production (Harman 1956; Harman 1983). An attractive hypothesis is that production of ROS during the long period of oocyte arrest in the ovary may cause accumulation of mtDNA mutations resulting in defective mitochondria. This could lead to the inability of the oocyte to produce sufficient ATP for development, thus contributing to decreased developmental competence and increased rates of aneuploidy in older women (Schon *et al.* 2000; Eichenlaub-Ritter *et al.* 2004; Tarin 1996). Indeed, oocytes and embryos are particularly sensitive to oxidative stress, and induction of oxidative stress in MII oocytes leads to a reduction in $\Delta\Psi_m$ and ATP levels via opening of the mitochondrial

permeability transition pore (mPTP), causing disruption of the spindle (Zhang *et al.* 2006). Similar results have been found when oxidative stress is induced in mouse zygotes leading to developmental arrest and apoptosis (Liu *et al.* 2000).

The presence of antioxidants has been shown to counteract the negative effects of ROS induction on spindle morphology and chromosome segregation in oocytes (Zhang *et al.* 2006; Tarin *et al.* 1998; Tarin *et al.* 2002) and to prevent telomere shortening, chromosome fusion and apoptosis in 1-cell mouse zygotes with induced mitochondrial damage (Liu *et al.* 2002). Moreover, oocytes from aged mice and older women display reductions in the expression of genes involved in mitochondrial function and antioxidant defence (Hamatani *et al.* 2004; Steuerwald *et al.* 2007) and the activity of the antioxidant enzyme superoxide dismutase is reduced in the cumulus cells of oocytes from older donors (Matos *et al.* 2009; Tatone *et al.* 2006), suggesting a reduction in antioxidant defence may contribute to some of the defects observed in aged oocytes. Thus, ROS production, decreased antioxidant defence and dysfunctional mitochondria may contribute to decreased developmental competence in reproductive aging; however further work will be required to demonstrate the link conclusively.

1.5 Synopsis

It is clear from the themes reviewed in this introduction that the mitochondria contained within the fully grown oocyte may play a crucial role in determining developmental competence. Mitochondrial replication is halted in the fully grown oocyte and does not resume until after implantation. Moreover, glycolysis is blocked in the oocyte and early embryo, requiring mitochondria to carry the burden of energy provision throughout early development. Thus, the mitochondria of the oocyte must be capable of sustaining the earliest stages of life. Indeed, disruptions to both mitochondrial distribution and function have been implicated in developmental arrest.

The aim of this thesis is therefore to investigate the distribution and function of mitochondria during maturation of the mouse oocyte. In particular, it has been more than 25 years since mitochondria were observed to undergo redistribution during murine oocyte maturation, and since it was suggested that translocation to the spindle region may be necessary to maintain spindle function. Nonetheless, it remains unclear whether this is the case, and whether compartmentalisation of mitochondria in this manner results in heterogeneity in ATP supply in different areas of the oocyte. Furthermore, although it is now clear that mitochondrial ATP provision is crucial for successful fertilisation, little is known about mitochondrial ATP supply and demand during oocyte maturation and how this may impact upon developmental competence.

Chapter 3 therefore describes the time course and mechanism of mitochondrial translocations during oocyte maturation and relates these both to the location and

function of the meiotic spindle. In Chapter 4, the role of mitochondrial adapter proteins in establishing mitochondrial distribution is examined, with a view to modulating their function in order to examine what the effects of mitochondrial redistribution may be on the formation and function of the spindle. Chapter 5 describes the use of a novel Förster resonance energy transfer (FRET) based ATP probe to monitor ATP levels and consumption in the maturing oocyte, and links these observations to the progression of oocyte maturation. Furthermore, this novel method for measuring ATP is also employed to attempt to elucidate whether heterogeneity in ATP supply in the spindle region can be observed. Finally, Chapter 6 summarises the data presented and discusses the findings in the context of our current understanding of contribution of mitochondria to the establishment of developmental competence.

2. Materials and Methods

2.1 Oocyte and embryo collection

Germinal vesicle stage oocytes were collected from 21-24 day old female MF1 mice that had been administered 7 international units (IU) pregnant mare's serum gonadotropin (PMSG) (Intervet) by intraperitoneal injection 48 hours earlier. Mice were culled by cervical dislocation and ovaries were collected into warmed M2 medium (Sigma-Aldrich) maintained at 37ºC. To release the oocytes, ovaries were punctured with a 27 gauge needle, oocytes were collected with a mouth pipette, and placed in drops of M2 under mineral oil (Sigma-Aldrich) to prevent evaporation. Only oocytes with an intact layer of cumulus cells were selected and these were subsequently removed by repeated pipetting with a narrow bore pipette. Oocytes were maintained in GV arrest where necessary by the addition of 200 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich) to the medium. Subsequent release from GV arrest was achieved by washing the oocytes through three drops of M2 without IBMX.

For *in vitro* maturation, denuded oocytes were incubated in drops of M16 medium (Sigma-Aldrich) under mineral oil at 37°C in an atmosphere of 5% CO_2, in air. For experiments examining *in vivo* maturation, oocytes were collected 6 hours after administration of hCG by the same method as GV stage oocytes but without the addition of IBMX to the medium.

To recover mature (MII) oocytes, mice were injected intraperitoneally with 7.5 IU human chorionic gonadotropin (hCG) (Intervet) 46-48 hours after PMSG injection.

Oviducts were collected from culled mice 12-14 hours after hCG administration. Cumulus enclosed oocytes were released into M2 medium by tearing the oviduct with a 27-gauge needle and cumulus cells were removed by the addition of 300 µg/ml hyaluronidase (Sigma-Aldrich) to the M2 medium. Oocytes were subsequently washed through three drops of M2 without hyaluronidase and maintained in M2 medium under mineral oil at 37 ºC.

Two-cell embryos were collected by mating female mice with MF1 male mice at the time of hCG administration. Oviducts were collected from mice culled 48 hours after hCG administration. Embryos were released into warmed HKSOM medium by tearing the oviduct, and placed in drops of HKSOM under mineral oil at 37 ºC.

2.2 *In vitro* fertilisation

MF1 male mice were culled by cervical dislocation and sperm were released from the epididymi into T6 media containing 10 mg/ml bovine serum albumin (BSA) (Sigma-Aldrich) which had been pre-equilibrated to pH 7.6 at 37 ºC in an atmosphere of 5% CO_2 in air. Following a 20 to 30 minute swim-out period, 100 µl of sperm solution was added to 100 µl T6 medium under mineral oil and placed at 37 ºC in an atmosphere of 5% CO_2 in air for 2 hours to allow capacitation. In order to carry out IVF, the zona was removed from MII oocytes by brief exposure to acidified Tyrode's solution (Sigma-Aldrich) at 37 ºC followed by repeated washing in M2 medium. Oocytes were then placed into a chamber on a glass coverslip in 0.5 ml of BSA-free M2 medium to allow the oocytes to adhere. After approximately five minutes, 0.5 ml of complete M2 medium containing BSA was added to the chamber,

followed by 50 µl of capacitated sperm. IVF was carried out 14 to 16 hours after hCG administration.

2.3 Microinjection

Oocytes were pressure injected using a micropipette and micromanipulators mounted on an Axiovert 135 inverted microscope (Leica). Oocytes were placed in a drop of M2 under mineral oil to prevent evaporation. A holding pipette was used to position the oocytes for microinjection and the micropipette was inserted into the oocyte. A brief over compensation of negative capacitance was used to penetrate the plasma membrane and microinjection was performed using a fixed pressure pulse through a pico-pump (World Precision Instruments). Microinjection volume was estimated at 5% by cytoplasmic displacement. Following microinjection, oocytes were placed in a fresh drop of M2 medium under mineral oil at 37 ºC and allowed to recover for several minutes before any further manipulations were carried out.

2.4 Immunofluorescence staining

Oocytes were fixed in 3.7% paraformaldehyde (PFA) (Sigma-Aldrich), made up in phosphate buffered saline (PBS), under mineral oil for 50 minutes at room temperature. Oocytes were subsequently permeabilised using 0.25 % Triton (Sigma-Aldrich) in PBS for 10 minutes at room temperature. For antibody staining, oocytes were blocked against non-specific interactions using 3% BSA in PBS under mineral oil either for 1 hour at 37°C or overnight at 4°C. Mitochondria were stained using an anti-cytochrome c antibody (Santa Cruz Biotechnology) or an anti-TOM20 antibody (gift from J.Kittler, UCL), both at a concentration of 1 in 500 in PBS containing 1% BSA. Tubulin was stained using an anti-β-tubulin antibody (Sigma-Aldrich) at a

concentration of 1 in 1000 in PBS containing 1% BSA. Miro and Trak1 antibodies were generous gifts from J. Kittler, UCL, used at concentrations of 1 in 500 and 1 in 25 respectively. Following repeated washing in drops of PBS containing 1% BSA, appropriate fluorescently-conjugated secondary antibodies were then used at a concentration of 1 in 1000 (Alexa Fluor 488 or 546, Molecular Probes). All antibody incubations were carried out at 37°C for 1 hour. DNA was stained using Hoechst 33342 (Invitrogen) at a concentration of 10 µg/ml for 10 minutes at room temperature.

2.5 Cloning

Trak1, Trak1-KDM(467-698), Trak2, Trak2-KDM(283-913) and Miro1 in pML5myc vectors (gifts from J. Kittler, UCL) were cloned into either pcDNA3.1-EGFP or pCS2-mCherry-F/A vectors to allow expression of fluorescently-tagged proteins since preliminary experiments with the original myc-tagged constructs revealed significant background staining. Cloning was carried out by Jenny Bormann.

2.6 Production of mRNA

mRNA was produced from linearised template DNA using either the mMessage mMachine SP6 kit, T7 kit or the T3 kit (Applied Biosystems) following the manufacturer's instructions. A summary of the mRNA produced can be found in Table 2.1.

2.7 Confocal imaging

Confocal imaging was carried out on a LSM 510 confocal microscope (Carl Zeiss) using a 63x oil-immersion lens with a numerical aperture of 1.4. Fixed oocytes were placed in a small drop of PBS in a glass-bottomed plastic dish. For imaging of live oocytes, a chamber with a glass coverslip base containing 1ml BSA-free M2 to allow oocytes to adhere, covered with a layer of mineral oil was used. Live oocytes were maintained at a temperature of 37°C by use of a heated stage. Images were acquired every 30 seconds or every minute. Drugs and inhibitors were added at 10-100x concentrations. A summary of those used can be found in Table 2.2

Imaging of Alexa 488-conjugated antibody staining or EGFP-tagged constructs was carried out using the 488 nm laser line and a 505-550 band pass filter and images of Alexa 546-conjugated antibody staining, mCherry- or dsRed2-tagged constructs were obtained using the 543 nm laser line and a 560 long pass filter. Hoechst staining was imaged using the 405 nm laser line and a 420-480 band pass filter. The estimated optical slice thickness was 2 µm.

In order to image the ATP FRET probe on the confocal, the 405 nm laser was used for excitation and a lambda scan was carried out between 443 nm and 572 nm with a step size of 20 nm for emission. Peak emission images of the fluorophores were then selected from the resulting images, at 476 nm for mseCFP (hereafter CFP) and 540 nm for cp173-mVenus (hereafter YFP). Images were acquired every minute. For acceptor photobleaching experiments, five consecutive images were acquired before bleaching and five images after (Fig. 2.1A). Bleaching was carried out with the 514 nm laser at 100% power and the duration of the bleaching was approximately 1

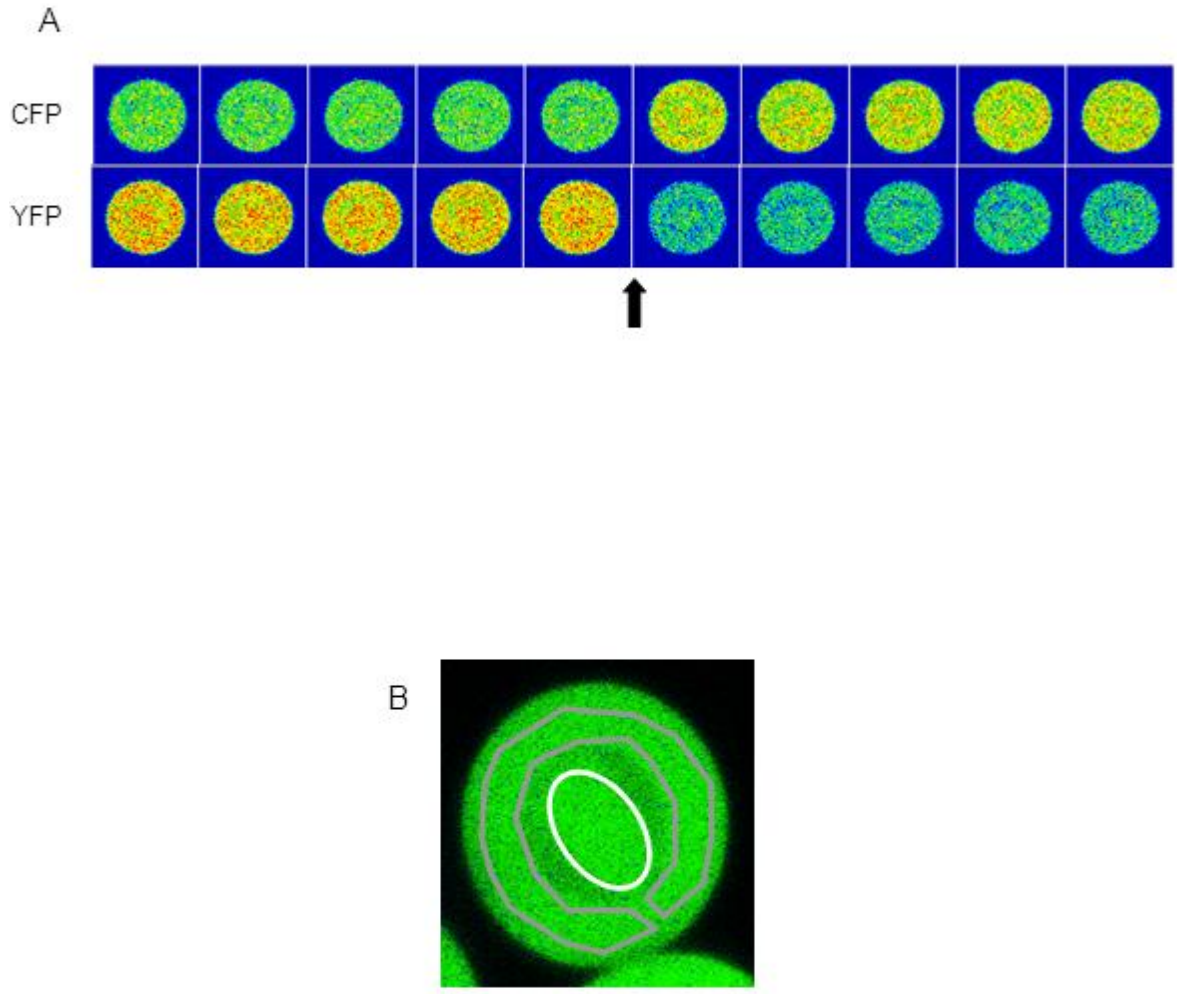

Figure 2.1. FRET acceptor photobleaching. A) The YFP signal was bleached (arrow) and the resulting percentage change in CFP and YFP fluorescence was measured. FRET efficiencies were calculated by dividing the percentage change in CFP by the percentage change in YFP to account for incomplete bleaching of the YFP signal. Warmer colours indicate higher fluorescence, cooler colours indicate lower fluorescence. B) FRET efficiencies in spindle (white) and non-spindle (grey) regions were compared by selecting ROIs in the appropriate locations. An area directly outside the spindle was excluded from both ROIs due to the low levels of fluorescence observed here in both the CFP and YFP channels.

minute, sufficient to reduce the YFP signal by at least 50%. Preliminary experiments showed that photobleaching time to attain a complete bleaching of the YFP signal was high and could be damaging to the oocytes, therefore this was not carried out. Instead, to control for incomplete bleaching of the YFP signal, FRET efficiency was calculated after background subtraction by dividing the percentage change in CFP signal by the percentage change in YFP signal. For time-lapse experiments, the FRET ratio was calculated by dividing the YFP signal by the CFP signal, after background correction. To measure spindle and non-spindle regions, ROIs were drawn in the appropriate areas of the oocyte. An area directly outside the spindle was excluded from both spindle and non-spindle ROIs as fluorescence of the probe in this region was low, likely due to the large aggregation of organelles present here (Fig. 2.1B).

2.8 Epifluorescence imaging

An Axiovert 200 microscope (Carl Zeiss) equipped with a CoolSnapHQ cooled CCD camera (Photometrics) and an XBO 75 xenon short-arc lamp microscope illuminating system (Carl Zeiss) was used for imaging, with a 20x objective with a numerical aperture of 0.75. MetaFluor software was used to control acquisition. Oocytes were placed in a chamber with a glass coverslip base containing 1ml BSA-free M2 covered with a layer of mineral oil, and maintained at 37°C by use of a heated stage. Drugs were added at 10-100x concentrations. Images were acquired every minute.

For imaging of the ATP FRET probe, a 430/25 filter for excitation of CFP, a CFP/YFP dichroic mirror, a 470/30 emission filter for CFP and a 535/30 emission

filter for YFP (Chroma Technology) were used. The FRET ratio was calculated by dividing the YFP signal by the CFP signal, after background correction. For imaging of mito-GFP, a 480/40 excitation filter was used, together with a FITC dichroic mirror and a 520 long pass emission filter (Chroma Technology). For imaging of mitochondrial membrane potential, oocytes were loaded with 50 nM TMRM (Invitrogen) in M2 medium for 30 minutes and imaged using a 546/12 excitation filter, TRITC dichroic mirror and 590 long pass emission filter. Imaging was carried out with 50 nM TMRM present in the medium.

2.9 Image analysis

Comparing the area occupied by mitochondria in the oocyte and polar body

In order to calculate the percentage area occupied by mitochondria in the oocyte or polar body, MetaMorph software was used. Confocal sections where the chromosomes at opposing ends of the spindle had been aligned in the horizontal plane were used. Thresholds were first set to distinguish mitochondria and regions were then drawn around either the oocyte or polar body. The *integrated morphometry analysis* function was used to calculate the area occupied by mitochondria and this number was divided by the area of the region to give a measure of the proportion of the oocyte or polar body occupied by mitochondria. Values were expressed as a percentage.

Spindle-associated mitochondria

In order to calculate the percentage of spindle associated mitochondria, a similar process to that described above was carried out, using MetaMorph software and setting a threshold before using the *integrated morphometry analysis* function to

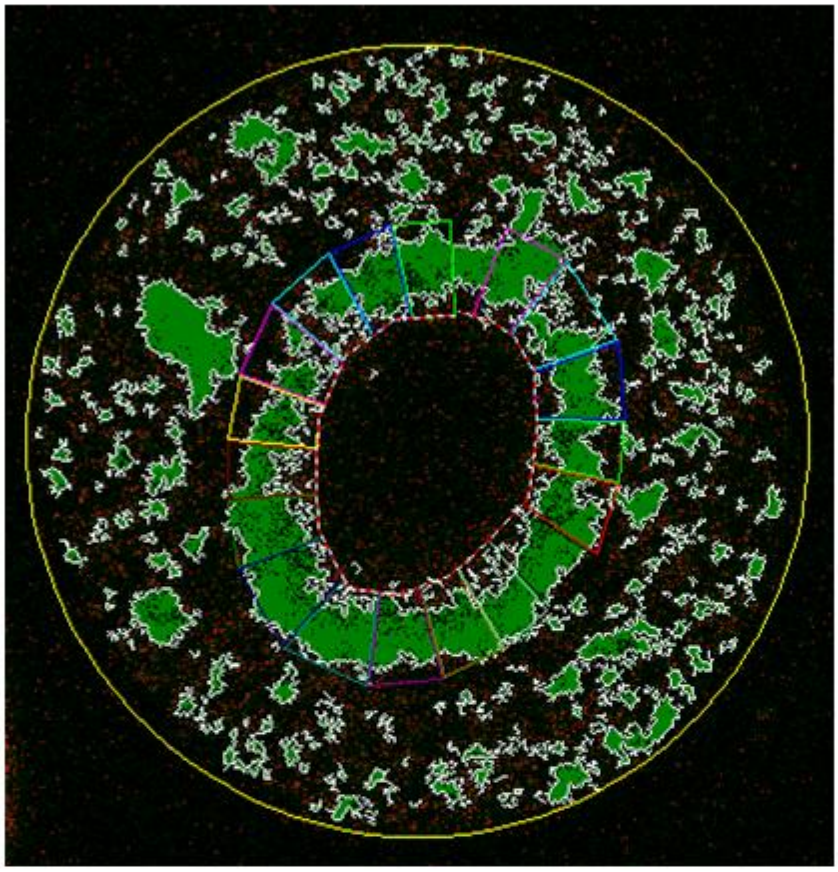

Figure 2.2. Measurement the proportion of spindle-associated mitochondria. MetaMorph analysis software was used to measure the percentage of mitochondria associated with the spindle. The spindle-associated mitochondria were defined by segment regions created around a central spindle region. Green areas are those which were recorded by the integrated morphometry analysis function.

measure the area occupied by mitochondria. Spindle-associated mitochondria were defined in a standardised manner from equatorial confocal sections by drawing a region in the central area of the oocyte, containing the spindle and devoid of mitochondria, termed the spindle region. The *draw segment region* function was then used to draw multiple segment regions around the perimeter of the spindle region, encompassing the spindle-associated mitochondrial ring. These segments had a set height of 8 μm as this size was found to include those mitochondria which were making up the mitochondrial ring associated with the spindle. Another region of interest was drawn to encompass the whole oocyte. The area occupied by mitochondria in all of the segment regions was divided by the area occupied by mitochondria in the whole oocyte to give a measure of the percentage of mitochondria which were associated with the spindle. An example can be seen in Figure 2.2.

Measurement of the rate of spindle collapse

Measurement of the rate of spindle collapse upon disruption of mitochondrial function was carried out by drawing a region of interest around the spindle and monitoring the change in fluorescence intensity in that region over time.

2.10 Statistical analysis

Statistical analysis of experiments with two groups was carried out using the unpaired t-test, except for comparison of ATP in spindle and non-spindle regions for which the paired t-test was used. Analysis of variance (ANOVA) with the Tukey-Kramer post-hoc test was used for experiments with three or more groups. * denotes a p value of <0.05, ** denotes a p value of <0.01 and *** denotes a p value of

<0.001. Error bars on column graphs indicate standard error of the mean (SEM). On box and whisker plots, the central line indicates median, the top and bottom of the box indicate 25th and 75th percentiles, and bars indicate maximum and minimum values.

Table 2.1 mRNA produced

Protein	Plasmid	Promoter used for mRNA production
MAP7-GFP	pRNA3	T3
Mito-GFP	pRNA3	T3
dsRed2ER	pcDNA3.1	T7
P50-GFP	pcDNA3.1	T7
Trak1-EGFP	pcDNA3.1	T7
Trak2-EGFP	pcDNA3.1	T7
Trak1KDM-mCherry	pCS2-mcherry-F/A	SP6
Trak2KDM-mCherry	pCS2-mcherry-F/A	SP6
Miro-mCherry	pCS2-mcherry-F/A	SP6
AT1.03	pcDNA3.1	T7
AT1.03RK	pcDNA3.1	T7

Table 2.2 Drugs used and their concentrations

Drug	Source	Concentration
Nocodazole	Sigma-Aldrich	10 µM
Latrunculin A	Sigma-Aldrich	6 µM
Oligomycin	Sigma-Aldrich	5 µg/ml
FCCP	Sigma-Aldrich	1 µM
Thapsigargin	Invitrogen	20 µM
Ouabain	Sigma-Aldrich	0.5mM
Roscovitine	Calbiochem	50 µM
U0126	Promega	50 µM
18-α-glycyrrhetinic acid	Sigma-Aldrich	10 µM
Cyclosporin A	Sigma-Aldrich	5 µM
Iodoacetic acid	Sigma-Aldrich	1mM
Cycloheximide	Sigma-Aldrich	50 µM

3. Mitochondrial Reorganisation During Oocyte Maturation

3.1 Introduction

In order to produce an egg which is competent to be fertilised and develop successfully, both nuclear and cytoplasmic maturation of the oocyte must occur. Nuclear maturation involves the resumption of meiosis I in the prophase-arrested GV oocyte, and progress to metaphase of the second meiotic division, prior to a second arrest which persists until fertilisation occurs. Cytoplasmic maturation encompasses a number of metabolic and structural changes including alterations in protein synthesis, mRNA stability, transcriptional regulation, cell signalling pathways and a reorganisation of the cytoplasm. An alteration in the distribution of mitochondria is one of the components of cytoplasmic maturation and has been described in a number of species (Barnett *et al.* 1996; Yu *et al.* 2010; Valentini *et al.* 2010; Brevini *et al.* 2005; Wilding *et al.* 2001; Sun *et al.* 2001; Bavister & Squirrell 2000; Calarco 1995; Van Blerkom & Runner 1984; Stojkovic *et al.* 2001).

Mitochondrial function in the oocyte is thought to be an important factor in establishing developmental competence (Thouas *et al.* 2006; Thouas *et al.* 2004; Wilding *et al.* 2003; Van Blerkom *et al.* 1995). However, although mitochondrial redistributions during oocyte maturation have been proposed to be a factor in determining developmental success, it is not yet clear how they play a role in producing a competent oocyte (Brevini *et al.* 2005; Bavister & Squirrell 2000; Van Blerkom & Runner 1984; Stojkovic *et al.* 2001; Sun *et al.* 2001; Brevini *et al.* 2005;

Bavister & Squirrell 2000). It has been suggested that mitochondrial accumulation in the region of the first meiotic spindle may be necessary for formation and maintenance of the spindle structure which, in a large cell such as the oocyte, may not receive sufficient ATP supply from diffusion alone (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004). Additionally, movement of mitochondria to regions of high energy demand many serve to minimise production of damaging ROS by concentrating mitochondria in areas where ATP requirements are greatest, rather than increasing overall mitochondrial activity and therefore ROS production, in order to meet ATP demand (Dumollard *et al.* 2009). Mitochondrial dysfunction has been linked to the generation of aberrant spindles (Zhang *et al.* 2006; Johnson *et al.* 2007; Zeng *et al.* 2007; Wang *et al.* 2009), however experimental evidence linking spindle-associated mitochondrial accumulation with spindle formation and function is lacking.

In animal cells, mitochondrial transport generally occurs along microtubule tracks, although actin filaments may play also play a role in positioning mitochondria in some cell types (Boldogh & Pon 2007). In oocytes, microtubule-based transport is thought to be responsible for redistributing mitochondria (Van Blerkom 1991; Sun *et al.* 2001), although a role for actin in mitochondrial clustering has recently been described (Yu *et al.* 2010). Movement along microtubule tracks occurs via the action of the motor proteins kinesin and dynein, with kinesin driving movement in the anterograde direction and dynein driving movement in the retrograde direction (Hirokawa *et al.* 1998).

The ER has been previously shown to undergo redistributions during oocyte maturation, notably with an accumulation observed around the first meiotic spindle (Mehlmann *et al.* 1995; Fitzharris *et al.* 2007). In the mature MII oocyte, mitochondria and ER have been demonstrated to be in close apposition and functionally linked, with fertilisation-triggered calcium oscillations being transmitted to the mitochondria resulting in increased mitochondrial activity (Dumollard *et al.* 2003; Campbell & Swann 2006; Dumollard *et al.* 2004). The apparently similar redistributions observed for the ER and mitochondria raise the possibility that a similar mechanism of ER triggered up-regulation of mitochondrial activity may be functional during oocyte maturation if the mitochondria and ER are similarly in close apposition.

In this chapter, the distribution of mitochondria in relation to the spindle is described throughout oocyte maturation. Mitochondria are shown to be in close proximity to the spindle, and aggregation of mitochondria around the first meiotic spindle is found to be regulated by the activities of dynein and kinesin and the presence of microtubules. Live time-lapse imaging confirms the distribution of mitochondria observed in fixed oocytes, and movement of mitochondria at the time of first polar body extrusion suggests a mechanism for retention of the organelles in the oocyte. The ER is found to be in close apposition to the mitochondria throughout oocyte maturation and disruption of mitochondrial function reveals mitochondrial ATP production is essential for maintenance of the first meiotic spindle.

3.2 Results

3.2.1 Time course of mitochondrial reorganisation during oocyte maturation

In order to examine the time-course of mitochondrial redistributions and their relationship to the meiotic spindle during oocyte maturation in more detail than has previously been described, oocytes were fixed at 2 hour intervals from GV stage to MII arrest. They were then stained for both mitochondria and tubulin, and examined by confocal microscopy (Fig. 3.1A). At GV stage, mitochondria were distributed throughout the oocyte with a small accumulation around the GV. As maturation progressed, mitochondrial accumulation in the central region of the oocyte increased such that by six hours after release from meiotic arrest, a substantial accumulation of mitochondria was observed around the metaphase I spindle.

As the spindle migrated to the cortex prior to Pb1 extrusion, mitochondria remained clustered around the spindle and followed the path of its migration. After Pb1 extrusion, an accumulation of mitochondria was observed around the spindle pole located in the centre of the oocyte and the mitochondria appeared to have retracted from the cortical spindle pole. At MII arrest mitochondria had redistributed throughout the oocyte although a small accumulation was sometimes observed around the MII spindle. Throughout maturation, mitochondria were observed clustered around cytoplasmic microtubules, likely MTOCs, as well as closely associated with spindle microtubules (Fig. 3.1B).

3.2.2 Live time-lapse imaging of mitochondrial redistributions

Live time-lapse imaging of mitochondria in oocytes during maturation has not previously been described. Therefore, in order to more examine mitochondrial

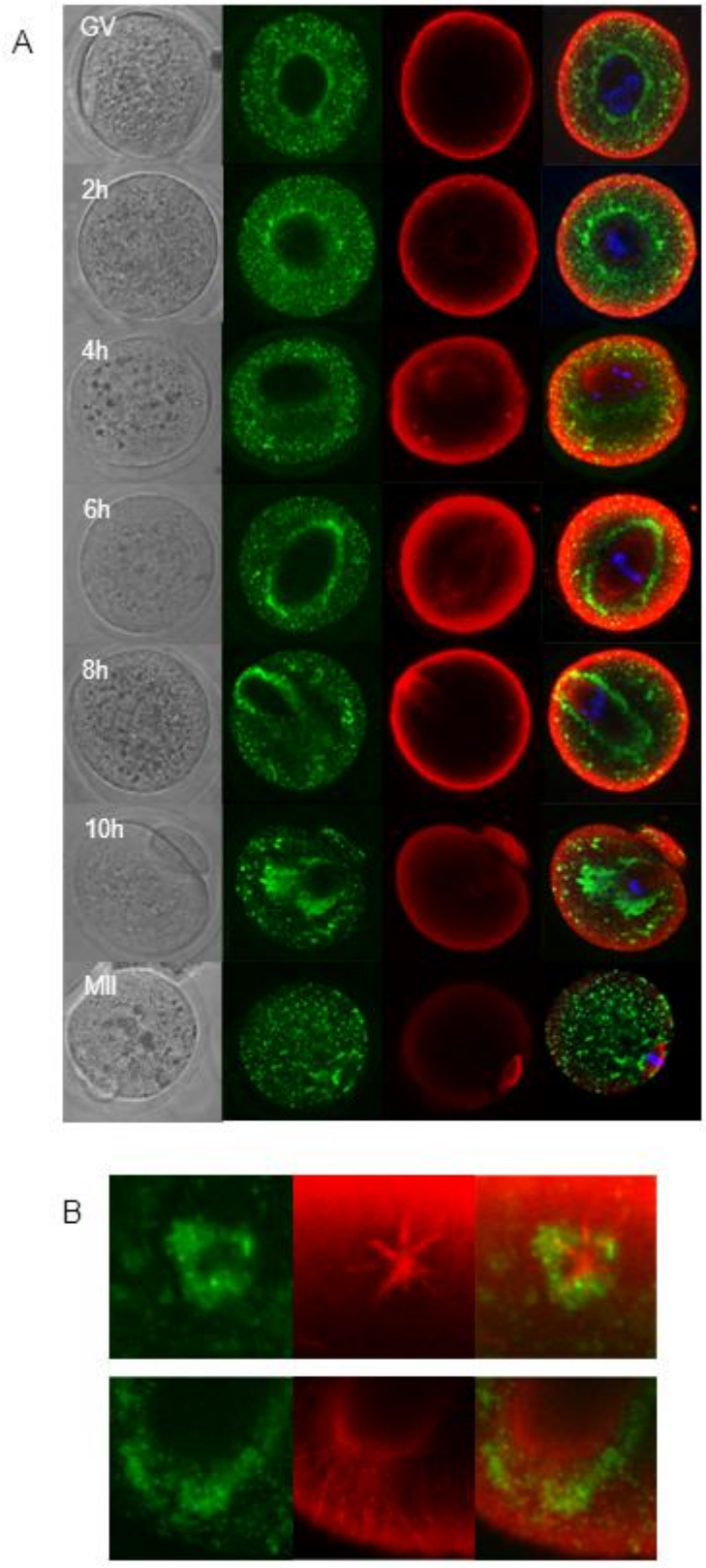

Figure 3.1. Reorganisation of mitochondria during oocyte maturation. A) GV oocytes collected and fixed at 2 hour intervals during maturation were stained for mitochondria (green), tubulin (red) and DNA (blue) and imaged by confocal microscopy. Images represent at least 7 oocytes from a minimum of two separate experiments. B) Mitochondria are closely associated with cytoplasmic microtubules (top row) and spindle microtubules (bottom row).

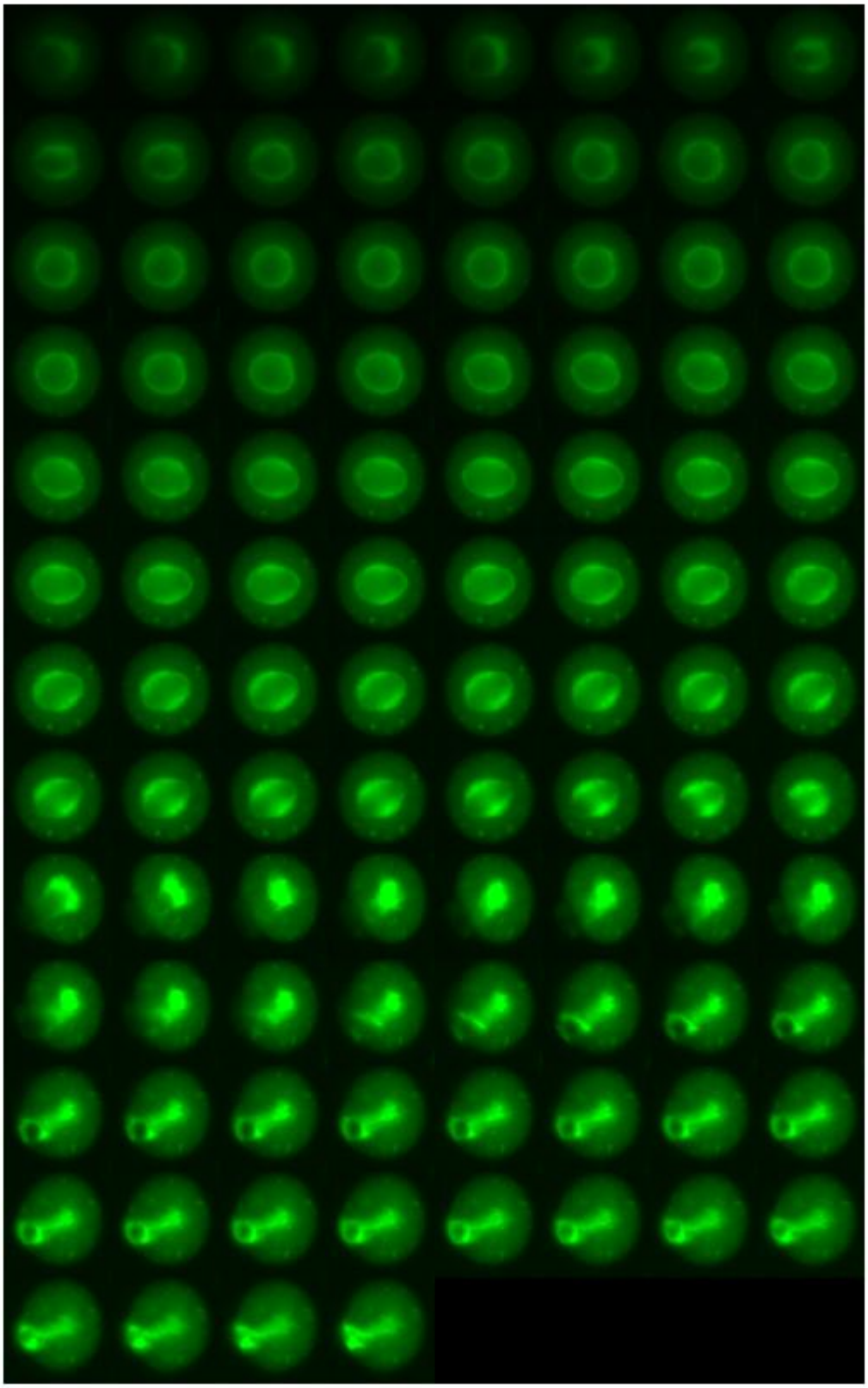

Figure 3.2. Time-lapse imaging of mitochondrial distribution during oocyte maturation. GV oocytes were microinjected with mRNA for mitochondria-targeted GFP. Following incubation in the presence of IBMX for 2-3 hours to allow expression, oocytes were released from meiotic arrest and imaged at 10 minute intervals for a period of 16 hours. Images shown are from one of three independent experiments with a total of 24 oocytes.

movements in real time throughout oocyte maturation, GV oocytes were microinjected with a GFP-tagged mitochondrial marker (mito-GFP) and imaged at 10 minute intervals for a period of 16 hours, from GV stage to MII arrest (Fig. 3.2). This confirmed that a progressive accumulation of mitochondria occurred around the developing first meiotic spindle, and accompanied the migration of the spindle to the cortex. As the first polar body was extruded, a remarkable retraction of mitochondria from the cortical spindle pole occurred and mitochondria clustered around the pole of the spindle directed towards the centre of the oocyte, possibly reflecting a mechanism for retention of the organelles in the oocyte during this asymmetric cell division. Surprisingly, the mitochondria were then trafficked towards the cortex again and another ring formation could be seen, probably around the developing second meiotic spindle, before the mitochondria began to redistribute once again. Some mitochondria dispersed around the cortex and throughout the oocyte, whilst others were retained around the metaphase II spindle, or in the central region of the oocyte.

3.2.3 Mitochondria are retained in the oocyte during polar body extrusion

In order to investigate the apparent retention of mitochondria observed during live imaging of polar body extrusion, oocytes were fixed at the time of polar body extrusion, stained for mitochondria and DNA, and imaged by confocal microscopy. By positioning the oocytes during imaging such that the separating chromosomes were aligned in the horizontal plane, it was possible to see that whilst mitochondria were clustered around the chromosomes to be retained in the oocyte, presumably enveloping this portion of the spindle, very few were observed in the polar body (Fig. 3.3A). Analysis of the percentage area occupied by mitochondria within the

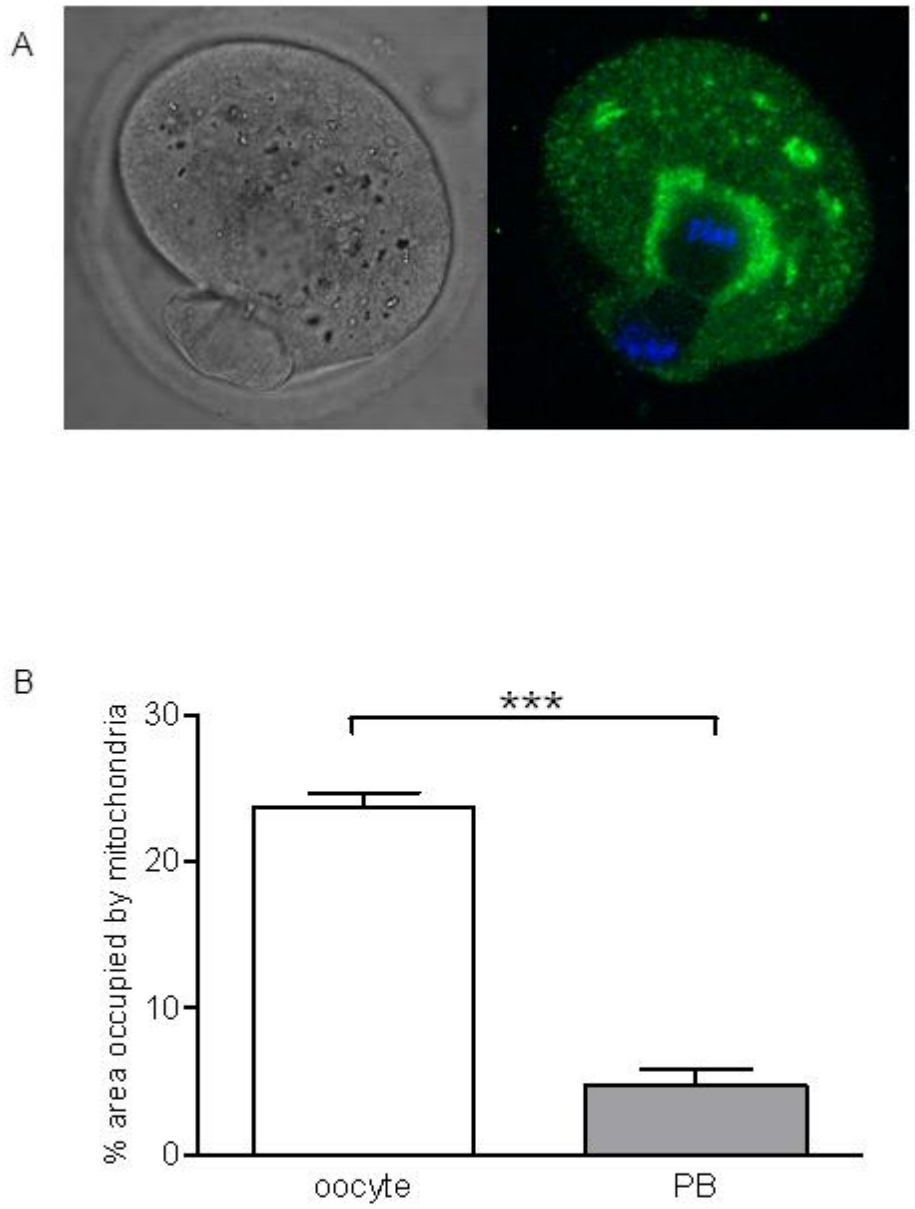

Figure 3.3. Mitochondria are retained in the oocyte during polar body extrusion. A) Oocytes were fixed at the time of polar body extrusion and stained for mitochondria (green) and DNA (blue). Confocal imaging shows mitochondria are retained in the oocyte and very few are observed in the polar body. Images are representative of results obtained from n=18 oocytes. B) Analysis of the percentage area occupied by mitochondria reveals a significant difference between the oocyte and polar body ($p<0.0001$).

oocyte or polar body in a single confocal slice where the separating chromosomes were aligned revealed that the percentage area occupied by mitochondria in the oocyte was significantly higher than in the polar body (Fig. 3.3A). This suggests that a mechanism may exist in the oocyte for retracting mitochondria located at the cortical spindle pole, such that they will be retained in the oocyte and not be lost to the polar body.

3.2.4 Mitochondrial reorganisation occurs both *in vivo* and *in vitro*

To confirm that the mitochondrial redistributions observed during *in vitro* maturation reflect those which occur during *in vivo* maturation, oocytes were also collected 6 hours (MI) and 12-14 hours (MII) after administration of hCG to initiate oocyte maturation *in vivo*. Mitochondrial distribution patterns at both MI (Fig. 3.4A) and MII stages (Fig. 3.4B) were similar in *in vitro* and *in vivo* matured oocytes. Therefore, redistribution of mitochondria occurs both *in vivo* and *in vitro*, and the changes in distribution observed here are not artefacts of *in vitro* maturation.

3.2.5 Mitochondrial redistribution is associated with oocyte maturation

To establish whether changes in mitochondrial distribution are linked to oocyte maturation, GV stage oocytes were maintained in GV arrest for 6 hours by the addition of 200 μM IBMX to the medium, prior to fixing and staining to observe mitochondrial distribution. In oocytes arrested at GV for 6 hours, mitochondrial distribution was not significantly changed from that observed in freshly isolated GV oocytes (Fig. 3.4C).

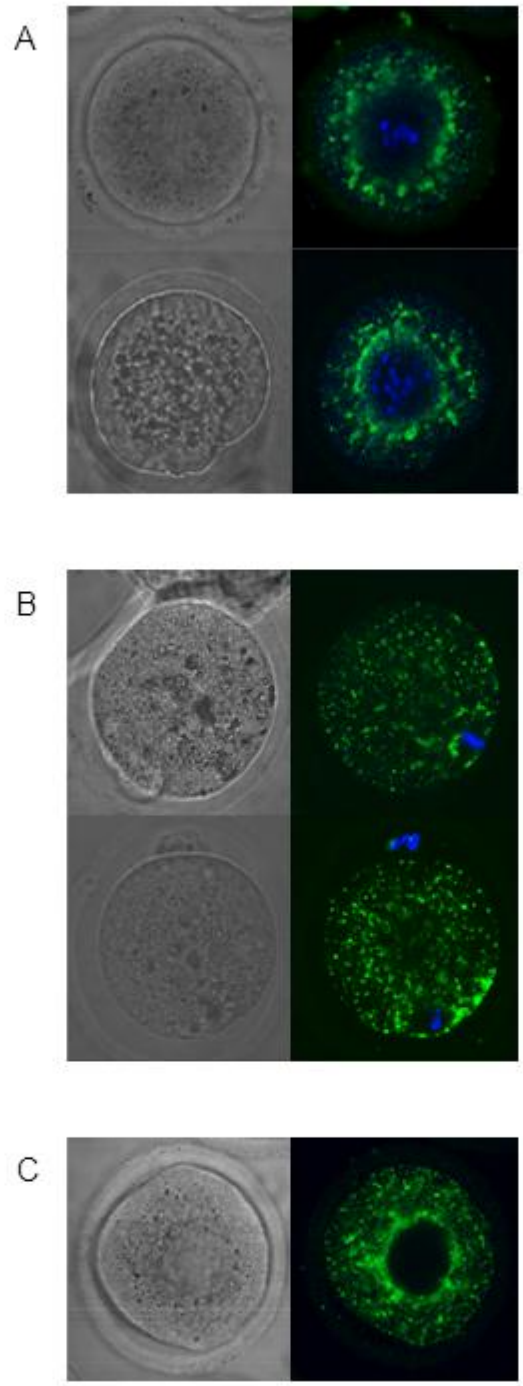

Figure 3.4. Mitochondrial reorganisation occurs *in vitro* and *in vivo* and is associated with the onset of maturation. Oocytes were fixed 6 hours after the onset of maturation (A) and at MII arrest (B) and stained for mitochondria (green) and DNA (blue). The distribution of mitochondria was examined and compared for oocytes matured *in vitro* (top rows) and *in vivo* (bottom rows). (C) Oocytes were maintained in GV arrest for 6 hours by incubation with IBMX to confirm that mitochondrial redistribution is associated with the onset of maturation. Images are representative of at least 8 oocytes for each condition.

3.2.6 Mitochondrial accumulation around the spindle is disrupted by nocodazole but not latrunculin A

To confirm if microtubules are responsible for the accumulation of mitochondria around the spindle which is observed during oocyte maturation, microtubules were disrupted using the microtubule depolymerising agent nocodazole, which has previously been used extensively in oocytes, including to assess changes to the structure of the ER (Fitzharris *et al.* 2007). GV stage oocytes were incubated with 10 μM nocodazole and fixed 6 hours after release from meiotic arrest. In agreement with previous reports (Van Blerkom 1991), mitochondria in these oocytes were distributed throughout the oocyte with no significant accumulations present in any region (Fig. 3.5B). In contrast, oocytes in which actin filaments had been disrupted by treatment with 6 μM latrunculin A (Spector *et al.* 1989) displayed mitochondrial distributions which were indistinguishable from control oocytes (Fig. 3.5C and Fig. 3.5A respectively). Efficacy of both nocodazole and latrunculin A treatment was confirmed by allowing some oocytes from each group to proceed through maturation. Control oocytes extruded a polar body whereas no polar bodies were observed when either microtubules or actin filaments were disrupted, consistent with previous reports (Soewarto *et al.* 1995; Fitzharris *et al.* 2007; Maro *et al.* 1984).

3.2.7 Accumulation of mitochondria around the first meiotic spindle is disrupted by dynein inhibition and enhanced by kinesin inhibition

Having established that mitochondrial accumulation in the spindle region requires the presence of intact microtubules, experiments were carried out to ascertain if this movement is dynein or kinesin-dependent. Inhibition of dynein was achieved using two different approaches; over-expression of p50, the dynamitin subunit of the

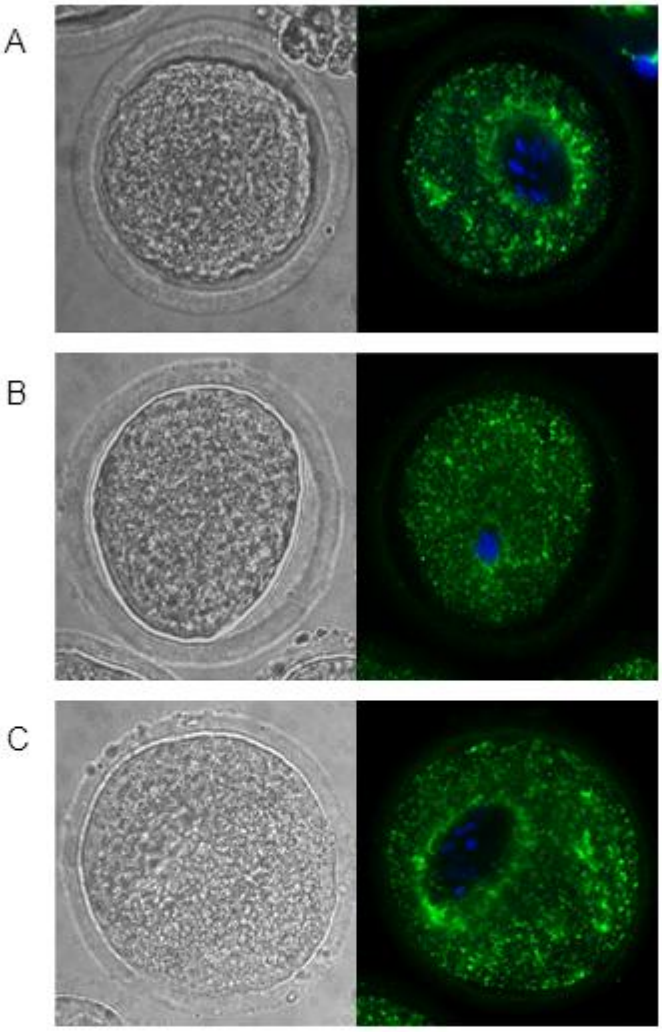

Figure 3.5. Mitochondrial accumulation around the spindle is disrupted by nocodazole but not latrunculin A. GV oocytes were transferred into media containing either (A) vehicle control, (B) 10 μM nocodazole or (C) 6 μM latrunculin A, and matured for 6 hours prior to fixing and staining for mitochondria (green) and DNA (blue). Latrunculin A treated oocytes (n=13) displayed a mitochondrial distribution indistinguishable from that of control oocytes (n=32) whereas nocodazole-treated oocytes failed to display the characteristic mitochondrial ring (n=23).

dynactin complex, which disrupts the dynein-dynactin interaction, thereby inhibiting dynein activity (Echeverri *et al.* 1996; Burkhardt *et al.* 1997), and a function-blocking antibody, 70.1, directed against the dynein intermediate chain (Varadi *et al.* 2004; Heald *et al.* 1997). Distribution of mitochondria from oocytes in which either dynein or kinesin had been inhibited was analysed as described in Chapter 2.9. Microinjection of GFP-tagged p50 into GV oocytes resulted in a decreased percentage of spindle-associated mitochondria during MI (Fig. 3.6A). This result was confirmed by the dynein function-blocking antibody, 70.1, which also resulted in a decrease in accumulation of mitochondria around the meiotic spindle (Fig. 3.6B). Inhibition of kinesin activity by microinjection of the function-blocking antibody SUK4 (Ingold *et al.* 1988) caused an increase in spindle-associated mitochondria (Fig. 3.6C) suggesting that a balance between activities of dynein and kinesin motors regulates mitochondrial distribution in the oocyte.

In p50 experiments, there was an overall higher proportion of spindle-associated mitochondria, both in the control oocytes and the p50-injected oocytes. This likely reflects a slightly different experimental approach since p50-injected oocytes and their control vehicle-injected counterparts were incubated overnight at GV stage to allow p50 expression to occur before maturation commenced, whereas 70.1 and SUK4 injected oocytes and their controls were released from meiotic arrest shortly after microinjection. However, the difference between the proportion of mitochondria associated with the spindle compared to the equivalently treated control oocytes was similar whether p50 or 70.1 was used to inhibit dynein activity. The reason for the increased accumulation in oocytes incubated overnight at GV stage prior to release is not clear. The finding that no significant accumulation of

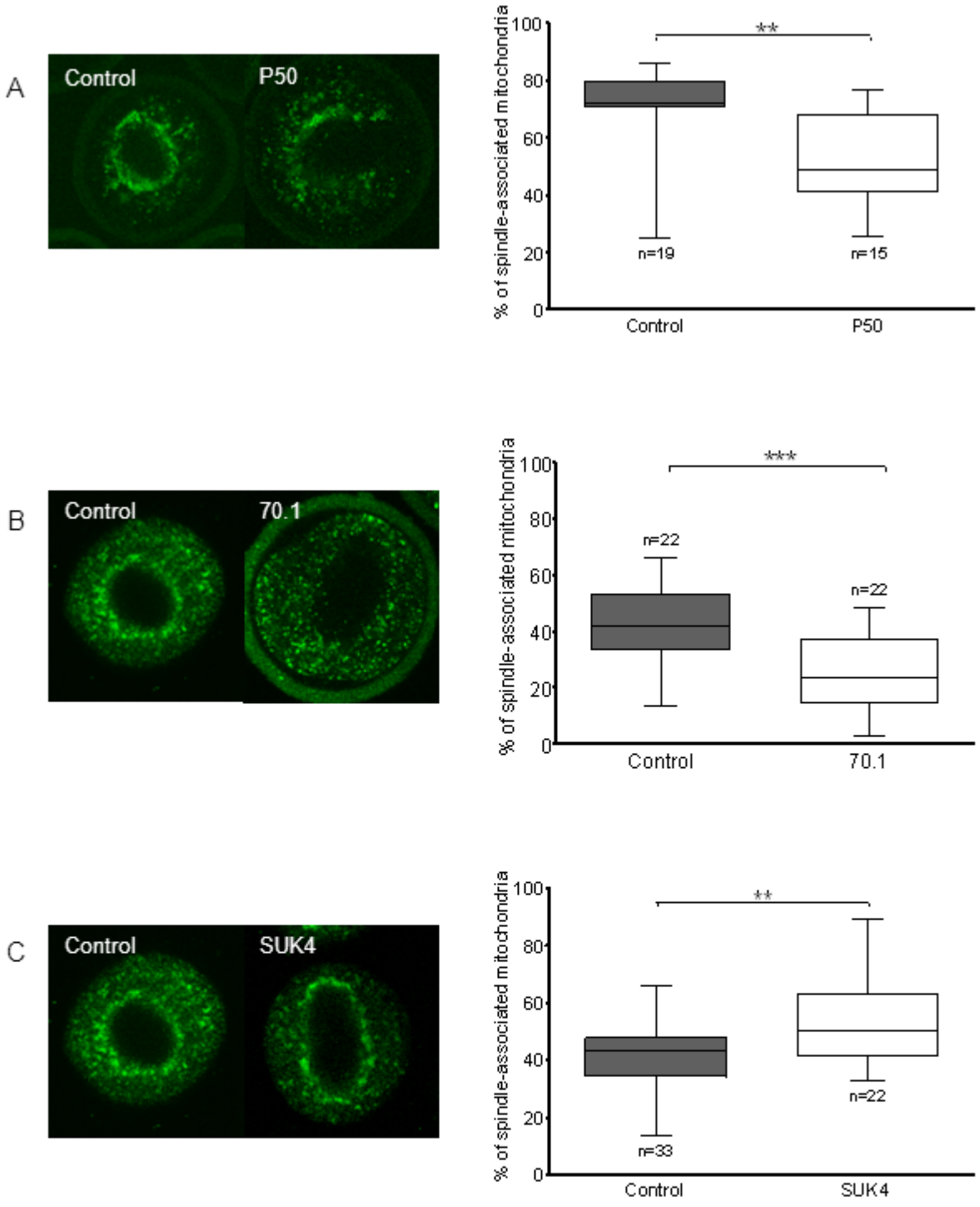

Figure 3.6. Mitochondrial accumulation around the spindle is disrupted by dynein inhibition and enhanced by kinesin inhibition. Oocytes arrested at GV stage were microinjected with (A) mRNA for p50 or (B) 70.1 to disrupt dynein function or (C) SUK4 to inhibit kinesin (KIF5) function. Microinjected oocytes were either maintained in GV arrest overnight to allow for mRNA expression and then released to undergo maturation (p50), or released directly after microinjection (70.1 and SUK4). Oocytes were fixed 6 hours after the onset of maturation, and stained for mitochondria (green). Dynein inhibition with both p50 and 70.1 decreased mitochondrial aggregation around the spindle ($p=0.0044$ and $p=0.0002$ respectively) whilst disruption of kinesin function with SUK4 increased mitochondrial aggregation ($p=0.0018$).

mitochondria around the GV is observed in oocytes arrested at GV stage for 6 hours (Fig. 3.4C) suggests that mitochondria may be aggregating in the spindle region more quickly in oocytes arrested overnight once they are released from meiotic arrest, rather than accumulating mitochondria whilst maintained at GV stage. Nonetheless, whilst both p50 and counterpart controls exhibited a higher proportion of spindle associated mitochondria, the difference between p50-injected oocytes and control vehicle-injected oocytes was maintained.

3.2.8 Maintenance of the meiotic spindle requires functional mitochondria

It has been proposed that mitochondrial accumulation around the spindle during oocyte maturation may serve to provide a localised supply of ATP to the spindle region (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004). In order to examine the ATP-dependence of the meiotic spindle, oocytes were microinjected with MAP7-EGFP to visualise the spindle, and mitochondrial inhibitors were applied. Confocal microscopy revealed that application of oligomycin, a blocker of the mitochondrial ATP-synthase, resulted in a rapid and complete disassembly of the spindle architecture. Carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), a protonophore and uncoupler of mitochondrial oxidative phosphorylation caused a similar but significantly faster collapse of the spindle. This may be attributable to a more rapid cellular depletion of ATP with FCCP as a result of reversal of the ATP synthase in an attempt to maintain mitochondrial membrane potential (Budd & Nicholls 1996).

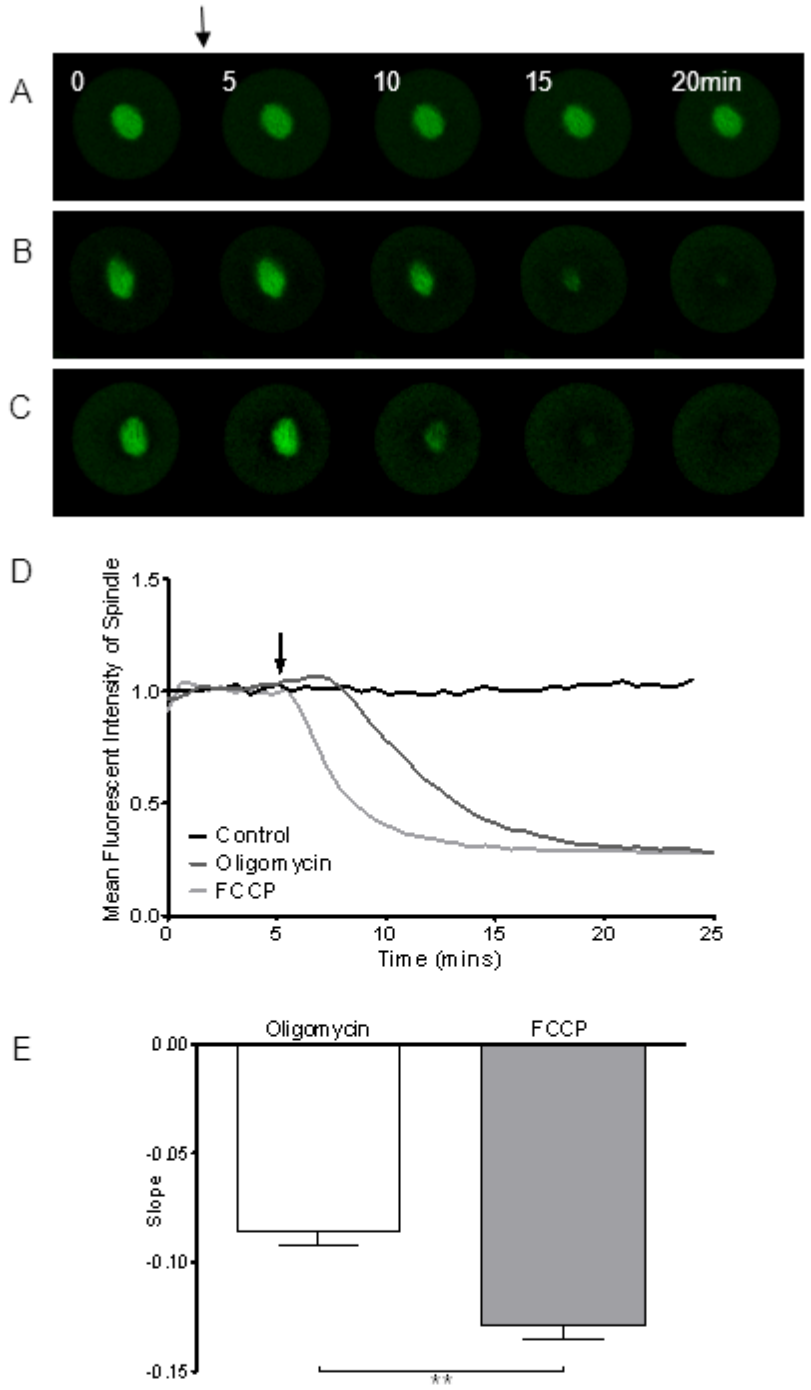

Figure 3.7. Maintenance of the meiotic spindle requires functional mitochondria. GV arrested oocytes were microinjected with MAP7-EGFP to visualise the spindle and then allowed to undergo maturation. Time-series experiments were performed between 4 and 8 hours after the onset of maturation, using a confocal microscope, and images were collected every 30 seconds. Representative images showing no change to the spindle on addition (arrow) of (A) vehicle control (n=7), and collapse of the spindle on application of (B) oligomycin (n=9) or (C) FCCP (n=11). (D) Normalised traces of fluorescence of the spindle region from the representative oocytes in (A), (B) and (C) on addition (arrow) of oligomycin, FCCP or vehicle control. Addition of FCCP causes a more rapid onset and a faster rate of spindle collapse than application of oligomycin. (E) Linear regression analysis of the linear portion of the graph for oligomycin and FCCP reveals that collapse of the spindle on application of FCCP is significantly faster than with oligomycin ($p=0.0003$).

3.2.9 Mitochondria are located in close apposition to the endoplasmic reticulum during oocyte maturation

Previous studies have shown that the ER also undergoes changes in distribution during oocyte maturation which are similar to those observed here for mitochondria (Fitzharris *et al.* 2007). In addition, at MII arrest mitochondria and ER are in close apposition and functionally linked (Dumollard *et al.* 2003; Dumollard *et al.* 2004; Campbell & Swann 2006). ER and mitochondria were therefore examined at GV stage prior to the initiation of maturation, during maturation (MI), and at MII stage. Confocal microscopy of oocytes which had been microinjected with dsRed2ER and mito-GFP to visualise both ER and mitochondria revealed that mitochondria and ER were similarly distributed at all stages of oocyte maturation (Fig. 3.8). At GV stage, both were present throughout the oocyte but exhibited a small accumulation in the GV region. Six hours after release from meiotic arrest, both mitochondria and ER had accumulated in the spindle region although interestingly, the ER was located closer to the spindle, forming a layer between the mitochondria and the spindle. In MII oocytes, mitochondria and ER were once again found in close apposition, in agreement with previous reports (Dumollard *et al.* 2004). Interestingly, whilst mitochondria were rarely observed in the polar body, in agreement with the findings in Figure 3.3, the ER was readily detectable in the polar body, suggesting that the retention mechanism which appears to be functioning at polar body extrusion may be specific to mitochondria.

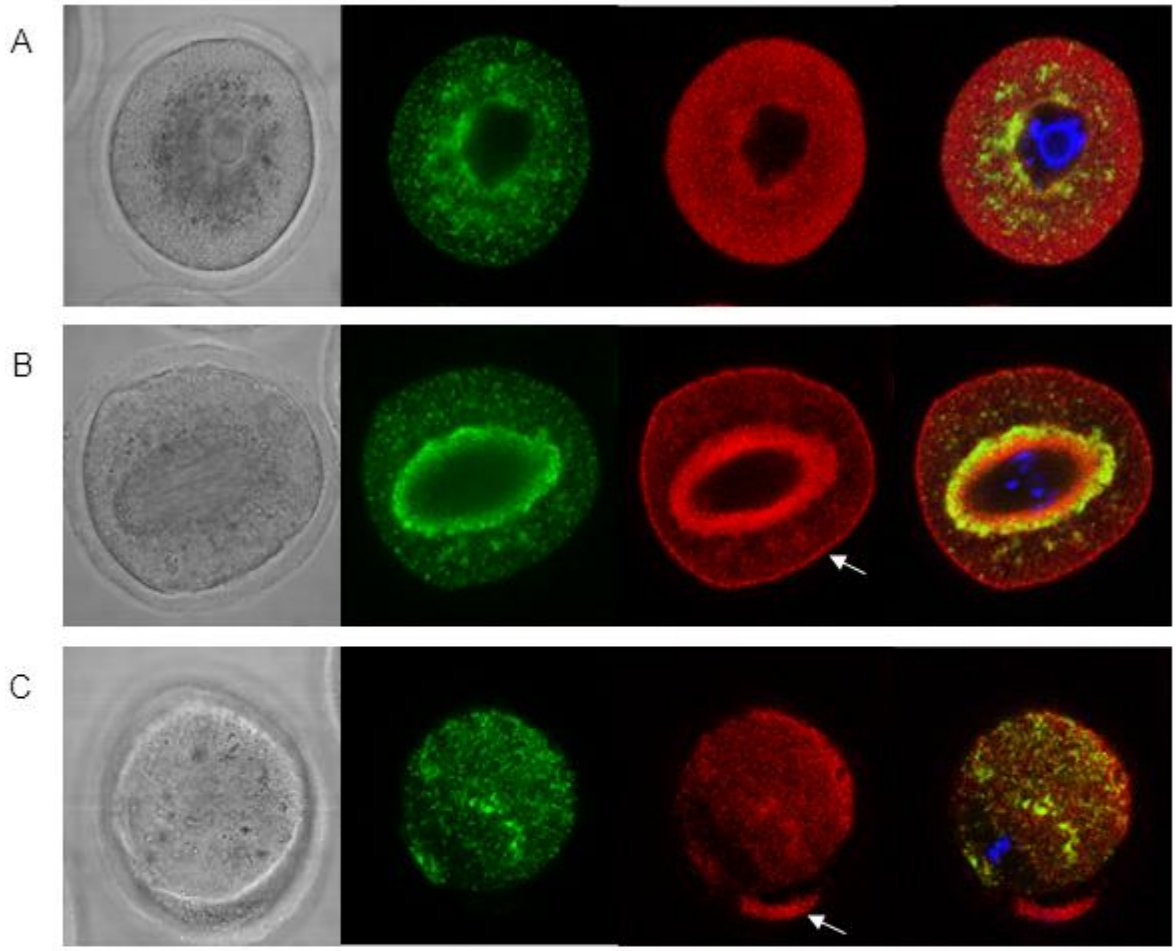

Figure 3.8. Mitochondria are located in close apposition to the ER throughout oocyte maturation. Oocytes microinjected with dsRed2ER (red) and Mito-GFP (green) and stained for DNA (blue) were examined by confocal microscopy at GV (A) (n=23), MI (B) (n=31)and MII (C) (n=14). Mitochondria and ER both show a striking accumulation around the spindle region at MI, with mitochondria enveloping the ER ring. Mitochondria at MI were not located at the plasma membrane but ER was (arrow in B). ER but not mitochondria were observed in the polar body at MII (arrow in C).

3.3 Discussion

Results presented in this chapter describe stage-specific redistributions of mitochondria during oocyte maturation which are inhibited by disassembly of the microtubule network and disruption of dynein activity. The distribution of mitochondria during maturation in the oocyte correlates closely with that of the ER, and functionally intact mitochondria are shown to be necessary for maintenance of the first meiotic spindle.

Changes in the distribution of mitochondria were first described in the mouse oocyte by Van Blerkom (Van Blerkom & Runner 1984) and have been subsequently found to occur in a number of species (Stojkovic *et al.* 2001; Wilding *et al.* 2001; Valentini *et al.* 2010; Brevini *et al.* 2005; Barnett *et al.* 1996; Sun *et al.* 2001; Calarco 1995; Yu *et al.* 2010). Accumulation of mitochondria around the first meiotic spindle has been proposed to provide greater ATP supply to meet the energetic requirements of a dynamic spindle (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004) but although deficits in mitochondria-derived ATP have been linked to aberrant spindle structure, (Zhang *et al.* 2006; Johnson *et al.* 2007; Zeng *et al.* 2007) evidence linking aggregation of mitochondria in the spindle region with formation and maintenance of the spindle has remained elusive.

The redistribution of mitochondria described here is largely in agreement with that previously described for the maturing mouse oocyte (Van Blerkom & Runner 1984; Calarco 1995). Mitochondria were found to be present throughout the oocyte at GV stage, although a limited accumulation was observed around the GV and in the central region shortly after GVBD. By 6 hours after the onset of maturation, there

was a dramatic accumulation of mitochondria which formed a ring in the central region of the oocyte. Immunofluorescence imaging of tubulin together with mitochondria revealed that the mitochondrial ring was enveloping and closely associated with the first meiotic spindle. The formation of the mitochondrial ring is linked with oocyte maturation since it does not occur in oocytes arrested at GV stage for 6 hours.

Inhibition of microtubules prevented formation of a mitochondrial ring around the spindle, whereas inhibition of the actin cytoskeleton has no apparent effect on the localisation of mitochondria to the spindle region, in agreement with previous reports (Sun *et al.* 2001; Yu *et al.* 2010; Van Blerkom 1991). A recent report described disruption of the ring structure at certain stages of maturation, and an actin-dependent change to the extent of mitochondrial clustering (Yu *et al.* 2010). The loss of the ring structure at the times described by Yu and colleagues was not observed here however, either by immunofluorescence imaging or during live time-lapse imaging. Additionally inhibition of actin filaments with Latrunculin A did not appear to have any obvious effects on mitochondrial clustering.

Transport along microtubules occurs via the actions of the motor proteins dynein and kinesin with kinesin moving cargoes in the anterograde direction, towards the plus ends of microtubules, and dynein moving cargoes in the retrograde direction, towards the minus ends of microtubules (Hirokawa *et al.* 1998). Although there are more data describing the association of mitochondria with kinesin motors, particularly in neurons (Hollenbeck & Saxton 2005), dynein motors have also been

shown to be involved in the trafficking of mitochondria along microtubules (Varadi *et al.* 2004; Pilling *et al.* 2006).

In the maturing oocytes, accumulation of mitochondria in the region of the first meiotic spindle appears to be dependent on dynein function, similar to the dynein-dependent ER movement seen at this stage of oocyte maturation (Fitzharris *et al.* 2007). Kinesin also seems to be involved in determining the distribution of mitochondria in the maturing oocyte, since inhibition of kinesin increased the percentage of spindle associated mitochondria. Thus, mitochondrial distribution in the oocyte would appear to be regulated by a balance of dynein and kinesin activity, with dynein dominating in order to drive formation of a mitochondrial ring around the first meiotic spindle.

In the mouse oocyte, the assembly of the acentriolar spindle is achieved by the formation of multiple MTOCs in the cytoplasm which then migrate towards the centre of the oocyte where they cluster to form the two poles of the spindle (Schuh & Ellenberg 2007). The observation of mitochondria clustered around what appear to be MTOCs in the cytoplasm during maturation raises the possibility that rather than being trafficked directly along cytoplasmic microtubules towards the spindle, mitochondria may accumulate at MTOCs via the action of dynein and then be transported towards the forming spindle together with the MTOCs as they migrate. Indeed, dynein function has also been demonstrated to be necessary for the inward movement of microtubule bundles during prophase/prometaphase in mitosis (Rusan *et al.* 2002) which is consistent with the timing and direction of movement of mitochondria during the initial phases of oocyte maturation.

In addition, evidence for cell cycle regulation of dynein function exists in Xenopus where phosphorylation by cdk1-cyclinB1 causes dissociation of the motor from membranes resulting in decreased dynein-mediated organelle transport at metaphase (Niclas *et al.* 1996; Addinall *et al.* 2001). This may be relevant in oocytes, where cdk1 activity gradually increases during meiosis I (Kishimoto 2003), in order to anchor mitochondria in the spindle region following their trafficking to this region during the early stages of maturation.

Following formation of the mitochondrial ring around the spindle, mitochondria were subsequently found to follow the path of the spindle as it migrated to the cortex, before being apparently drawn back into the cytoplasm during polar body extrusion. Analysis of mitochondrial distribution at polar body extrusion indicates the presence of a retention mechanism to maintain the mitochondrial complement of the oocyte and limit loss of mitochondria to the polar body. Previous studies have observed only a few mitochondria in the polar body (Calarco 1995) and the presence of a retention mechanism seems plausible given that mitochondrial replication does not resume until after implantation (Jansen 2000a; Piko & Taylor 1987). It was not however possible to examine the mechanisms of mitochondrial redistribution during and after polar body extrusion in more detail since interventions which inhibit the microtubule and actin cytoskeletons also disrupt normal spindle dynamics and prevent polar body extrusion (Soewarto *et al.* 1995).

Surprisingly, following the drawing back of mitochondria towards the centre of the oocyte, they then move towards the periphery of the oocyte and form a ring around what was presumed to be the forming MII spindle, an observation which has not

previously been described. This suggests that mitochondrial accumulation in the spindle region may be a necessary feature of spindle formation during meiosis in the mouse oocyte. Mitochondria then largely dispersed throughout the oocyte although a number also remained in the central region of the oocyte and around the MII spindle in some cases.

In some cell types, mitochondria redistribute to specific cellular locations which have high energy use, a process which has been most extensively described in neurons where mitochondria move bidirectionally throughout the axon (Hollenbeck & Saxton 2005). This process may be particularly important in large cells where diffusion of ATP over large distances may not be sufficient to meet demand. It has been suggested that a similar mechanism may be at play in oocytes, with mitochondrial redistributions reflecting regional differences in ATP requirements which may be fulfilled by sequestration of mitochondria in specific areas of the oocyte (Van Blerkom & Runner 1984; Van Blerkom 1991; Eichenlaub-Ritter *et al.* 2004). The finding here that the first meiotic spindle is acutely sensitive to ATP depletion supports this notion although further work is needed to establish whether formation of the mitochondrial ring is necessary to maintain a functional spindle.

Indeed, although dynein inhibition depleted mitochondrial aggregation in the spindle region, it was not possible to ascertain whether this resulted in mitochondria-specific effects on the spindle architecture since disruption of dynein function both inhibits the transport of other organelles (Fitzharris *et al.* 2007) and affects the spindle itself (Gaglio *et al.* 1997; Heald *et al.* 1997; Merdes *et al.* 2000; Gaetz & Kapoor 2004; Rusan *et al.* 2002). A more targeted disruption of mitochondrial aggregation will be

necessary in order to ascertain whether the presence of the mitochondrial ring is required to form and maintain a functional spindle. Attempts were made to disrupt mitochondria specifically in the spindle region either by targeted photosensitisation with confocal microscopy or by targeted ablation of mitochondria using a caged photo-releasable mitochondrial uncoupler, AG10, (Avlonitis *et al.* 2009) but these approaches were not successful.

A recent report has linked actin-dependent changes in the extent of mitochondrial clustering with changes to mitochondrial ATP generation (Yu *et al.* 2010). The authors observed "bursts" of ATP during oocyte maturation which were associated with the structure of the actin cytoskeleton but were unaffected by preventing the formation of the mitochondrial ring with nocodazole. This led to the conclusion that formation of the ring was not necessary to cause the "bursts" of ATP which were observed. However, this finding does not preclude the possibility that accumulation of mitochondria in the spindle region may provide a localised supply of ATP for formation and maintenance of a functional spindle whilst minimising production of harmful ROS. Indeed, aberrations in mitochondrial redistributions during oocyte maturation have been linked to compromised developmental competence (Bavister & Squirrell 2000; Wang *et al.* 2009) and mitochondrial dysfunction and insufficient provision of ATP have been related to disruption of the spindle (Zhang *et al.* 2006; Johnson *et al.* 2007; Zeng *et al.* 2007; Wang *et al.* 2009) suggesting that mitochondrial distribution and activity may well be important for successful development.

The microtubule-based and dynein-dependent movement of mitochondria towards the meiotic spindle observed here is similar to that described for the ER (Fitzharris *et al.* 2007) and co-labelling of the mitochondria and ER reveals that they are closely associated throughout maturation. A close apposition between mitochondria and ER has previously been described at the MII stage when mitochondrial ATP production is essential for sustaining calcium oscillations at fertilisation (Dumollard *et al.* 2004). These calcium oscillations are transmitted to the mitochondria where they stimulate mitochondrial activity thereby matching ATP supply with demand (Dumollard *et al.* 2004; Campbell & Swann 2006). An attractive hypothesis is that the spatial correlation of the mitochondria and the ER throughout maturation, and particularly around the MI spindle, may mean a similar mechanism is functioning throughout oocyte maturation. Although no calcium oscillations have been measured later than 4 hours after the release from the follicle (Carroll *et al.* 1994), a recent report finds that mitochondria take up Ca^{2+} released by constitutive low level activity of the IP_3 receptor and that this transfer of Ca^{2+} from the ER to the mitochondria is essential to maintain cellular bioenergetics (Cardenas *et al.* 2010). This suggests that absence of detectable calcium transients throughout maturation does not necessarily preclude the existence of a functional as well as spatial correlation between the mitochondria and the ER during oocyte maturation, which may serve to balance ATP supply and demand.

3.4 Summary

Mitochondria accumulate in the region of the first meiotic spindle dependent on the presence of microtubules, and regulated by the activities of dynein and kinesin motor proteins. Movement of the spindle towards the cortex is accompanied by

mitochondria, which are subsequently retracted into the centre of the oocyte, reflecting the possible existence of a retention mechanism to avoid loss of these organelles to the polar body. Mitochondria then aggregate around the second meiotic spindle before becoming redistributed by the time of MII arrest. In light of the sensitivity of the spindle to ATP depletion, co-localisation of ER and mitochondria in the spindle region may reflect a mechanism for localised provision of ATP to an area of high demand. Confirmation of this proposal will require a mechanism for specifically disrupting movement of mitochondria to this region.

4. Mitochondrial Adapter Proteins During Oocyte Maturation

4.1 Introduction

Mitochondrial trafficking in mammalian cells generally occurs along microtubule tracks, via the actions of the motor proteins dynein and kinesin (Hollenbeck & Saxton 2005). Most kinesin family members transport cargoes towards the plus ends of microtubules, whilst dynein motors are responsible for transport towards the minus ends of microtubules (Hirokawa *et al.* 1998). Both motors can be simultaneously bound to a bidirectional cargo and activities of the motors may be balanced to determine the net direction of transport, although the precise way in which this is coordinated is not fully clear (Welte 2004).

A role is emerging for adapter complexes linking microtubule motor proteins to specific cargoes in order to regulate trafficking processes. For mitochondrial transport, recent work has shown the Miro and Trak proteins to be involved. The Trak protein family has two members in mammalian species; Trak1 and Trak2, which share 47% amino acid identity (Iyer *et al.* 2003), and a single *Drosophila* orthologue termed Milton (Stowers *et al.* 2002). A role for Milton in mitochondrial trafficking was first described in *Drosophila* nerve terminals where photoreceptors mutant for Milton lacked mitochondria at synapses and displayed abberant synaptic transmission (Stowers *et al.* 2002). This was attributed to an interaction of Milton with kinesin heavy chain which, when disrupted, prevented trafficking of mitochondria towards microtubule plus ends at the distal ends of neuronal processes.

The involvement of the *Drosophila* Milton protein in mitochondrial transport along microtubules in the anterograde direction, via an interaction with kinesin, has now been demonstrated in a number of neuronal cell types (Gorska-Andrzejak *et al.* 2003; Guo *et al.* 2005; Glater *et al.* 2006), but of particular interest is the finding that Milton has a role in the trafficking of mitochondria into the *Drosophila* oocyte to form the Balbiani body (Cox & Spradling 2006). Disruption of two classes of Milton alleles had opposing effects on the timing and extent of mitochondrial acquisition by the oocyte from surrounding germ cells, which was attributed to their differing effects on the levels of the two major Milton transcripts, RA and RB. The $milt^{92}$ allele abolished any detectable Milton protein, and resulted in mitochondrial movement being blocked, whereas the class II alleles only disrupted the transcription of RA, leading to increased transport towards the minus end of microtubules. This led to the suggestion that the Milt-PA isoform mediates mitochondrial movement via interaction with kinesin whilst Milt-PB regulates dynein-dependent transport, although a direct interaction with dynein was not demonstrated. The absence of both isoforms thus prevents mitochondrial movement in this system.

Reports describing the mammalian orthologues of *Drosophila* Milton, Trak1 and Trak2, reveal a somewhat more complex picture. Trak2 was initially identified as being involved in $GABA_A$ receptor trafficking, and was named GRIF-1 ($GABA_A$ receptor interacting factor 1) (Beck *et al.* 2002). Both Trak1 and Trak2 (also called OIP106 and OIP98) also interact with *O*-GlcNAc transferase (OGT), an enzyme catalysing the post-translational modification of proteins by ß-*O*-GlcNAc (Iyer *et al.* 2003; Brickley *et al.* 2011). Trak1 has also been shown to form a complex with RNA polymerase II, localised to nuclear punctae in HeLa cells by immunoflourescence

staining of the endogenous protein, whilst Trak2 has a role in the trafficking of the K^+ channel Kir2.1 (Grishin *et al.* 2006). A Trak1 mutation has been identified as a cause of hypertonia, proposed to be as a result of aberrant $GABA_A$ receptor endocytic trafficking (Gilbert *et al.* 2006) and both Trak1 and Trak2 have been localised to early endosomes by virtue of an interaction with Hrs, an endocytic protein, where they have a role in endosome-to-lysosome trafficking (Kirk *et al.* 2006; Webber *et al.* 2008).

Nonetheless, a role for Trak1 and Trak2 in mitochondrial trafficking in mammalian systems has also been reported. Both Trak1 and Trak2 co-localise and interact with mitochondria and kinesin, and alterations in Trak levels or activity alter mitochondrial morphology, distribution, and transport in both anterograde and retrograde directions (Brickley *et al.* 2005; Smith *et al.* 2006; Koutsopoulos *et al.* 2010; MacAskill *et al.* 2009a; Brickley & Stephenson 2011; Brickley *et al.* 2011).

Trak/Milton proteins link to mitochondria at least in some cases via an interaction with Miro (mitochondrial Rho) proteins located in the mitochondrial membrane (Glater *et al.* 2006; Fransson *et al.* 2006; MacAskill *et al.* 2009a; Wang & Schwarz 2009). Miro-1 and Miro-2 constitute a novel family of Rho GTPases localised to mitochondria, which can influence the organisation of the mitochondrial network (Fransson *et al.* 2003; Guo *et al.* 2005; Fransson *et al.* 2006; Saotome *et al.* 2008; MacAskill *et al.* 2009a; MacAskill *et al.* 2009b; Russo *et al.* 2009). Miro proteins contain two GTPase domains, the first of which has homology to typical Rho GTPases but the second of which is atypical. They also have two EF hand domains situated between the GTPase domains, which participate in Ca^{2+}-dependent

mitochondrial stopping (Fransson *et al.* 2003; Saotome *et al.* 2008; MacAskill *et al.* 2009b). A C-terminal transmembrane domain confers mitochondrial localisation (Fransson *et al.* 2006).

In mammalian systems, Miro proteins have been reported to recruit Trak2 to mitochondria to mediate mitochondrial trafficking, (MacAskill *et al.* 2009a), as well as interacting directly with kinesin, independently of Trak2; an association which is calcium sensitive and allows targeting of mitochondria to neuronal synapses (MacAskill *et al.* 2009b). An alternative mechanism for Ca^{2+}-dependent mitochondrial stopping has also been proposed whereby under conditions of high Ca^{2+}, Miro disrupts the association of kinesin motor domain with microtubules, resulting in the cessation of mitochondrial movement, although kinesin remains bound to mitochondria (Wang & Schwarz 2009). Importantly, although Miro has only been shown to interact directly with kinesin motors, the alteration of either levels or activity of Miro can affect both anterograde and retrograde transport (Russo *et al.* 2009; Wang & Schwarz 2009; Brickley & Stephenson 2011; MacAskill *et al.* 2009b). A summary of the interactions between Trak, Miro and kinesin can be found in Figure 4.1.

In light of the role of Trak and Miro proteins in mitochondrial trafficking in several cell types, this chapter describes attempts to elucidate the role of Miro and Trak family proteins in mitochondrial transport in the maturing oocyte. Localisation of both endogenous and over-expressed Trak and Miro proteins was investigated and their effects on mitochondrial distribution in the oocyte examined.

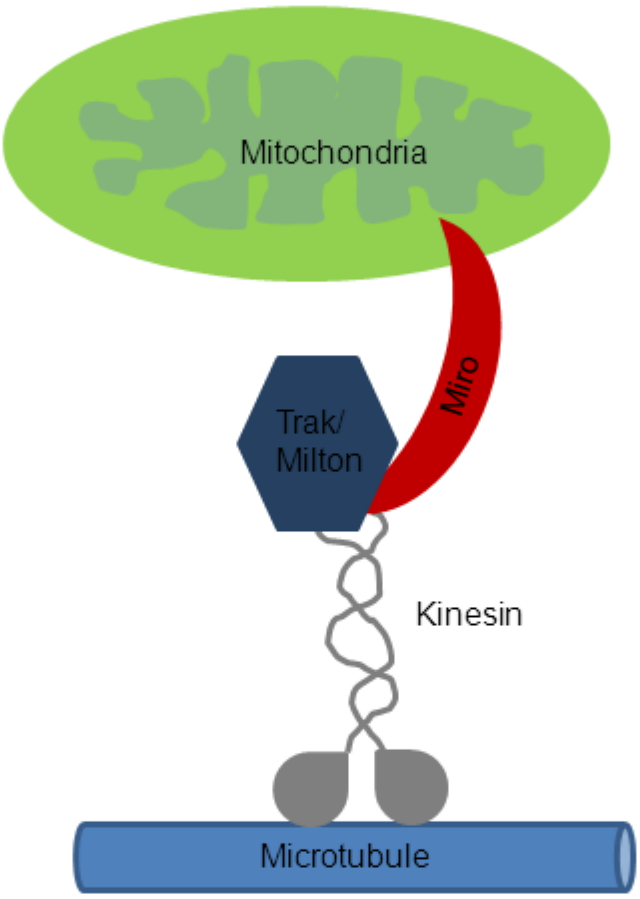

Figure 4.1. Miro, Trak and kinesins in mitochondrial trafficking. Miro is located in the mitochondrial membrane where it participates in mitochondrial trafficking either by interaction with Trak/Milton proteins which bind to kinesin, or by binding kinesin directly, in a Ca2+ dependent manner.

4.2 Results

4.2.1 Expression levels of mitochondrial adapter proteins

In order to investigate whether Trak and Miro proteins may be involved in mitochondrial trafficking during oocyte maturation, expression levels in the oocyte were examined using the Gene Atlas GNF1M expression profiles at the BioGPS portal (Wu *et al.* 2009; Su *et al.* 2004). This revealed that levels of Trak1 and Miro expression were high in both the oocyte and fertilised egg compared to other tissues, suggesting a potential role for these proteins in mitochondrial movement at these developmental stages (Fig. 4.2). Conversely, levels of Trak2 expression were low in the oocyte and fertilised egg compared to other tissues (Fig. 4.2).

4.2.2 Endogenous localisation of mitochondrial adapter proteins in the oocyte

The localisation of the endogenous Miro and Trak1 proteins was investigated by immunofluorescence staining and confocal microscopy in oocytes and embryos from GV to 2-cell stage. Miro was found to be localised to the mitochondria at all developmental stages examined (Fig. 4.3A), appearing as punctate staining associated with mitochondria (Fig. 4.3B). Surprisingly, despite high levels of mRNA in the oocyte and egg according to expression profiles, Trak1 was not localised to the mitochondria at any of the stages investigated. Staining instead appeared to be largely cytosolic, although in some cases the signal was enriched in the spindle region (Fig. 4.4). The Trak1 antibody used here has previously been used successfully to examine Trak1 distribution (Gilbert *et al.* 2006). Nonetheless the localisation of both Trak1 and Miro by immunofluorescence was confirmed by over-expression of the fluorescently-tagged proteins. Localisation of endogenous Trak2 was also attempted but was not successful, possibly due to low levels of expression,

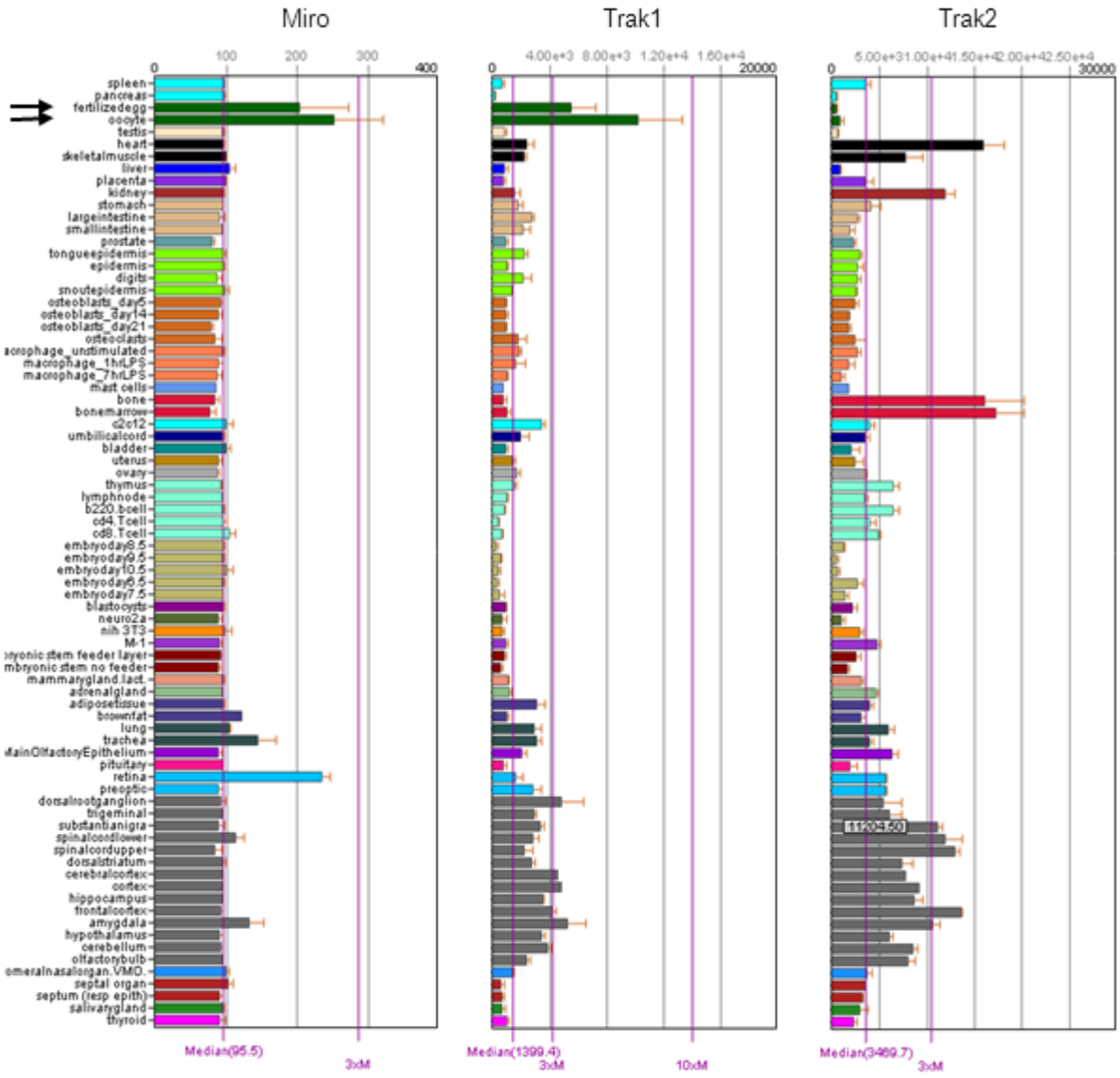

Figure 4.2. Expression levels of Miro, Trak1 and Trak2. Graphs of Gene Atlas GNF1M expression profiles for Miro, Trak1 and Trak2 from the BioGPS portal. Arrows indicate the data for the oocyte and fertilised egg.

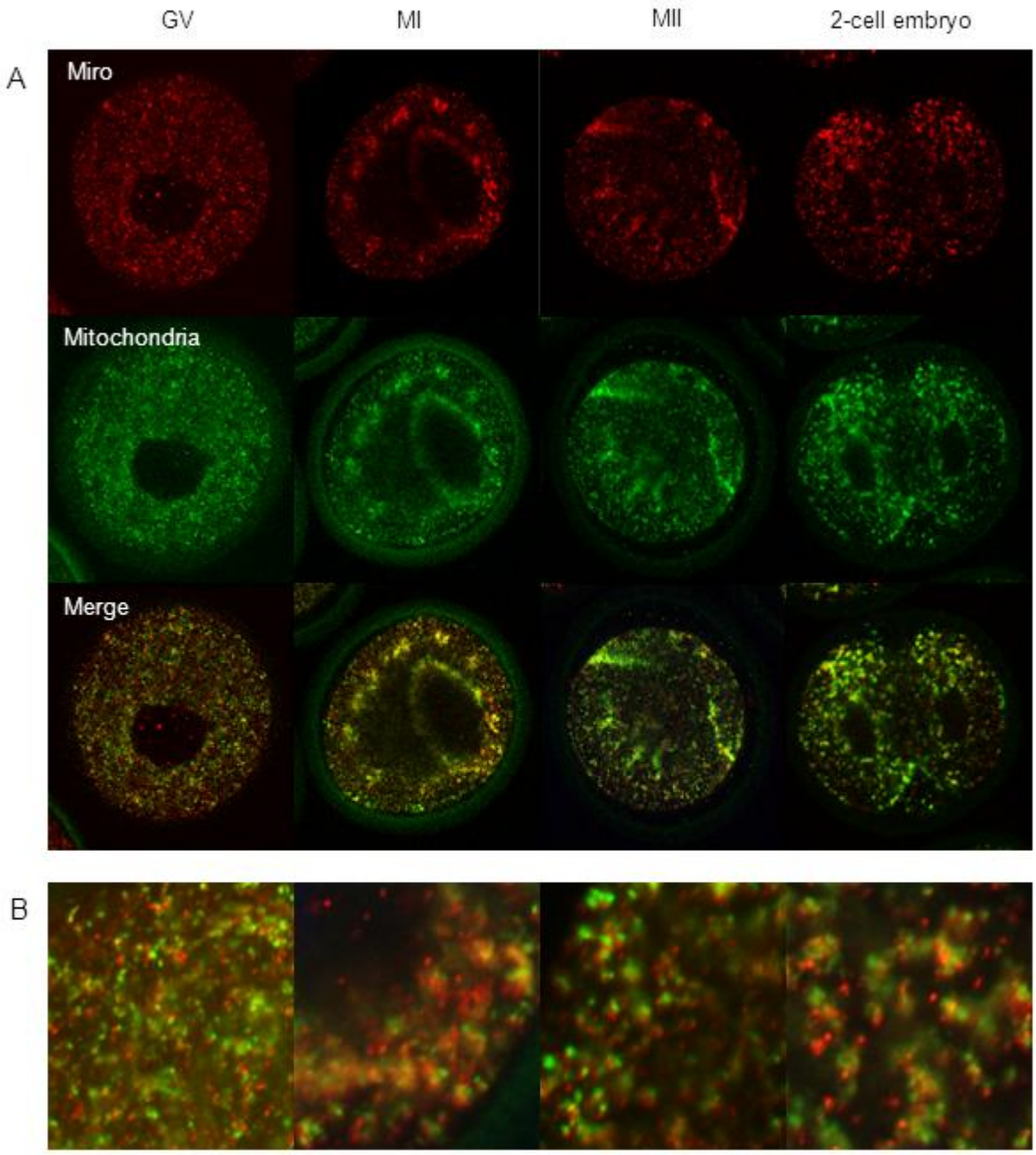

Figure 4.3. Endogenous Miro is localised to the mitochondria. A) Oocytes at GV, MI and MII stages and 2-cell embryos were stained for Miro and mitochondria. Punctate staining of Miro can be seen associated with individual mitochondria. B) Magnification of a section of the images shown in (A). Images are representative of at least 10 oocytes for each stage examined.

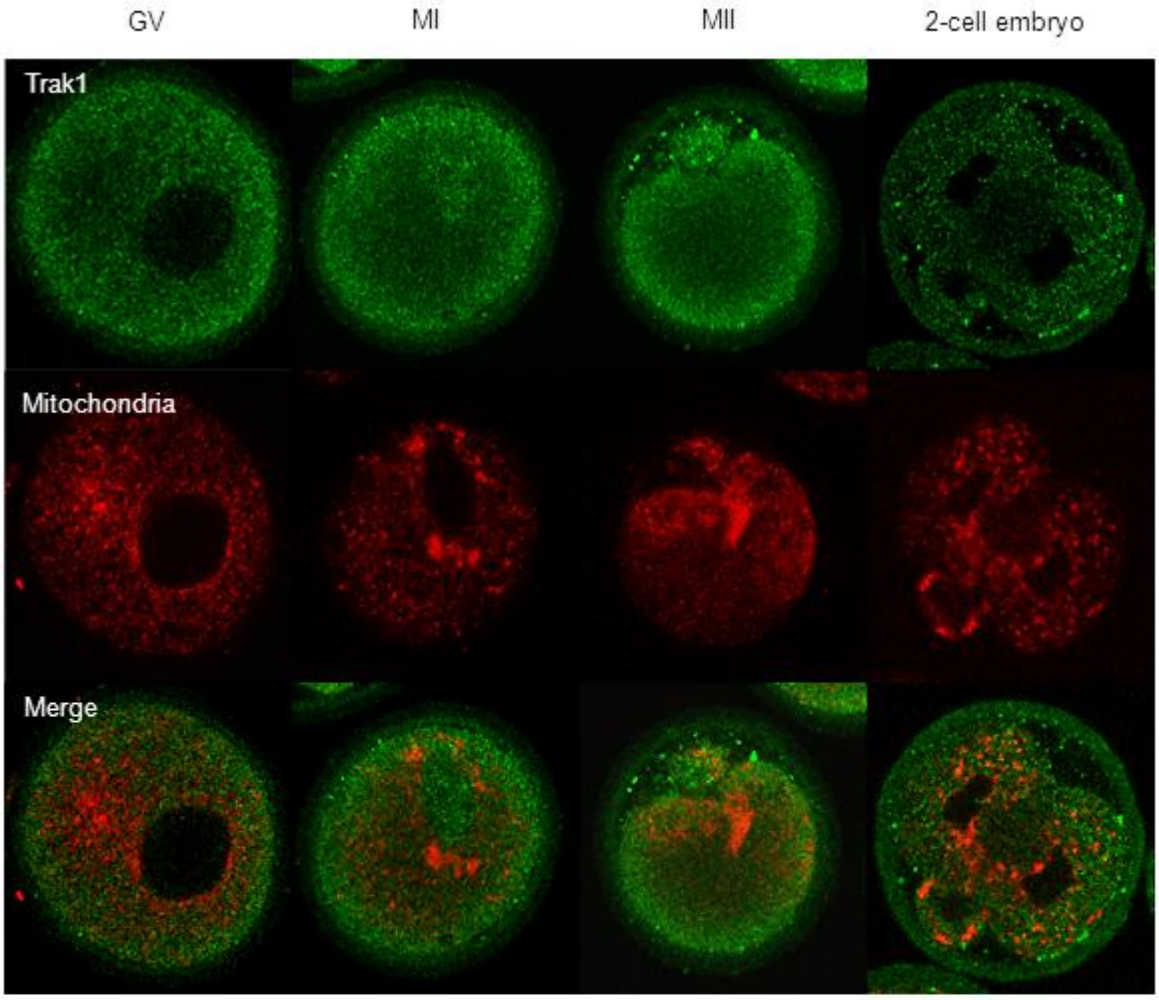

Figure 4.4. Endogenous Trak1 is not localised to mitochondria. Oocytes at GV, MI and MII stages and 2-cell embryos were stained for Trak1 and mitochondria. Trak1 is largely cytosolic, with some enrichment apparent in the spindle region. Images are representative of at least 10 oocytes for each stage examined.

since the antibody employed has been used successfully in other studies (Beck *et al.* 2002; Brickley *et al.* 2005; MacAskill *et al.* 2009a). Thus, it was only possible to determine the localisation of a fluorescently-tagged Trak2 protein.

4.2.3 Exogenous Miro and Trak2, but not Trak1, are localised to oocyte mitochondria

In order to confirm the localisations observed with antibodies directed against the endogenous proteins for Trak1 and Miro, and in order to investigate the localisation of Trak2, the proteins were fluorescently tagged and over-expressed in oocytes by microinjection of mRNA. Miro-mCherry was localised to the mitochondria at GV, MI and MII (Fig. 4.5), in agreement with the endogenous localisation. Trak1-EGFP was largely cytosolic, although enrichment was detected within the spindle (Fig. 4.6); observations which were again similar to those detected by immunolocalisation of the endogenous protein. Trak2-EGFP was localised both to mitochondria and at structures within the spindle (Fig. 4.7).

4.2.4 Over-expression of Trak2, but not Miro or Trak1, alters mitochondrial distribution

Over-expression of Miro-mCherry did not appear to have any influence on the overall distribution of mitochondria in the oocyte. Trak1-EGFP over-expression also had no effect, an unsurprising finding given its non-mitochondrial localisation (Fig. 4.5 and Fig. 4.6 respectively). In contrast, Trak2 over-expression caused a dramatic increase in mitochondrial aggregation at all stages examined (Fig. 4.7). Indeed, very few mitochondria could be found dispersed within the cytosol. Nonetheless, mitochondria accumulated in the spindle region at MI, and appeared to be trafficked

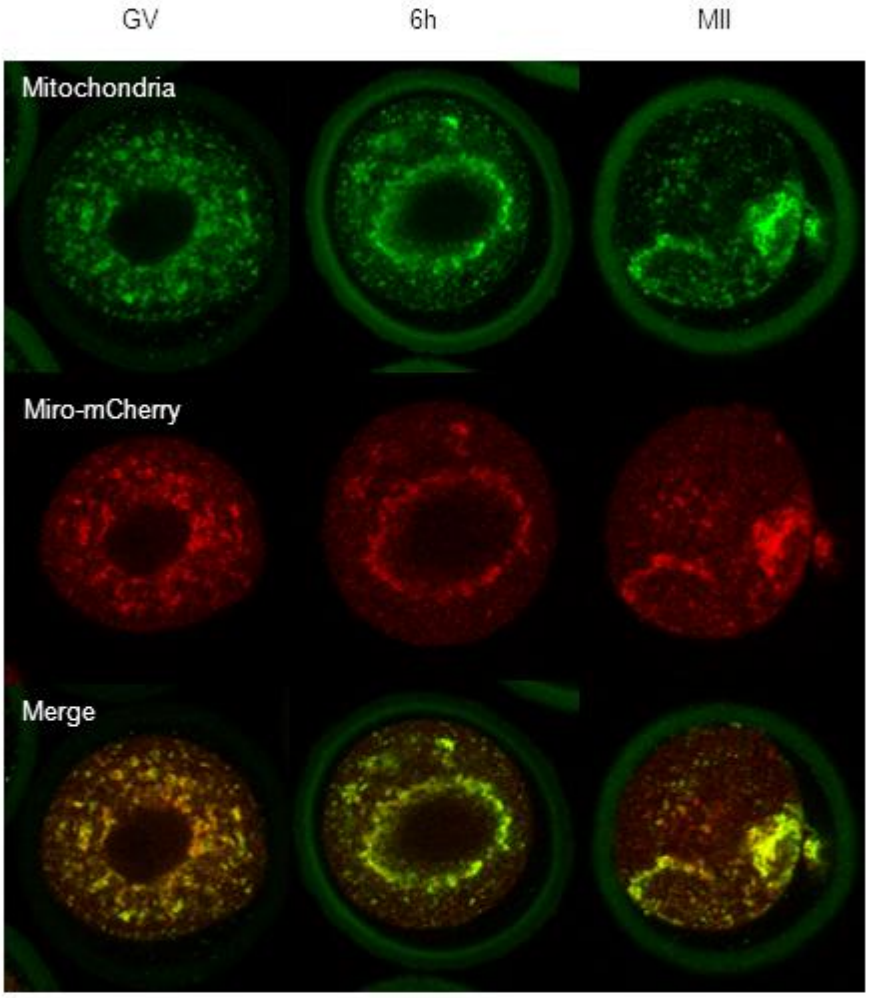

Figure 4.5. Miro-mCherry is localised to the mitochondria. Oocytes at GV stage were microinjected with mRNA for Miro-mCherry. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were either fixed at GV stage or matured to MI or MII before fixing and staining for mitochondria. The localisation of the over-expressed protein is consistent with that observed by immunofluorescence staining of the endogenous protein. Images are representative of at least 10 oocytes for each stage examined.

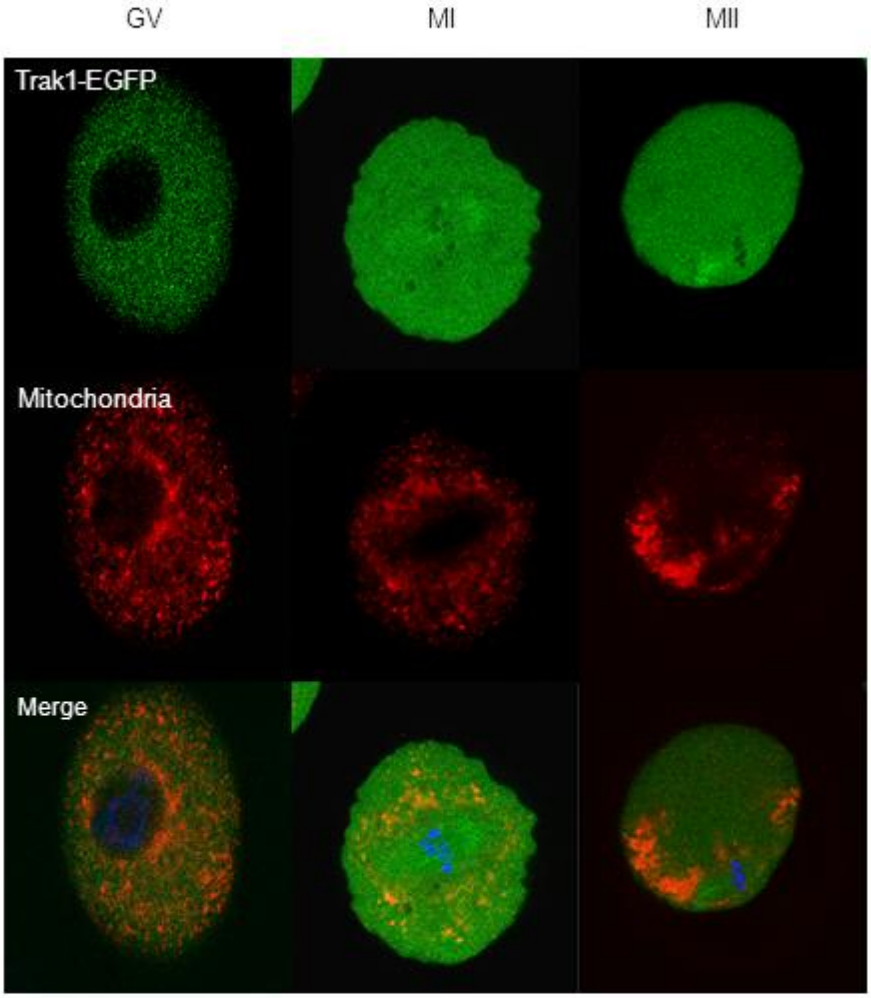

Figure 4.6. Trak1-EGFP is not localised to the mitochondria. Oocytes at GV stage were microinjected with mRNA for Trak1-EGFP. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were either fixed at GV stage or matured to MI or MII before fixing and staining for mitochondria. The localisation of the over-expressed protein is consistent with that observed by immunofluorescence staining of the endogenous protein. Images are representative of at least 10 oocytes for each stage examined.

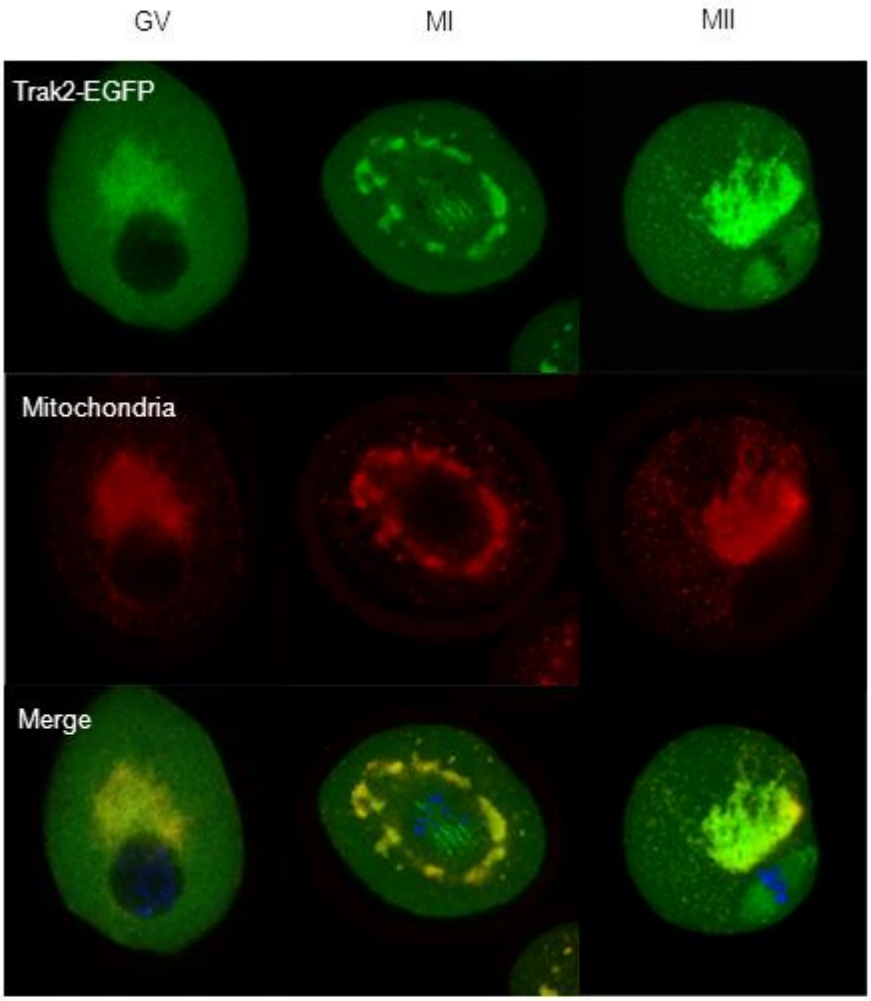

Figure 4.7. Trak2-EGFP is localised to the mitochondria and at the spindle. Oocytes at GV stage were microinjected with mRNA for Trak2-EGFP. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were either fixed at GV stage or matured to MI or MII before fixing and staining for mitochondria. Mitochondrial accumulation is increased at all stages examined. Images are representative of at least 10 oocytes for each stage examined.

towards the cortex at polar body extrusion, since mitochondria were found in the vicinity of the spindle at MII. Interestingly, the organisation of the mitochondrial network seemed to have been altered. Individual mitochondria were largely no longer distinguishable and mitochondria were instead clumped together into large aggregates, suggesting a possible alteration in fission and fusion dynamics in addition to, or because of, a transport defect.

4.2.5 Trak1 and Trak2 at the spindle align with microtubules

The spindle localisation observed for both Trak1 and Trak2 was examined in more detail by staining oocytes over-expressing Trak1-EGFP or Trak2-EGFP for tubulin to visualise the spindle. This revealed that both proteins were localised within the spindle. However, neither Trak1 nor Trak2 appeared to be binding directly to microtubules and were instead aligned with microtubules (Fig. 4.8). This was particularly clear with Trak2 where the protein could be seen to lie in between and alongside spindle microtubules.

4.2.6 Trak1 and Trak2 localise to the spindle independently of kinesin binding

Both Trak1 and Trak2 have been reported to bind to kinesin and the domains responsible for this interaction have been mapped (Brickley *et al.* 2005; Smith *et al.* 2006). Since both Trak1 and Trak2 were found within the spindle, alongside microtubules, it is plausible that the kinesin-binding domain of the Trak proteins is responsible for this localisation. In order to examine whether these domains are important for localisation of the two proteins to the spindle region, fluorescently tagged deletion mutants lacking this domain were over-expressed. These constructs retain the ability to bind to Miro but are unable to interact with kinesin (MacAskill *et*

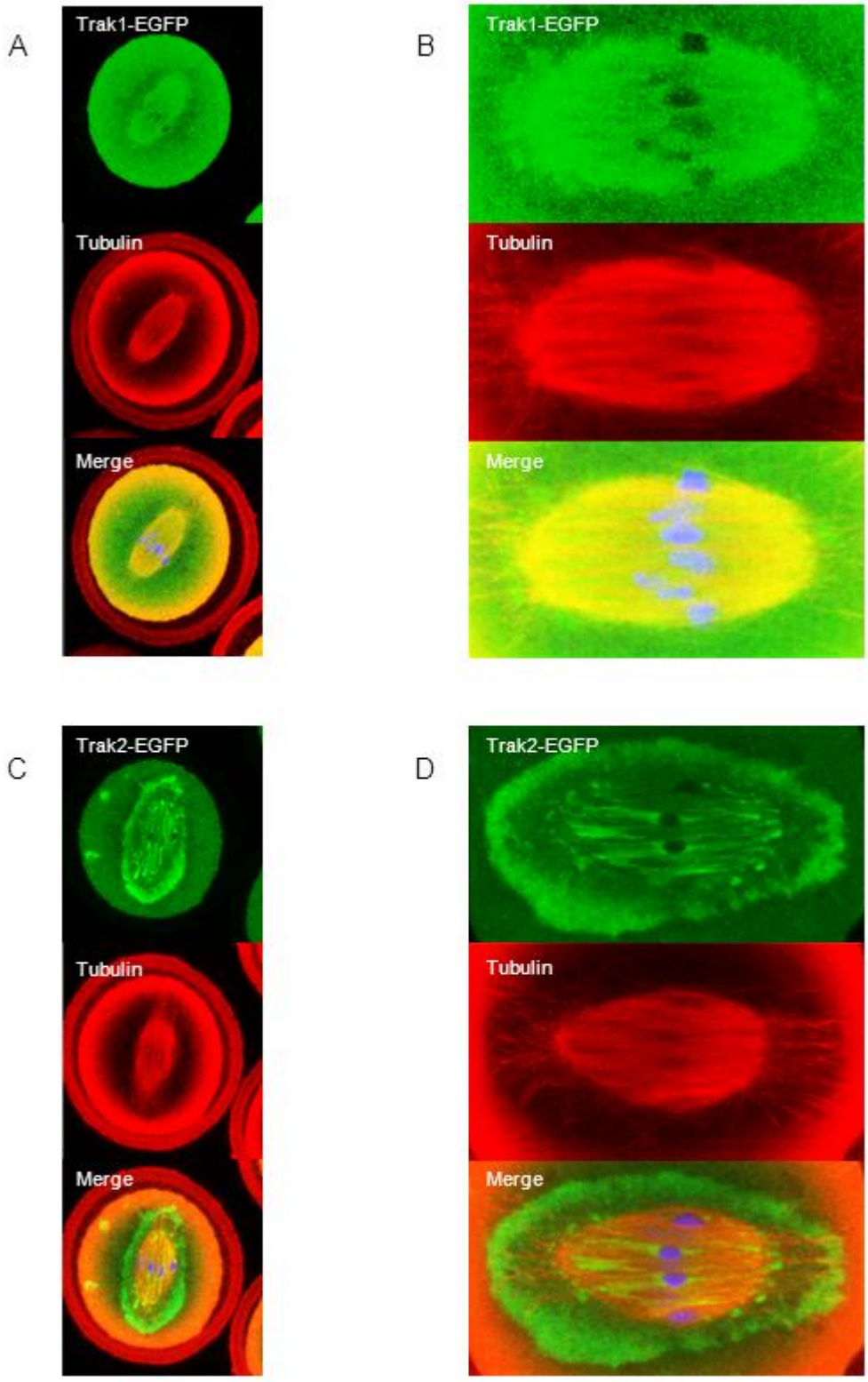

Figure 4.8. Trak1 and Trak2 align with microtubules at the spindle. Oocytes at GV stage were microinjected with mRNA for (A) Trak1-EGFP or (C)Trak2-EGFP. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were released from meiotic arrest and matured to MI before fixing and staining for tubulin. (B) and (D) are higher magnification images of the spindle region from (A) and (C) respectively. Images are representative of results obtained from at least 27 oocytes.

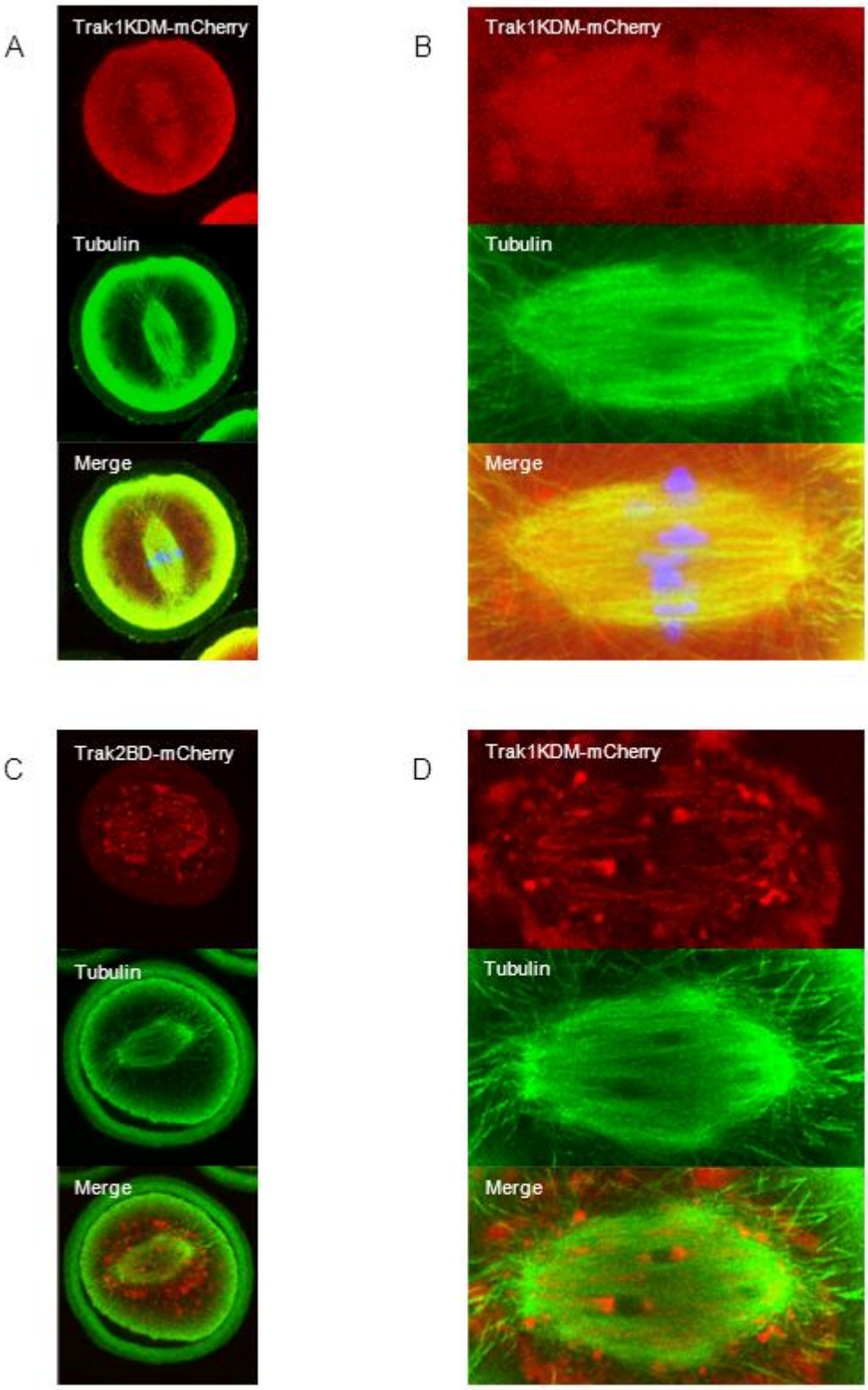

Figure 4.9. Trak1KDM and Trak2KDM retain a spindle localisation. Oocytes at GV stage were microinjected with mRNA for (A) Trak1KDM-mCherry or (C)Trak2KDM-mCherry. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were released from meiotic arrest and matured to MI before fixing and staining for tubulin. (B) and (D) are higher magnification images of the spindle region from (A) and (C) respectively. Images are representative of results obtained from at least 25 oocytes.

al. 2009a). For both Trak1 and Trak2, the absence of the kinesin binding domain did not alter the localisation of the protein at the spindle (Fig. 4.9). This suggests that the localisation of both proteins to the spindle area is not dependent on kinesin binding and occurs via an unknown mechanism. The localisation of Trak2 at the mitochondria was also unchanged by the absence of the kinesin binding domain, suggesting that this interaction occurs independently of kinesin binding.

4.2.7 Manipulation of mitochondrial adapter proteins

Attempts were made to knock down Trak and Miro proteins using a morpholino approach in order to ascertain their function in the oocyte. However, preliminary experiments with Trak1 showed that knockdown was unsuccessful and this, together with reports of shRNA knockdown of Miro in neurons requiring one week to deplete the protein successfully (A.F. Macaskill, personal communication), led to this approach being abandoned since the proteins were likely to be too stable for morpholino knockdown to work in the limited time for which oocytes can be cultured.

As an alternative approach to begin investigating the role of Trak and Miro proteins within the oocyte, the Trak2 kinesin-deletion mutant was employed (MacAskill *et al.* 2009a; Brickley & Stephenson 2011). Miro has been reported to couple to kinesin by two different mechanisms; it is able to bind to kinesin either directly, in a Ca^{2+}-dependent manner as has been demonstrated in neuronal synapses (MacAskill *et al.* 2009b), or as part of a complex involving Trak, Miro and kinesin, an interaction which is independent of Ca^{2+} levels (Brickley & Stephenson 2011). If Miro is participating in mitochondrial transport as part of the Miro/Trak/Kinesin complex,

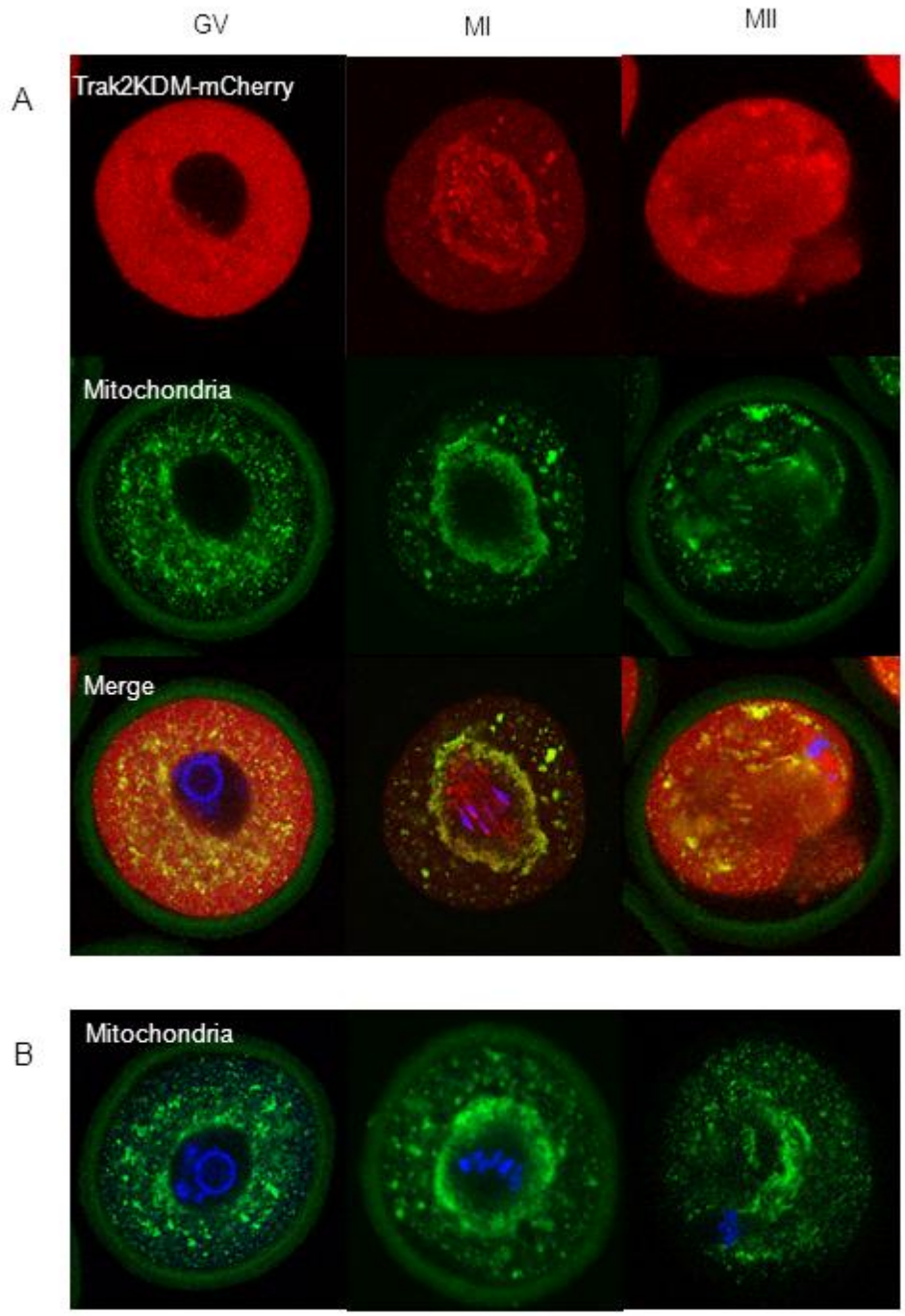

Figure 4.10. Trak2KDM does not affect mitochondrial distribution. (A) Oocytes at GV stage were microinjected with mRNA for Trak2KDM-mCherry. After overnight incubation in the presence of IBMX to allow protein expression, oocytes were either fixed at GV stage or matured to MI or MII before fixing and staining for mitochondria. (B) Control oocytes showing normal mitochondrial distribution. Images are representative of results obtained from between 11 and 41 oocytes for each stage examined.

then over-expression of a Trak kinesin-deletion mutant which retains the ability to bind Miro but is unable to couple to kinesin will act as a dominant negative and prevent mitochondrial transport that is dependent on the interaction of Miro and Trak, by uncoupling the complex from kinesin (MacAskill *et al.* 2009a; Brickley & Stephenson 2011).

The mutant protein was retained at the mitochondria (Fig. 4.10), as well as within the spindle (see Fig. 4.9), however the increased mitochondrial aggregation observed on over-expression of the wild type protein was prevented by over-expression of the Trak2 kinesin-deletion mutant, indicating that the interaction of Trak2 with kinesin is required for this effect (Fig. 4.10). Indeed, the overall distribution of mitochondria in oocytes expressing the Trak2 kinesin-deletion mutant was broadly unchanged when compared to control oocytes, suggesting that the Miro/Trak/Kinesin complex may not be required to establish normal distribution of mitochondria in the maturing oocyte.

4.3 Discussion

Experiments in this chapter describe the expression and localisation of the mitochondrial adapter proteins Trak1 and Trak2, and the mitochondrial Rho GTPase Miro, together with the effects of over-expression of the proteins, and disruption of the trafficking complex, on the distribution of mitochondria in the maturing oocyte.

Both Trak1 and Miro proteins are highly expressed in the oocyte according to the BioGPS portal, suggesting a potential role in mitochondrial transport during oocyte maturation. However, whilst Miro was localised to the mitochondria in oocytes and embryos at all stages examined, both by immunofluorescence staining and over-expression of fluorescently-tagged protein, Trak1 did not exhibit mitochondrial localisation, either endogenously or when it was over-expressed, and was instead observed at vesicular structures within the spindle. Trak2, which appears to be poorly expressed in oocytes, and was undetectable by immunofluorescence staining was localised both within the spindle and to the mitochondria when over-expressed.

The finding that Miro is localised to mitochondria in oocytes is in agreement with its reported localisation and function in other cell types (Fransson *et al.* 2006; Fransson *et al.* 2003; Glater *et al.* 2006; MacAskill *et al.* 2009a; MacAskill *et al.* 2009b; Guo *et al.* 2005). With regard to Trak proteins, whilst the *Drosophila* Trak orthologue Milton has only been localised to mitochondria where it interacts with Miro and kinesin (Glater *et al.* 2006; Stowers *et al.* 2002; Gorska-Andrzejak *et al.* 2003), mammalian Trak proteins have been shown to participate in the trafficking of a number of cargoes, including endosomes, $GABA_A$ receptors, and mitochondria, as well as interacting with the enzyme OGT and RNA polymerase II, in addition to

Miro and kinesin (Kirk *et al.* 2006; Webber *et al.* 2008; Gilbert *et al.* 2006; Beck *et al.* 2002; Iyer *et al.* 2003; Brickley *et al.* 2005; Smith *et al.* 2006; Fransson *et al.* 2006; MacAskill *et al.* 2009a; Brickley & Stephenson 2011; Koutsopoulos *et al.* 2010). It has been suggested therefore that Trak proteins act as adapter proteins able to recruit kinesin to specific cargoes for trafficking, regulated by interaction with adapter proteins such as Miro at mitochondrial sites and Hrs at endosomes (MacAskill *et al.* 2009a). The non-mitochondrial localisation of Trak1 within the spindle, and the localisation of Trak2 both at the mitochondria and at non mitochondrial sites within the spindle is in line with this notion, and suggests that the Trak protein family may have a role other than, or in addition to, mitochondrial trafficking in the maturing oocyte.

This is the first demonstration of the presence of the Trak proteins within the spindle, although Trak1 has previously been found to interact with RNA polymerase II within the nucleus (Iyer *et al.* 2003). Nonetheless, both proteins were observed in the spindle region when exogenously expressed and endogenous Trak1 was also found to be enriched in this area. The presence of fluorescent tags on the exogenously expressed proteins is unlikely to have influenced localisation of the proteins since Koutsopoulos et al. have demonstrated that the presence of a GFP tag on either the C- or N-terminus of both Trak1 and Trak2 does not alter the localisation when the proteins are over-expressed in COS-7 cells (Koutsopoulos *et al.* 2010).

Both proteins aligned with microtubules within the spindle region, and were additionally enriched around the spindle poles and at sites of microtubule-kinetochore interaction in some cases. This localisation was maintained when Trak

proteins lacking the kinesin-binding domain were expressed, suggesting this localisation is not dependent on binding of the Trak proteins to kinesin, a surprising finding since this is the only domain in Trak proteins which has reported microtubule interactions. Furthermore, the Trak2-kinesin deletion mutant also remained localised to mitochondria suggesting that Trak2 is able to localise to mitochondria independently of an interaction with kinesin. Both Trak1 and Trak2 have been shown to interact with endosomes (Webber *et al.* 2008; Kirk *et al.* 2006) which are also located in the spindle region during oocyte maturation (R. Dale, unpublished observations). However, the localisation of endosomes is not consistent with that of Trak1 and Trak2 within the spindle since endosomes are found only outside the spindle. The same is true of the ER, which also localises in the spindle region. Indeed, although a limited amount of ER is seen to invade the spindle and surround the chromosomes, the majority is found around the spindle rather than within it (Fitzharris *et al.* 2007). It is therefore unlikely that the striking accumulation of Trak proteins within the spindle is primarily associated with either endosomes or ER.

It is interesting to note that as well as having diverse sub-cellular localisations and cargo interactions in different cell types examined, in studies where Trak1 and Trak2 have been investigated together, the two proteins exhibit some differing localisations and functions. In two different cultured cell types, although both proteins localised to the mitochondria when exogenously expressed, this was more apparent for Trak1, whereas Trak2 was also observed in the cytosol (Koutsopoulos *et al.* 2010; Brickley *et al.* 2005). In another study, Trak1 but not Trak2 shRNAi knock-down resulted in decreased mitochondrial mobility, although the Trak1 shRNAi-induced arrest of mitochondrial movement could be rescued by exogenous expression of either Trak1

or Trak2 (Brickley & Stephenson 2011). This suggests that Trak1 and Trak2 may serve distinct functions in different cell types and is consistent with the differing expression levels and localisations observed for Trak1 and Trak2 in oocytes.

Over expression of Miro, Trak1, Trak2, and Trak2 kinesin-deletion mutants appears to show that the Miro/Trak/Kinesin complex is not required for mitochondrial trafficking to the spindle region during oocyte maturation. In the maturing oocyte, no overall effect on mitochondrial distribution was observed when Miro was over-expressed. This is in contrast to the reported effects of Miro over-expression in other cell types, which include collapse of the mitochondrial network and the aggregation of mitochondria (Fransson *et al.* 2003; Fransson *et al.* 2006; Glater *et al.* 2006; Saotome *et al.* 2008), increased mitochondrial length (Saotome *et al.* 2008; Russo *et al.* 2009), transport into neuronal processes (Guo *et al.* 2005; MacAskill *et al.* 2009a), mitochondrial motility (Saotome *et al.* 2008; MacAskill *et al.* 2009b), Ca^{2+}-dependent mitochondrial stopping (Saotome *et al.* 2008) and recruitment of Trak to the mitochondria (MacAskill *et al.* 2009a), and decreased mitochondrial motility in both the anterograde and retrograde directions (Russo *et al.* 2009).

It is possible that mitochondrial motility in the oocyte may have been affected without any change to mitochondrial distribution, if for example the motility change occurred for both anterograde and retrograde movements. Additionally, whilst no obvious effects on mitochondrial length or morphology were detected, these parameters were not examined in detail therefore it is possible a change may have occurred. However, one study reports no effect mitochondrial distribution, morphology, motility or length when Miro is over-expressed in hippocampal neurons

(Wang & Schwarz 2009), consistent with the results observed in the maturing oocyte. In such a scenario, it is possible that Miro is not a limiting factor in mitochondrial motility, thus over-expression does not have any effect.

The observation that mitochondrial distribution in unaffected by expression of a Trak2 kinesin deletion mutant in the oocyte, which functions as a dominant negative, suggests that in addition to Miro over-expression having no significant effects on mitochondrial transport in the oocyte, the Miro/Trak complex is not required for normal distribution of mitochondria. Over-expression of the kinesin deletion mutant uncouples the recruitment of kinesin from the Trak/Miro complex at the mitochondria thus disrupting the Trak/Miro/Kinesin complex.

In contrast to the results observed in oocytes, expression of the dominant negative protein in neurons results in reduced transport of mitochondria into neuronal processes (MacAskill *et al.* 2009a). Additionally, mutation or knock-down of Miro proteins has been found to cause aberrant transport of mitochondria to distal neuronal processes (Guo *et al.* 2005; Russo *et al.* 2009), decreased mitochondrial motility in both directions (Saotome *et al.* 2008; Russo *et al.* 2009; MacAskill *et al.* 2009b), decreased mitochondrial length (Russo *et al.* 2009), and impaired Ca^{2+}-dependent mitochondrial stopping (Saotome *et al.* 2008; Wang & Schwarz 2009; MacAskill *et al.* 2009b). It is possible that these differences can be accounted for by cell type specificity in Miro function, since much of the work elucidating the role of Miro in mitochondrial trafficking has been carried out in neuronal cell types. Alternatively, since Miro has also been reported to bind directly to kinesin, without the need for Trak proteins (MacAskill *et al.* 2009b), then Trak-independent Miro

function may have been maintained in the presence of Trak2KDM, resulting in no change to mitochondrial distribution.

However, over-expression of Trak2 in oocytes results in increased mitochondrial aggregation, with very few mitochondria observed outside the central region of mitochondria, either around the germinal vesicle at GV stage, or around the spindle at MI and MII. This effect is consistent with that observed in other cell types, both with *Drosophila* Milton (Stowers *et al.* 2002) and the mammalian Trak proteins (Brickley *et al.* 2005; Smith *et al.* 2006; Koutsopoulos *et al.* 2010; Brickley *et al.* 2011).

Interestingly, the distribution of mitochondria when Milton/Trak proteins are over-expressed is similar to that observed in Milton/Trak mutant or knock-down experiments (Stowers *et al.* 2002; Gorska-Andrzejak *et al.* 2003; Glater *et al.* 2006; Brickley *et al.* 2011), in that mitochondria aggregate and clump at the minus ends of microtubules rather than being increasingly trafficked towards the plus ends as one might expect for a kinesin-associated trafficking protein. In the absence of any evidence for a direct interaction of Milton/Trak proteins with dynein (Stowers *et al.* 2002; Brickley *et al.* 2005), this has been attributed to an inhibitory effect on kinesin, similar to the inhibition of dynein-mediated transport by over-expression of a component of the dynein adapter complex, dynamitin (Burkhardt *et al.* 1997). This is supported by the finding that co-expression of Milton/Trak and kinesin can re-distribute mitochondria to plus-ends of microtubules, suggesting that the respective level of the two proteins is critical for determining transport (Glater *et al.* 2006; Smith *et al.* 2006; Brickley *et al.* 2011). Indeed, when the Trak2-kinesin deletion

mutant was over-expressed, mitochondrial distribution was normal, suggesting that the aggregation of mitochondria on over-expression of Trak2 is a result of the interaction between Trak2 and kinesin.

Alternatively, kinesin has been shown to interact with dynein, thus the binding of Milton/Trak to kinesin may influence the association with dynein to coordinate bi-directional transport (Deacon *et al.* 2003; Ligon *et al.* 2004). Interestingly, in Trak1 shRNAi knockdown experiments, both anterograde and retrograde mitochondrial movement is decreased, in line with this notion (Brickley & Stephenson 2011). Additionally, Milton/Trak proteins contain a region in the N-terminus which has homology to HAP-1 (Stowers *et al.* 2002; Beck *et al.* 2002; Webber *et al.* 2008; Gilbert *et al.* 2006), a protein which associates with microtubules and binds to the dynactin subunit p150/Glued (Li *et al.* 1998; Engelender *et al.* 1997), suggesting that HAP-1 and Milton/Trak proteins may share an interaction with dynein, albeit an indirect one in the case of Milton/Trak. An indirect interaction with dynein was proposed to explain why different Milton mutants, which resulted in altered expression of two major Milton transcripts which differed in their HAP1 domains, had opposing effects on the trafficking of mitochondria into the *Drosophila* oocyte (Cox & Spradling 2006).

In addition to mitochondrial trafficking defects, over-expression of Trak2 in oocytes resulted in clumping of mitochondria into large aggregates, suggesting a change in fission and fusion dynamics. Increased continuity of the mitochondrial network on Trak1 over-expression has been previously reported (Koutsopoulos *et al.* 2010), together with a decreased frequency of fission and fusion events in Trak1 shRNAi

knockdown experiments (Brickley & Stephenson 2011). Miro activity has also been linked to the fusion state of mitochondria, through regulation of Drp1 (Saotome *et al.* 2008), suggesting that function of the Miro/Trak trafficking machinery may be important to maintain mitochondrial fission-fusion dynamics.

4.4 Summary

It would appear that Miro, which is expressed in oocytes and localised to the mitochondria, may be dispensable for normal mitochondrial trafficking in the oocyte, at least as part of a Trak/Miro complex. However, although over-expression of a Trak2-kinesin deletion mutant can establish that the Miro/Trak complex is not required, in the absence of an effective morpholino knock-down approach, it was not possible to eliminate the possibility that Miro may participate in mitochondrial trafficking in the oocyte via a direct interaction with kinesin, without a requirement for Trak, as has been reported in other cell types. Thus, further work is needed to eliminate with certainty a role for Miro in mitochondrial trafficking in the oocyte.

Furthermore, despite apparently strong expression at the mRNA level in the oocyte, neither endogenous nor exogenously expressed Trak1 appeared to localise to the mitochondria, and the function of the protein within the spindle remains unclear. Exogenously expressed Trak2 localised to the mitochondria and led to trafficking defects, and was also found within the spindle, as with Trak1. Over-expression of a Trak2-kinesin deletion mutant did not disrupt mitochondrial trafficking, suggesting that the accumulation of mitochondria observed with Trak2 over-expression is dependent on the ability of the protein to bind kinesin, and that Trak2 is not required for normal mitochondrial distribution in the oocyte.

5. Mitochondrial Function During Oocyte Maturation

5.1 Introduction

Mitochondrial dysfunction and a deficit in mitochondria-derived ATP in the oocyte have been linked to compromised meiotic maturation and decreased developmental competence (Van Blerkom 2004; Dumollard *et al.* 2007b; Wilding *et al.* 2003; Thouas *et al.* 2004; Zhang *et al.* 2006; Thouas *et al.* 2006; Wang *et al.* 2009). Conversely, higher levels of ATP in oocytes have been correlated with an increased capacity for continued development (Van Blerkom *et al.* 1995; Stojkovic *et al.* 2001; Zeng *et al.* 2007), suggesting that mitochondrial function and ATP provision in the oocyte is of vital importance to the oocyte and early embryo.

Mitochondria are among the most abundant organelles in the oocyte but they appear structurally immature when compared with those at later developmental stages, exhibiting a rounded structure with a dense matrix and few cristae (Wassarman & Josefowicz 1978; Motta *et al.* 2000; Sathananthan & Trounson 2000). This morphology has led to the suggestion that oocyte mitochondria are relatively metabolically inactive. However, glycolytic rates are low in the oocyte and early embryo, and oxidative phosphorylation of pyruvate provides the primary source of energy, underlining the importance of mitochondrial function to successful maturation and early development (Biggers *et al.* 1967; Barbehenn *et al.* 1974; Johnson *et al.* 2007; Dumollard *et al.* 2007a; Leese & Barton 1984; Wilding *et al.* 2002; Brinster 1971).

Measurement of ATP in oocytes has shown an increase in ATP levels between GV and MII stages in cattle and pig, and a reduction in cat, (Stojkovic *et al.* 2001; Brevini *et al.* 2005; Freistedt *et al.* 2001; Sun *et al.* 2001). Additionally, a correlation between higher oocyte ATP content and successful developmental competence has been suggested in cattle and human oocytes, although not in pig (Stojkovic *et al.* 2001; Van Blerkom *et al.* 1995; Brevini *et al.* 2005). Furthermore, during the course of the work described in this chapter, a report was published which described dynamic changes in ATP levels during oocyte maturation in the mouse (Yu *et al.* 2010). In this study, peaks of ATP production were observed, accompanied by mitochondrial clustering, concomitant with the formation of long cortical microfilaments. Both the formation of long cortical microfilaments and the bursts of ATP production could be prevented by disruption of the actin cytoskeleton with cytochalasin B, leading to the proposal that the changes in ATP levels observed during maturation were driven by mitochondrial clustering, as a result of changes to the microfilament network.

Thus far, measurement of ATP levels in oocytes has been achieved either by indirect means such as by measurement of Mg^{2+}, or by bioluminescence measurements of luciferase. Using these methods, it has largely only been possible to measure ATP in populations of cells, although microinjection of luciferase protein into oocytes has also allowed the measurement of ATP levels in single oocytes (Dumollard *et al.* 2004; Dumollard *et al.* 2008). A recent report describes the establishment of a novel FRET-based method for measuring ATP in live cells in real time (Imamura *et al.* 2009). Imamura and colleagues created a series of recombinant probes, named ATeam, based around the ε subunit of the bacterial ATP-synthase and using the

modified CFP and YFP fluorophores mseCFP (hereafter CFP) and cp173-mVenus (hereafter YFP). The large conformational change in the ε subunit upon ATP binding was exploited to create a FRET probe where the binding of ATP results in the CFP and YFP fluorophores coming into close proximity, allowing FRET to occur.

The ε subunit binds ATP with high specificity without hydrolysing it and the FRET signal is unaltered in vitro by changes in the pH from 7.1 to 8.5. Rates of ATP binding and dissociation were found to allow measurement of changes in [ATP] with rates of up to 0.1 s^{-1} and in vivo, the probe was found to respond to changes in [ATP] in HeLa cells resulting from inhibition of glycolysis and oxidative phosphorylation. The probe has subsequently been used to measure ATP in yeast (Bermejo *et al.* 2010) and human fibroblasts (Liemburg-Apers *et al.* 2011). The ATeam probe employed here, AT1.03 has a reported dissociation constant of 3.3mM at 37°C, allowing measurement of ATP in the physiological range.

In recent years, the concept of compartmentalisation of ATP production and consumption has emerged (Kuiper *et al.* 2009; Weiss & Lamp 1989; Kennedy *et al.* 1999; de Groof *et al.* 2002; van Horssen *et al.* 2009). This has been particularly well studied in neurons where axonal transport of mitochondria serves to maintain ATP supply at synapses (Hollenbeck & Saxton 2005). In the oocyte, it has been proposed that the reorganisation of mitochondria observed during oocyte maturation serves to provide a greater supply of ATP to areas of high demand, such as the spindle (Van Blerkom & Runner 1984). However, thus far no attempts to detect subcellular heterogeneity of ATP in the oocyte have been described. The development of a FRET-based recombinant ATP probe provides not only a novel way of measuring

ATP levels in single live oocytes in real time, but also a method for attempting to measure compartmentalisation of ATP provision and consumption, which may exist in a large cell such as the oocyte.

This chapter describes the measurement of various aspects of mitochondrial ATP production and consumption during oocyte maturation using a novel FRET-based recombinant probe. Characterisation of the probe as a method for reliable ATP measurements in single oocytes is described together with attempts to investigate whether ATP supply and demand is differentially regulated in specific subcellular regions of the oocyte. In addition ATP levels are monitored throughout maturation, and consumption of ATP is compared in oocytes at GV, MI and MII. The chapter concludes by describing attempts to elucidate mechanisms governing changing rates of ATP consumption, and by considering the effects of cumulus cells on ATP levels in the oocyte.

5.2 Results

5.2.1 Characterisation of ATeam for ATP measurements in oocytes

ATP levels in live cells have been monitored using the ATeam FRET probe in a number of cell types including in HeLa cells on inhibition of glycolysis and oxidative phosphorylation (Imamura *et al.* 2009), yeast cells mutant for a hexose transporter (Bermejo *et al.* 2010) and human skin fibroblasts (Liemburg-Apers *et al.* 2011). However, the probe has not previously been used in oocytes. Therefore experiments were first carried out to establish whether the AT1.03 ATeam probe could effectively be used to measure ATP levels in single oocytes in real time. Oligomycin, an inhibitor of the mitochondrial ATP-synthase, was applied to oocytes which had been microinjected with the ATP probe and time-lapse imaging was carried out to monitor ATP levels. Glycolysis is largely inactive in oocytes due to a block in the glycolytic enzyme phosphofructokinase (Barbehenn *et al.* 1974), therefore inhibiting the mitochondrial ATP synthase should lead to a decline in ATP levels. Indeed, a rapid decrease in the FRET signal was seen upon application of oligomycin (Fig. 5.1A).

In order to confirm this result, a second mitochondrial inhibitor, FCCP, a protonophore which uncouples the electron transport chain from ATP synthesis was used. As with oligomycin, ATP levels were observed to decrease (Fig. 5.1B). In contrast, no change in FRET ratio was detected on addition of dimethyl sulfoxide (DMSO) as a vehicle control (Fig. 5.1A). To confirm that the probe was responding specifically to changes in ATP, a mutant form of the probe in which arginine residues at positions 122 and 126 were been replaced with lysine, and which is therefore unable to bind ATP (AT1.03RK) (Imamura *et al.* 2009) was used. When

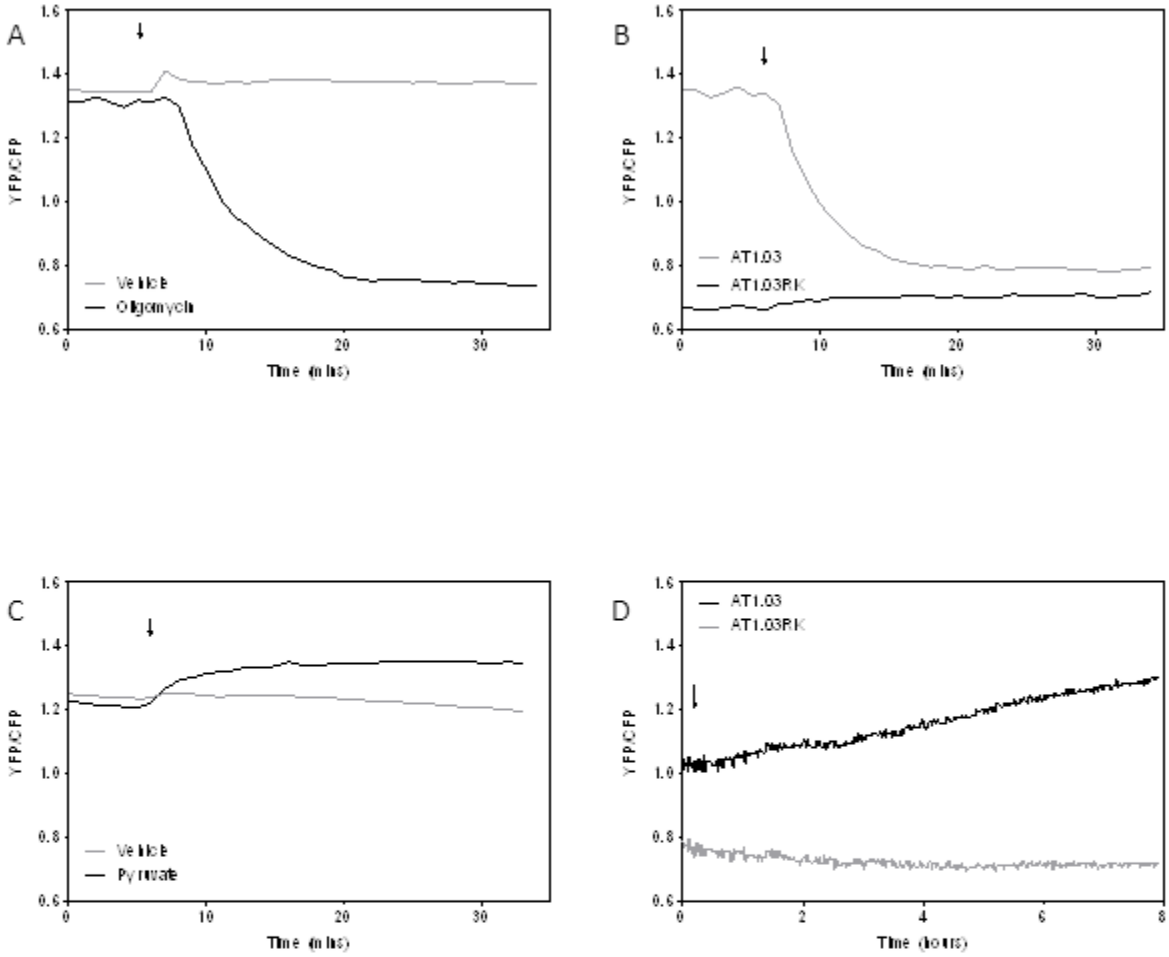

Figure 5.1. Validation of AT1.03 for ATP measurements in oocytes. GV oocytes were microinjected with mRNA encoding AT1.03 or the mutant probe AT1.03RK. Imaging was carried out during MI for (A), (B) and (C) and in ovulated MII arrested eggs for (D). A) 5 µg/ml oligomycin (n=23) or the equivalent volume of DMSO (n=19) (arrow) was applied to oocytes expressing AT1.03. B) 1 µM FCCP (arrow) was applied to oocytes expressing AT1.03 (n=29) or AT1.03RK (n=36). C) 2 mM pyruvate (n=14) or vehicle control (n=8) (arrow) was applied to substrate-depleted oocytes expressing AT1.03. D) Addition of sperm (arrow) to oocytes expressing AT1.03 (n=8) or AT1.03RK (n=3).

FCCP was applied to oocytes which had been microinjected with AT1.03RK, no change in the FRET signal was observed (Fig. 5.1B).

Next, to establish if the probe could also detect increasing ATP levels, oocytes were incubated in substrate-free media (without pyruvate, lactate or glucose) for one hour prior to the commencement of imaging to deplete oocyte ATP levels. Addition of pyruvate back to the media to stimulate the resumption of mitochondrial ATP production resulted in an increase in the YFP:CFP ratio, indicating a rise in ATP levels (Fig. 5.1C). Finally, since ATP levels have previously been shown to increase at fertilisation (Dumollard *et al.* 2004; Campbell & Swann 2006), experiments were carried out to ascertain if AT1.03 could detect this increase. In agreement with previous reports, an increase in ATP levels was detected at fertilisation in oocytes expressing AT1.03 (Fig. 5.1D). In contrast, in oocytes which had been microinjected with AT1.03RK, the FRET ratio remained unchanged throughout the imaging period.

5.2.2 Comparison of ATP levels in the spindle and non-spindle regions

It has been proposed that mitochondrial accumulation in the spindle region may provide a localised supply of ATP to an area of high energy consumption, but no experiments have thus far been reported which attempt to elucidate whether such a subcellular heterogeneity in ATP supply and demand exists. Previously used methods for ATP measurements in live cells such as the Mg^{2+} and luciferase assays would be unable to detect differences in ATP levels at the subcellular levels therefore the ATeam FRET probe provides, for the first time, the means with which to attempt to measure differences in ATP levels in subcellular regions.

Acceptor photobleaching, which measures the FRET efficiency and therefore reflects ATP levels, was employed to attempt to establish if a difference in ATP levels exists between the spindle and non-spindle regions in MI oocytes. These experiments were carried out by photobleaching the YFP signal throughout the oocyte and comparing the resulting change in CFP fluorescence between the spindle and non-spindle regions (Fig. 5.2A). The change in CFP signal was divided by the change in YFP signal in order to account for incomplete bleaching of YFP (see Methods for a more detailed description). However, this approach failed to reveal any differences in ATP levels between the spindle and non-spindle region (Fig. 5.2A).

Since the provision of higher levels of ATP proposed to occur in the spindle region is thought to provide a greater supply to an area of high demand, it is possible that increased ATP provision in this region is matched by higher ATP consumption, resulting in no difference in absolute levels of ATP between spindle and non-spindle regions. In order to investigate whether this could account for the inability to detect subcellular heterogeneity in ATP levels, ATP production was inhibited by application of oligomycin in order to block the mitochondrial ATP synthase. Since glycolysis is inactive in the oocyte, preventing mitochondrial ATP production allowed measurement of the rate of ATP consumption. However, no difference in the rate of ATP consumption was detected between the spindle and non-spindle regions (Fig. 5.2B) and, as with acceptor photobleaching, no difference in absolute ATP levels in the two regions was detected using this alternative approach (Fig. 5.2C).

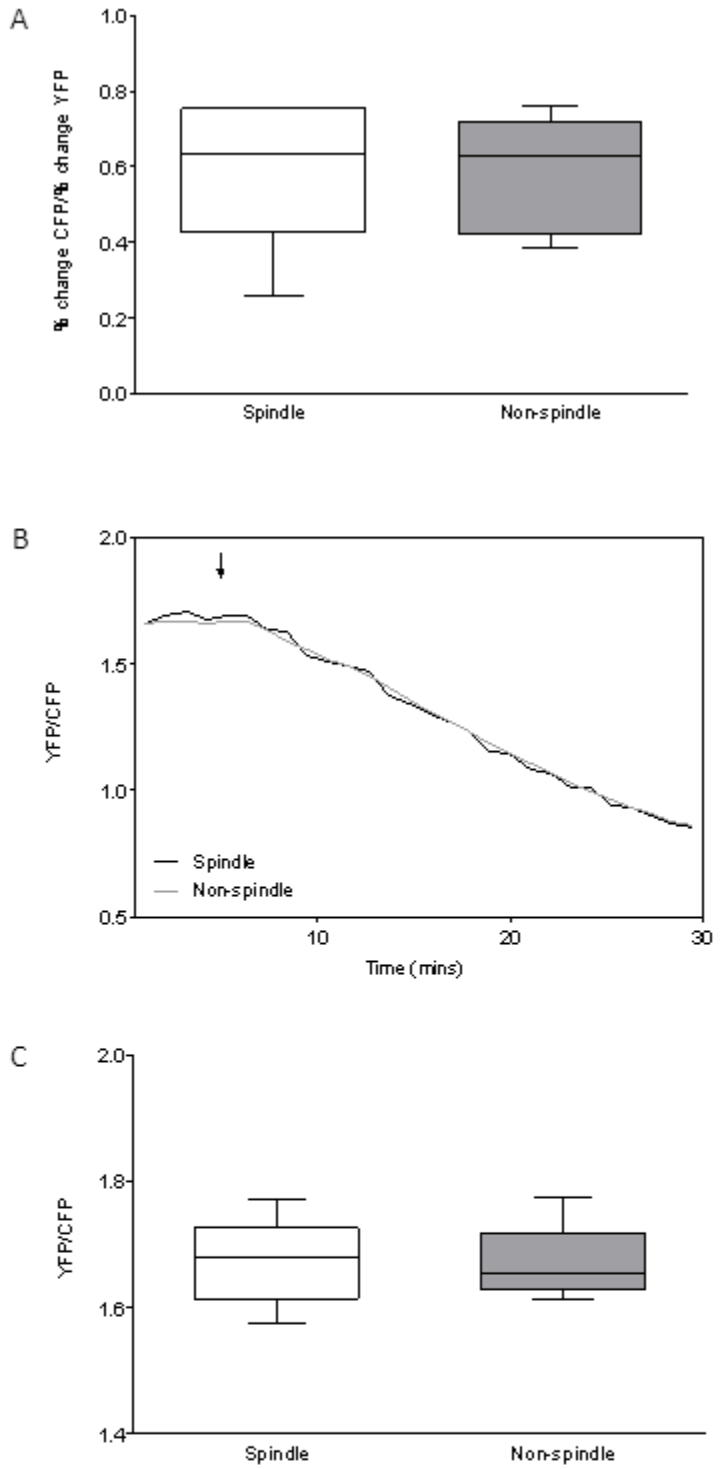

Figure 5.2. No difference in ATP levels and ATP consumption between spindle and non-spindle regions is detectable with AT1.03. GV oocytes were microinjected with mRNA encoding AT1.03. Experiments were carried out on a confocal microscope during MI, between 4 and 8 hours after release from meiotic arrest. A) FRET efficiency in spindle and non-spindle regions was measured by acceptor photobleaching. No difference was detected between spindle and non-spindle regions (n=8). B) 5 µg/ml oligomycin (arrow) was added to oocytes and ATP levels and ATP consumption was compared between spindle and non-spindle regions. Representative example from n=8 oocytes. C) Analysis of the ATP level in spindle and non-spindle regions does not reveal a significant difference between the two regions (n=8).

5.2.3 ATP levels are altered at different stages of maturation

ATP levels in oocytes of some species have been measured before and after maturation and found to be different at these two stages (Stojkovic *et al.* 2001; Freistedt *et al.* 2001; Brevini *et al.* 2005). In order to investigate whether progress of oocyte maturation has any effect on ATP levels in the mouse oocyte, ATP levels were measured at GV, MI and MII stages. These experiments revealed that ATP levels were significantly higher at GV stage than in those oocytes which were undergoing maturation (MI) and those arrested at MII (Fig. 5.3A). ATP levels were also significantly lower at MII arrest than during MI (Fig. 5.3A).

5.2.4 ATP consumption is altered at different stages of maturation

Since ATP levels were altered at different stages of maturation, experiments were carried out to establish if rates of ATP consumption were also changed. Mitochondria provide the primary source of ATP to the oocyte, therefore the rate of ATP consumption in the oocyte can be measured by monitoring the rate of ATP decline on addition of oligomycin, which inhibits the mitochondrial ATP synthase. Application of oligomycin to oocytes at GV, MI and MII stages revealed that ATP was consumed significantly more rapidly during MI and at MII than in oocytes arrested at GV stage (Fig. 5.3B and 5.3C). Glycolytic activity is low in oocytes but in order to confirm that differences in consumption observed by inhibiting the ATP synthase at different stages were not caused by altered levels of glycolysis, the glycolytic inhibitor iodoacetic acid (Downs 1995) was added after oligomycin (Fig. 4.5B). As expected, this led to no further change in ATP levels (Fig. 5.3B). Iodoacetic acid also had no effect on MI oocytes when added in the absence of oligomycin (Fig. 5.3D), confirming that glycolysis has no major impact upon ATP

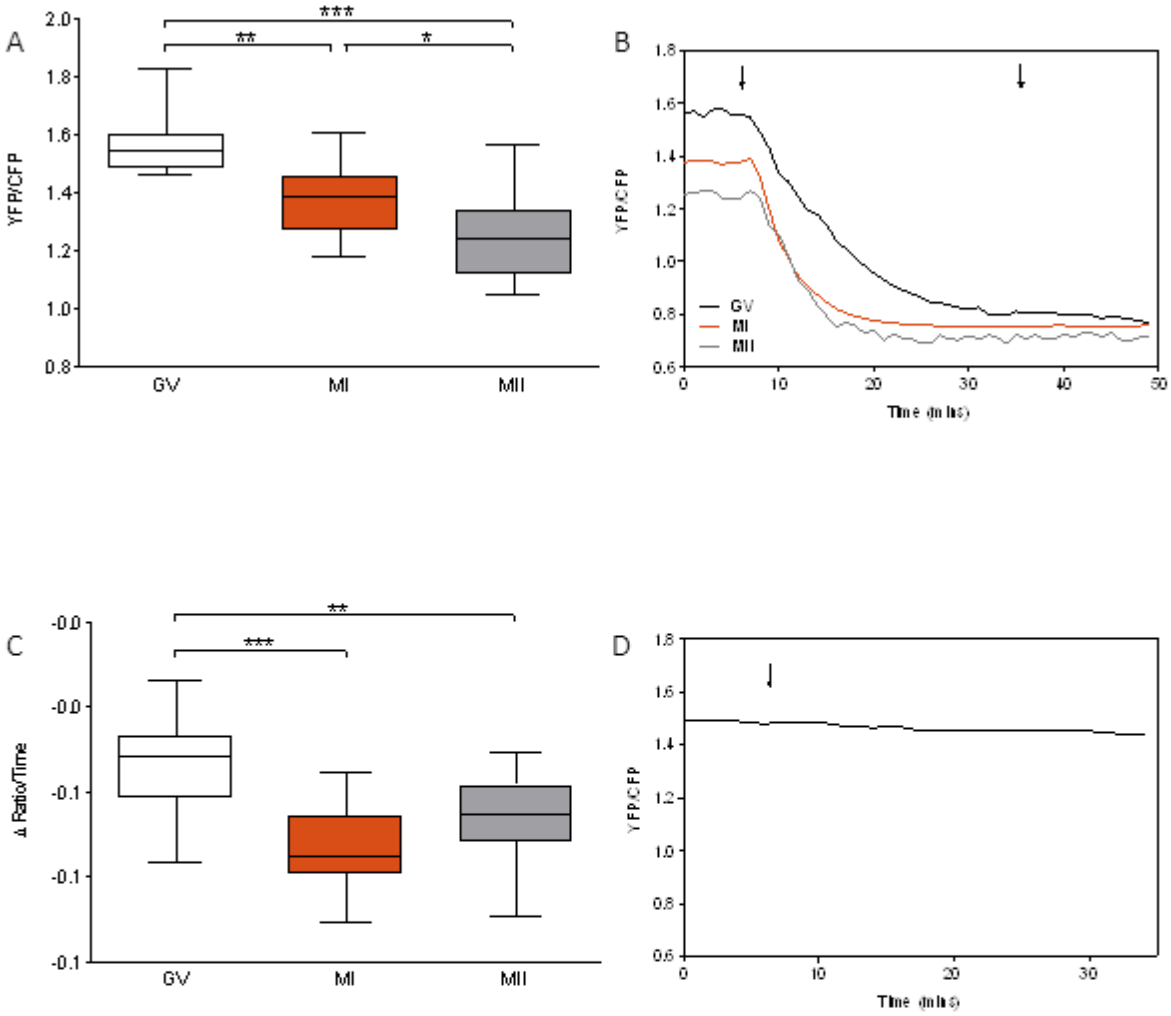

Figure 5.3. ATP levels and ATP consumption are altered at different stages of oocyte maturation. A) ATP levels were compared at GV (n=10), MI (n=20) and MII (n=18) stages by imaging oocytes microinjected with AT1.03. Levels are significantly higher in GV oocytes than those at MI and MII and in MI than MII. B) Addition of 5 μg/ml oligomycin (first arrow) reveals differences in rates of ATP consumption, which are not due to differences in glycolytic activity since addition of 1mM iodoacetic acid (second arrow) does not result in any further changes to ATP levels. C) Linear regression analysis of the rate of ATP decline over the first seven minutes after oligomycin addition (the linear portion) reveals that the rate of ATP consumption at MI and MII is significantly faster than at GV stage. D) Inhibition of glycolysis by addition of 1mM iodoacetic acid (arrow) does not result in any changes to ATP levels (n=29).

production during oocyte maturation and that the differences in rates of ATP decline can be attributed specifically to differing rates of ATP consumption.

5.2.5 ATP consuming processes during oocyte maturation

In order to try to establish which processes might be contributing to increasing rates of ATP consumption during oocyte maturation, ATP levels were monitored during inhibition of a number of ATP-consuming processes. Firstly, since it has been postulated that the spindle may have significant ATP requirements (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004), nocodazole was applied to MI oocytes to disassemble the spindle. However, this did not result in any alteration to ATP levels (Fig. 5.4A). It is possible that in light of a reduction in ATP requirements resulting from spindle disassembly, there may be an adjustment of ATP production such that a steady state level of ATP is maintained in the oocyte. In order to exclude this possibility, oligomycin was added to reveal any differences in ATP consumption after disassembly of the spindle. However, no change in the rate of ATP consumption was seen (Fig. 5.4A).

Protein synthesis can account for significant energy use in some cells types (Rolfe & Brown 1997; Muller *et al.* 1986; Siems *et al.* 1992), and occurs at a high rate in the maturing oocyte (Schultz & Wassarman 1977). The contribution of protein synthesis to ATP usage was therefore investigated by inhibiting protein synthesis using cycloheximide. There was however no change in either ATP levels or consumption when protein synthesis was halted (Fig 5.4B).

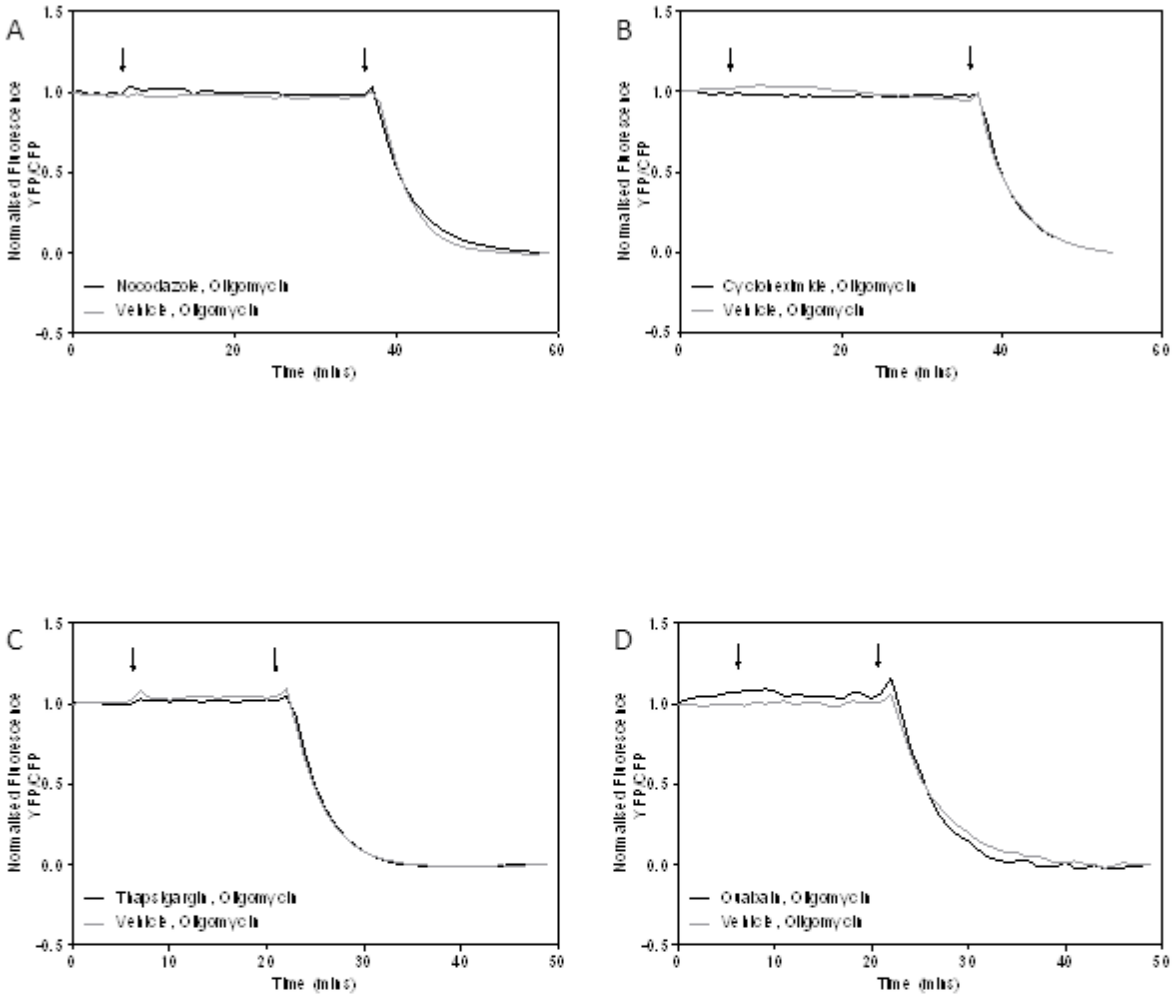

Figure 5.4. Inhibition of microtubule polymerisation, protein synthesis, SERCA pump activity or Na^+/K^+ ATPase activity has no effect on ATP levels or consumption. GV oocytes were microinjected with mRNA encoding AT1.03. Experiments were carried out during MI, between 4 and 8 hours after release from meiotic arrest. (A) 10 µM nocodazole (n=18) or the equivalent volume of vehicle control (n=7) was applied to oocytes (first arrow), followed by 5 µg/ml oligomycin (second arrow). (B) 50 µM cycloheximide (n=30) or the equivalent volume of vehicle control (n=17) was applied (first arrow), followed by 5 µg/ml oligomycin (second arrow). (C) 20 µM thapsigargin (n=13) or the equivalent volume of vehicle control (n=9) was applied to oocytes (first arrow), followed by 5 µg/ml oligomycin (second arrow). (D) 0.5mM ouabain (n=14) or the equivalent volume of vehicle control (n=5) was applied (first arrow) followed by 5 µg/ml oligomycin (second arrow).

It has been found that calcium signalling machinery is up-regulated during oocyte maturation and this includes an increase in the size of the ER Ca^{2+} stores (Jones *et al.* 1995; Tombes *et al.* 1992). Since the SERCA pump which re-sequesters Ca^{2+} into the ER is an ATPase, it is possible that increased calcium stores result in greater activity of the SERCA pump, which may account for the differences in rates of ATP consumption observed. Therefore, the SERCA pump was inhibited with thapsigargin and both ATP levels and ATP consumption were measured. However, no difference in either parameter was detected (Fig. 5.4C).

The Na^+/K^+ ATPase is known to consume significant amounts of ATP at blastocyst stage in order to facilitate formation of the blastocoel cavity (Houghton *et al.* 2003). Although energy usage by the Na^+/K^+ ATPase is significantly lower during oocyte maturation than in the blastocyst, some activity has been previously detected in the mouse egg (Van Winkle & Campione 1991). Therefore, the pump was inhibited to examine if it was making a contribution to the increased ATP consumption observed during oocyte maturation. Inhibition of the Na^+/K^+ ATPase with ouabain, at the same concentration used to detect activity in the mouse egg in a previous study (Van Winkle & Campione 1991), revealed no change in ATP levels, and ATP consumption upon application of oligomycin was also unchanged (Fig. 5.3D).

5.2.6 Effect of MPF and MAPK inhibition on ATP levels and consumption

MPF and MAPK are activated during, and indeed control the progress of oocyte maturation (Abrieu *et al.* 2001). MPF and MAPK were therefore inhibited to establish if the changes in ATP levels and consumption observed during oocyte maturation occur as a result of activation of these kinases. Inhibition of MPF with

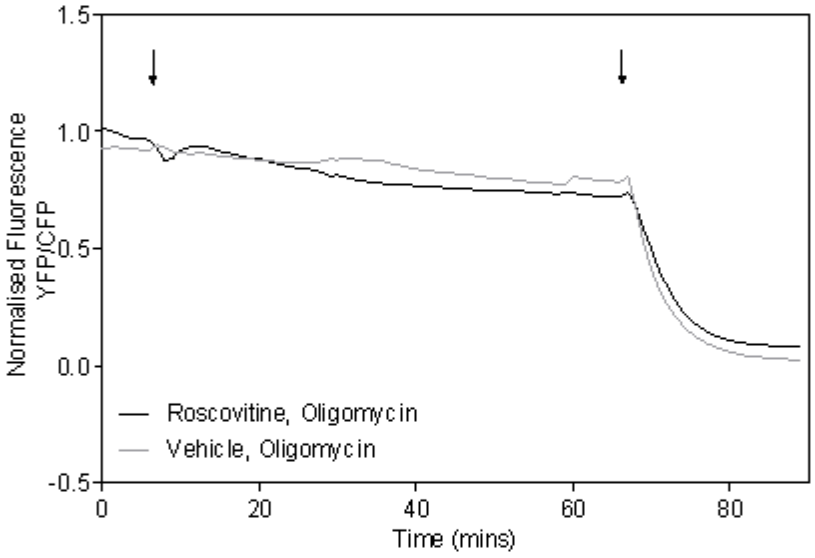

Figure 5.5. Inhibition of MPF has no effect on ATP levels or ATP consumption. GV oocytes were microinjected with mRNA encoding AT1.03. Experiments were carried out during MI, between 4 and 8 hours after release from meiotic arrest. 50 μM roscovitine or the equivalent volume of vehicle control was added to MI oocytes (arrow) followed by 5 μg/ml oligomycin (second arrow).

roscovitine had no effects on ATP levels and subsequent addition of oligomycin did not reveal any changes to ATP consumption (Fig. 5.5).

In contrast, inhibition of MAPK with U0126 resulted in a rapid decrease in ATP levels (Fig 5.6A). This was accompanied by a dissipation of the mitochondrial membrane potential, suggesting that the decrease in FRET was a genuine reflection of a change in ATP levels, caused by mitochondrial disruption (Fig. 5.6B). Indeed, it is also worth noting that there were no unexpected excursions of individual fluorescence intensity of either CFP or YFP, suggesting the changes are the result of a real change in the FRET ratio rather than an interaction with the sensor, or any other effect unrelated to ATP. A recent report provides evidence for regulation of mitochondrial membrane potential and ATP production by extracellular-signal-regulated kinase (ERK)/MAPK activity (Monick *et al.* 2008). In human alveolar macrophages, mitochondrial integrity was found to be essential to maintain ATP levels, as is the case in oocytes, and inhibition of ERK/MAPK, which was found to be constitutively active in this cell type, disrupted mitochondrial membrane potential resulting in ATP depletion and cell death. Additionally, in a human cancer cell model, constitutive activation of mitochondrial ERK was found to protect against cell death by desensitising the mitochondrial permeability transition pore, thereby maintaining mitochondrial integrity (Rasola *et al.* 2010).

Therefore, in order to investigate whether the mPTP has a role in the dissipation of mitochondrial membrane potential observed in oocytes on inhibition of MAPK, pore formation was inhibited using cyclosporin A (CsA) prior to application of U0126. However CsA failed to have a protective effect either on the mitochondrial

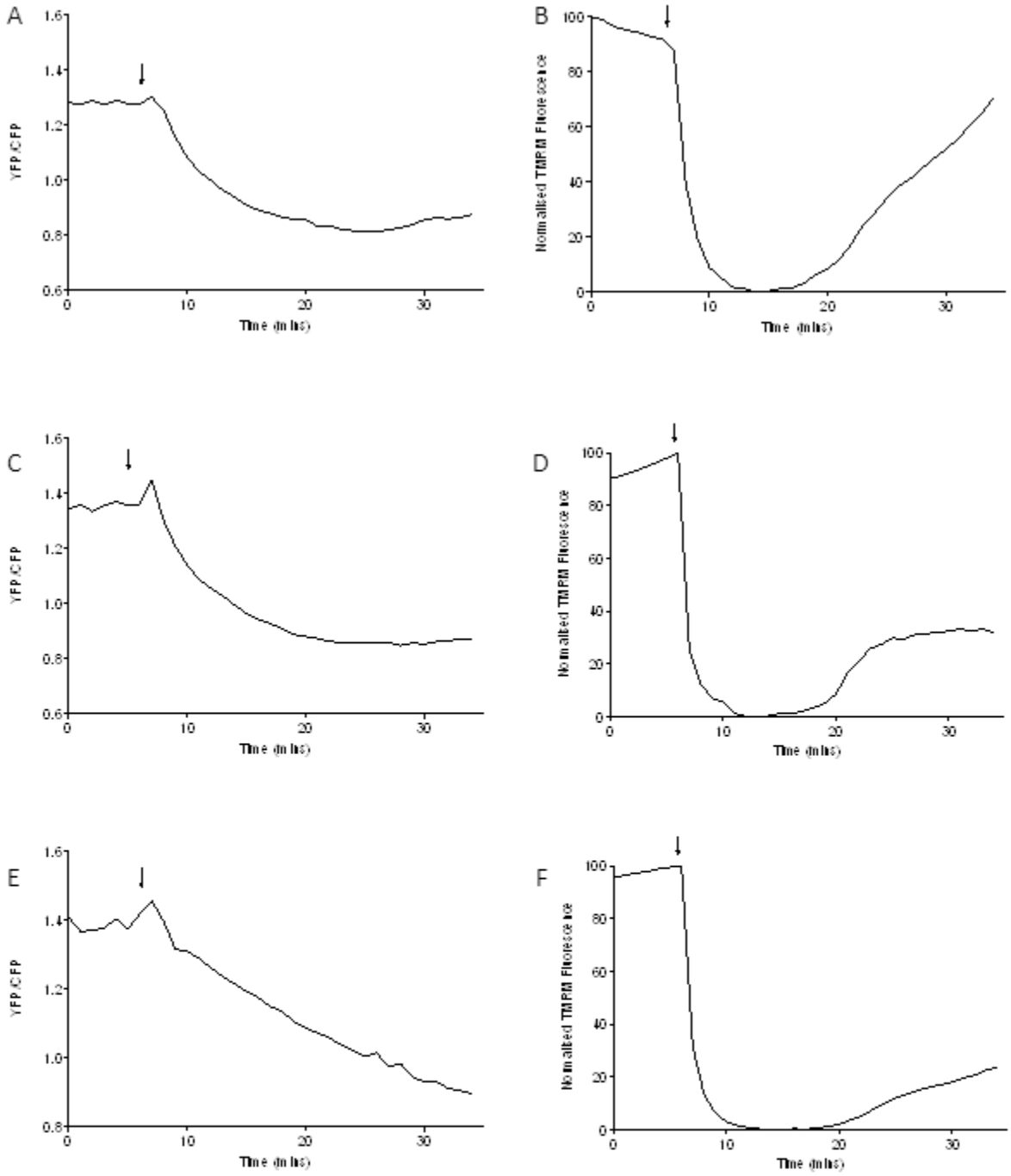

Figure 5.6. Inhibition of MAPK results in dissipation of mitochondrial membrane potential and a rapid decrease in ATP levels. A) MAPK activity was inhibited in MI oocytes by addition of 50 µM U0126 (arrow) resulting in a decline in ATP levels (n=29). B) Addition of 50 µM U0126 (arrow) results in dissipation of the mitochondrial membrane potential in MI oocytes, as measured by TMRM (n=8). C) Pre-treatment of MI oocytes with 5 µM CsA for 30 min fails to protect against the effect of 50 µM U0126 (arrow) on ATP levels (n=16). D) Dissipation of mitochondrial membrane potential persists after pre-treatment with 5 µM CsA on addition of 50 µM U0126 (arrow) (n=8). E) ATP levels decrease in GV stage oocytes on application of 50 µM U0126 (arrow) (n=9). F) Mitochondrial membrane potential is dissipated in GV oocytes on addition of 50 µM U0126 (arrow) (n=10).

membrane potential, as measured by TMRM (Fig. 5.6D), or on ATP levels (Fig. 5.6C) when MAPK was inhibited, indicating that the effect of MAPK on mitochondrial membrane potential and ATP production is not mediated via an effect on the mPTP.

MAPK is not active in GV stage oocytes (Verlhac *et al.* 1993; Verlhac *et al.* 1994), therefore one would expect to see no effect on application of U0126. Surprisingly however, application of U0126 to GV oocytes resulted in dissipation of mitochondrial membrane potential and a decrease in ATP levels in GV oocytes (Fig 5.6E and 5.6F). This suggests either an indirect effect of U0126 on mitochondria, or possibly, the existence of a mitochondrial pool of ERK/MAPK which is active even in the GV stage oocyte. Attempts were made to inhibit MAPK activity using the alternative inhibitors, PD98059 and ERK inhibitor peptide. However, PD98059 was found to be fluorescent and so could not be used during live cell imaging as it interfered with the FRET signal. The ERK inhibitor peptide was instead toxic to oocytes even at half the concentration used in another study examining the effects of MAPK activity on mitochondrial membrane potential (Rasola *et al.* 2010). It was therefore not possible to demonstrate conclusively that the effect of U0126 on mitochondrial membrane potential and cellular ATP levels is directly attributable to inhibition of MAPK activity.

5.2.7 Live time-lapse imaging of ATP levels during maturation

Finally, in order to examine the changes to ATP throughout maturation in more detail, oocytes which had been microinjected with AT1.03 were imaged at 15 minute intervals from GV to MII stage. This revealed that ATP levels undergo dynamic

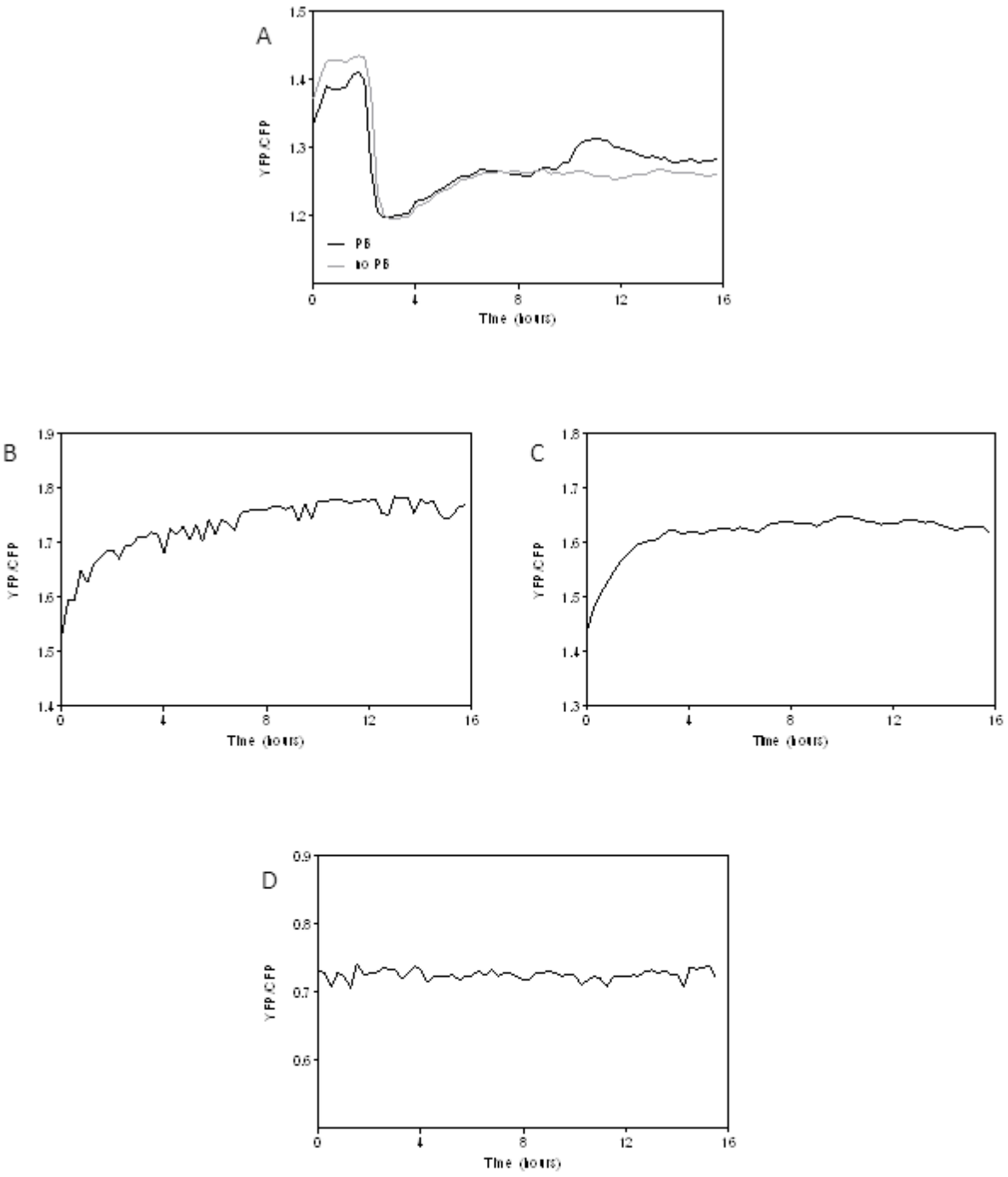

Figure 5.7. ATP levels are dynamically regulated during oocyte maturation. Oocytes expressing AT1.03 or AT1.03RK were imaged at 15 minute intervals from GV to MII. A) Representative examples of an oocyte which extruded a polar body and an oocyte which failed to extrude a polar body from n=22 and n=8 oocytes respectively. A peak in ATP levels was always seen at the time of polar body extrusion and no peak was observed when oocytes failed to extrude a polar body. B) Mean trace from n=30 oocytes expressing AT1.03, arrested at GV stage by 200 μm IBMX. C) Representative example of an oocyte arrested at GV stage by 200 μm IBMX showing that the changes in ATP levels exhibited by maturing oocytes are not displayed by those arrested at GV stage. D) Oocytes expressing the mutant probe, AT1.03RK, and which extrude a polar body, do not exhibit changes in the FRET ratio during oocyte maturation. Representative example from n=11 oocytes.

changes during oocyte maturation (Fig. 5.7A). A gradual increase in the first stages of maturation is followed by a sharp decrease in FRET ratio shortly after GVBD. ATP levels then gradually increase until the time of polar body extrusion, when a transient increase in ATP can be seen. Following polar body extrusion, ATP levels remain largely stable for the remaining imaging time. The increase in ATP observed at polar body extrusion was not seen in oocytes which commenced oocyte maturation but failed to extrude a polar body (Fig. 5.7A), suggesting this change in ATP levels is associated with the formation of the polar body.

To confirm that the changes in ATP levels were associated with oocyte maturation, oocytes which were maintained in GV arrest throughout the imaging time by incubation with IBMX (Fig. 5.7B). Surprisingly, a modest increase in ATP levels was seen during the early stages in some oocytes. However, the dynamic changes observed in maturing oocytes were not observed, as can be seen from a representative example of a single oocyte (Fig.5.7C), suggesting that these changes are attributable to altered levels of ATP which are associated with the progression of oocyte maturation Furthermore, no change to the FRET ratio was observed when oocytes which been microinjected with AT1.03RK were imaged during maturation (Fig. 5.7D), confirming the results observed in maturing oocytes were due to genuine changes in ATP level.

5.2.8 Effect of cumulus cells on oocyte ATP levels

Cumulus-oocyte coupling can provide metabolic support to oocytes during maturation by providing ATP or metabolic substrates to the oocyte (Leese & Barton 1985; Downs 1995; Johnson *et al.* 2007). Therefore, in order to investigate whether

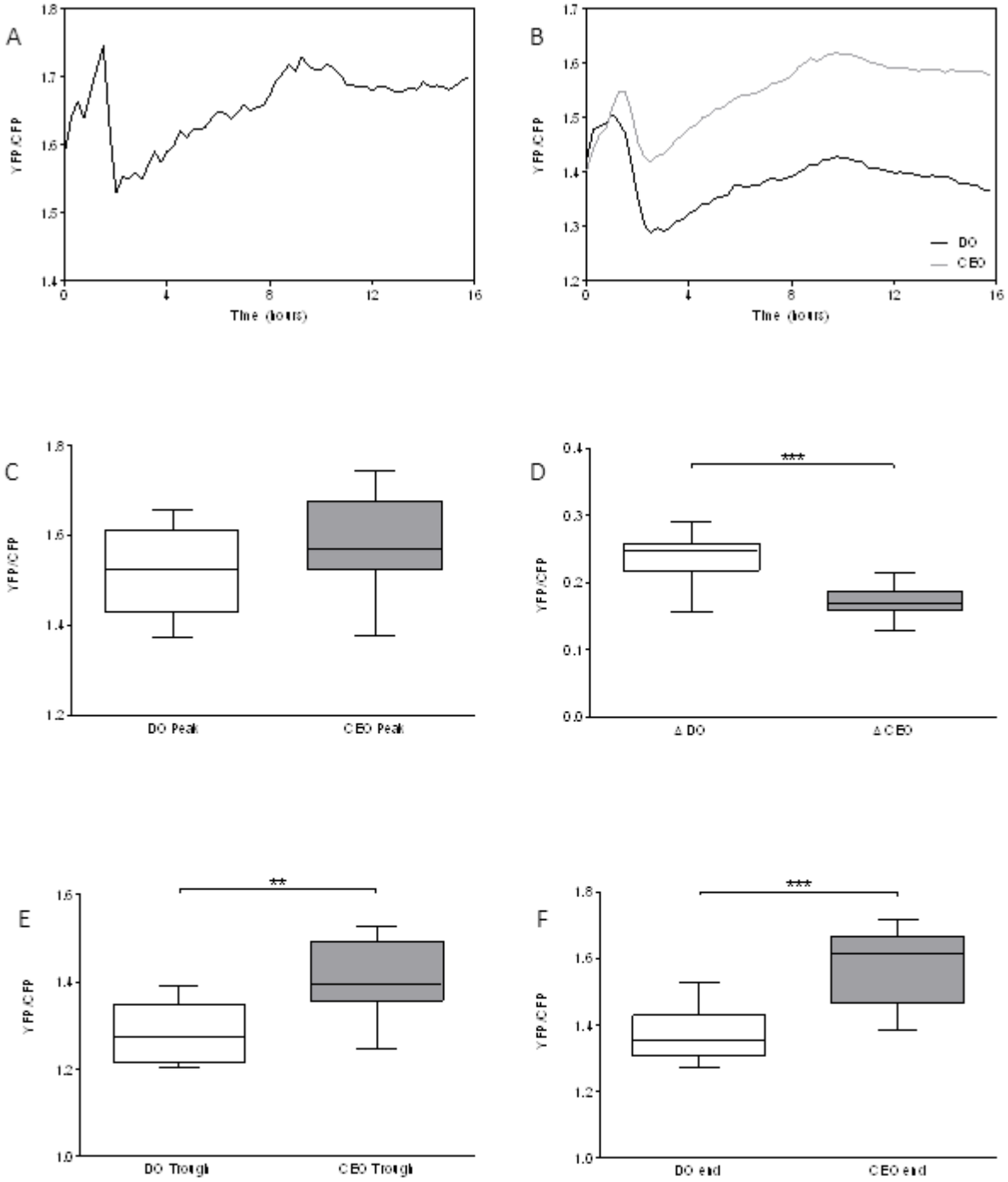

Figure 5.8. ATP levels are higher in cumulus-enclosed oocytes. Oocytes expressing AT1.03 were imaged at 15 minute intervals from GV to MII. Only those oocytes which extruded a polar body are included. A) CEOs exhibit the same pattern of ATP changes as DOs. Representative example from n=14 oocytes. B) Mean traces from DOs (n=9) and CEOs (n=14) reveal higher ATP levels in CEOs. Analysis of these oocytes reveals that (C) the mean peak in ATP levels prior to GVBD is not significantly higher in CEOs than DOs, (D) the mean fall in ATP levels after GVBD results in a trough which is significantly lower in DOs than CEOs ($p=0.0022$), (E) the mean change in ATP levels between the peak and the trough is significantly greater in DOs than CEOs ($p<0.0001$), (F) The mean ATP level at the end of the imaging time when oocytes are arrested at MII is significantly higher in CEOs than DOs ($p<0.0001$).

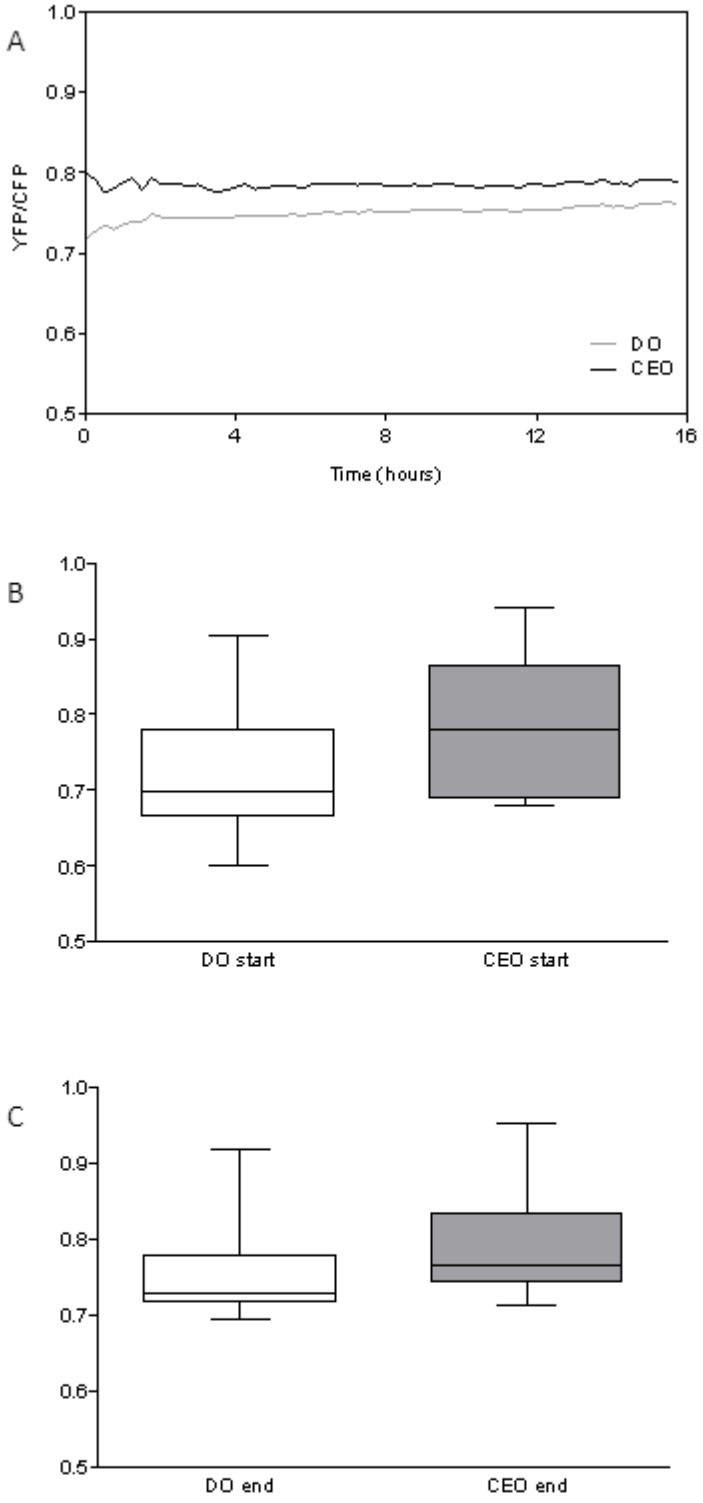

Figure 5.9 The FRET ratio is similar in denuded and cumulus-enclosed oocytes expressing AT1.03RK. Oocytes expressing AT1.03RK were imaged at 15 minute intervals from GV to MII. Only those oocytes which extruded a polar body are included. A) The FRET ratio is similar in DOs (n=14) and CEOs (n=16) throughout maturation. Analysis of the FRET ratio at (B) the start of the imaging time, calculated as the average FRET ratio from the first three images acquired for each oocyte, and at (C) the end of the imaging time, calculated as the average FRET ratio from the last three images acquired for each oocyte, reveals no significant difference between DOs and CEOs.

the dynamic changes in ATP levels observed during maturation are influenced by the presence of cumulus cells, ATP levels were simultaneously measured in cumulus enclosed oocytes (CEO) and denuded oocytes (DO). Interestingly, whilst the pattern of ATP changes was maintained (Fig. 5.8A), cumulus enclosed oocytes exhibited higher ATP levels than their denuded counterparts (Fig. 5.8B). More detailed analysis indicated that whilst the peak of ATP levels observed in the early stages of maturation tended to be higher in CEOs than DOs, the difference was not significant (Fig. 5.8C). However, the drop in ATP levels after GVBD was significantly greater in DOs than CEOs (Fig. 5.8D) such that the level of ATP in the trough was significantly lower in DOs than CEOs (Fig. 5.8E). A higher level of ATP was then maintained in the CEOs for the remainder of the imaging period (Fig. 5.8F). In oocytes expressing AT1.03RK, the ATP levels were not significantly different in DOs and CEOs at any stage of maturation, indicating that the difference observed in oocytes expressing AT1.03 was not attributable to an imaging artefact resulting from the imaging of CEOs (Fig. 5.9).

Cumulus cells are thought to contribute to oocyte metabolism via gap junctions (Anderson & Albertini 1976; Downs 1995; Gilula *et al.* 1978). Gap junctional communication was therefore inhibited using 18-α-glycyrrhetinic acid and ATP levels of DOs and CEOs matured in parallel was monitored (Fig. 5.10A). The concentration of 18-α-glycyrrhetinic acid used here, 10 μM, has previously been shown to reduce gap junctional coupling between the oocyte and cumulus cells to 0.2% (Downs 2001). Oocytes matured normally in the presence of the gap junction inhibitor and extruded polar bodies. The dynamic changes in ATP levels were also maintained in both DOs and CEOs. Remarkably however, the higher level of ATP

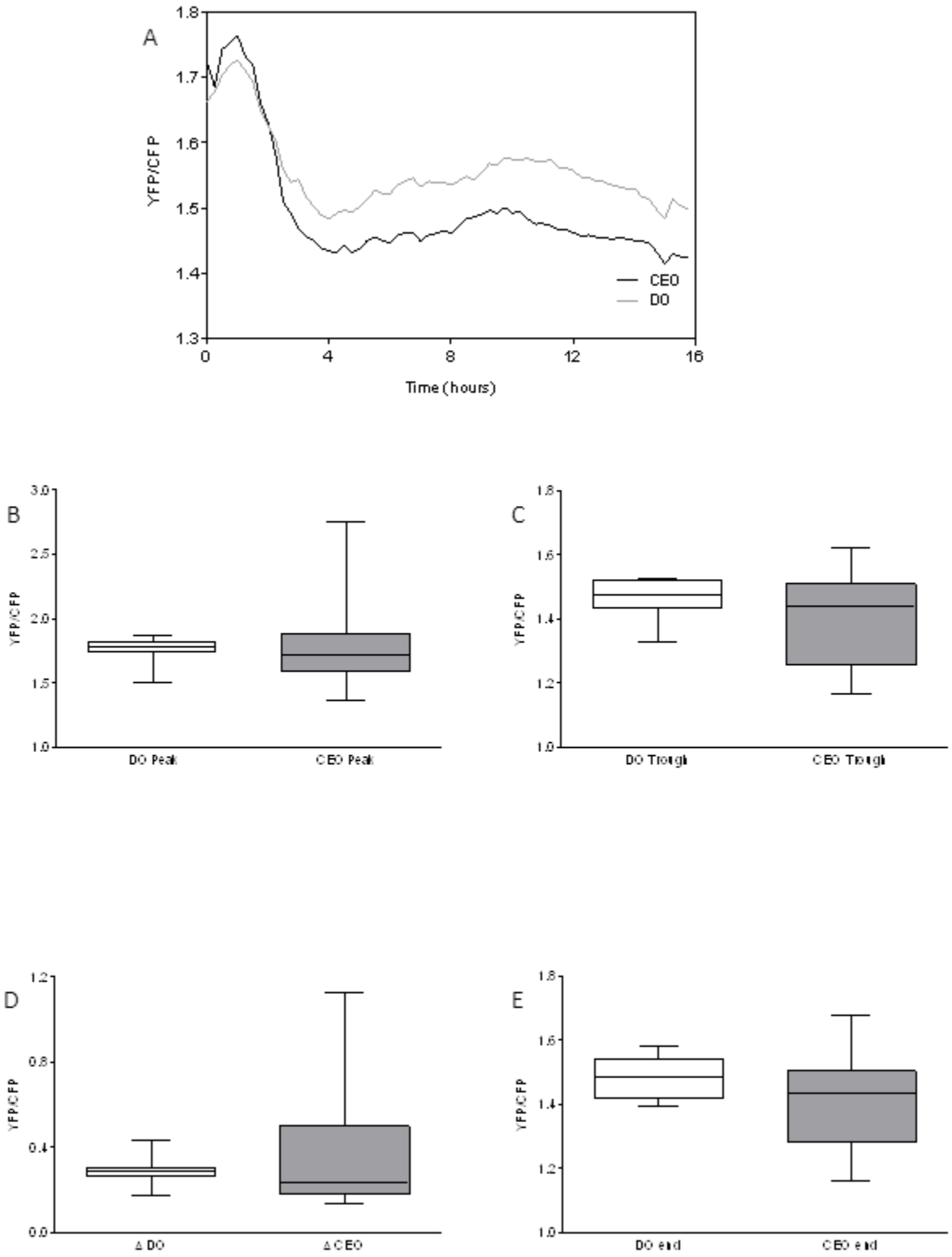

Figure 5.10 Inhibiting gap junctions results in similar ATP levels in denuded and cumulus-enclosed oocytes. Oocytes expressing AT1.03 were imaged at 15 minute intervals from GV to MII. Only those oocytes which extruded a polar body are included. A) In the presence of 10 μM AGA, ATP levels are similar in CEOs (n=15) and DOs (n=8). The mean peak (C), trough (D), change (E) and end (F) ATP levels are not significantly different between CEOs and DOs.

observed in CEOs was completely abolished when oocytes were matured in the presence of 18-α-glycyrrhetinic acid, such that there was no longer a significant difference in ATP levels between DOs and CEOs at any of the stages of maturation tested (Fig. 5.10 B, C, D and E).

5.3 Discussion

Work in this chapter has described the characterisation and use of a novel FRET-based recombinant probe for measurement of ATP levels in individual live oocytes. Changes in both ATP levels and consumption rates in oocytes at different stages of maturation were observed, and live time-lapse imaging of maturing oocytes revealed dynamic changes to ATP levels throughout maturation. Finally, the presence of cumulus cells was found to influence ATP levels in the maturing oocyte.

The importance of mitochondrial ATP production for energy provision in the oocyte and early embryo is now well established. Indeed, at fertilisation, mitochondria-derived ATP is essential for sustaining calcium oscillations, which in turn up-regulate mitochondrial ATP production in an elegant linking of supply and demand (Dumollard *et al.* 2004; Campbell & Swann 2006). However, whilst it has been suggested that ATP content of the oocyte may be relevant for establishing developmental competence (Van Blerkom *et al.* 1995; Stojkovic *et al.* 2001; Tamassia *et al.* 2004; Zeng *et al.* 2007), a detailed examination of ATP supply and consumption during maturation has not been described. Furthermore, whilst the distribution of mitochondria in the oocyte is thought to be important for provision of ATP to areas of high demand (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004), experimental evidence supporting this proposal is limited.

In order to examine ATP dynamics in the maturing oocyte, a newly developed FRET probe, AT1.03, was used (Imamura *et al.* 2009). The probe was validated for use in oocytes by examining whether processes known to influence oocyte ATP levels could be measured. Indeed, a decrease in oocyte ATP was detected when

mitochondrial activity was disrupted using oligomycin or FCCP, and increases in oocyte ATP levels were detected both when pyruvate was restored to substrate-free medium and at fertilisation, consistent with previous reports where ATP levels were measured by alternative methods (Dumollard *et al.* 2004; Campbell & Swann 2006). The prompt onset of ATP decline on addition of either FCCP or oligomycin is consistent with evidence that mitochondrial oxidative phosphorylation provides the primary source of ATP to the oocyte, and is supported by the finding that inhibition of glycolysis with iodoacetic acid has no effect on ATP levels. The rapid decline in ATP suggests that consumption rates are high in the oocyte which, together with the fact that ATP provision is dependent on mitochondrial oxidative phosphorylation, indicates the importance of mitochondrial function during oocyte maturation.

One of the advantages of measuring ATP levels using a FRET-based indicator is the potential to measure subcellular heterogeneity in ATP provision and usage, something which it has not been possible using previously available methods for monitoring ATP (Liemburg-Apers *et al.* 2011). Therefore, the probe was next used to attempt to resolve the question of whether accumulation of mitochondria in the spindle region during oocyte maturation results in the differential supply of ATP to this area. Studies have reported movement of mitochondria to the spindle region in several species (Van Blerkom & Runner 1984; Calarco 1995; Barnett *et al.* 1996; Bavister & Squirrell 2000; Wilding *et al.* 2001; Sun *et al.* 2001; Stojkovic *et al.* 2001; Brevini *et al.* 2005; Yu *et al.* 2010; Valentini *et al.* 2010) and defects in mitochondrial reorganisation have been linked to compromised developmental competence (Bavister & Squirrell 2000; Wang *et al.* 2009; Muggleton-Harris & Brown 1988). The presence of aberrant spindles has been correlated with

mitochondrial dysfunction (Zhang *et al.* 2006; Johnson *et al.* 2007; Zeng *et al.* 2007; Wang *et al.* 2009) and this, together with the finding that the spindle is acutely sensitive to ATP decline (see 3.2.8), suggests that such an aggregation of mitochondria in the spindle region may well be necessary to maintain a functional spindle by ensuring a sufficient supply of ATP.

Examining ATP levels in the spindle and non-spindle region by both standard ratiometric FRET imaging and acceptor photobleaching did not however reveal any regional differences in ATP supply, and nor did halting ATP production with oligomycin in order to examine rates of ATP consumption. This does not preclude that a differential supply and demand exist since it is possible that the probe is unable to detect sub-cellular variation in ATP levels. Indeed, whilst the generation of FRET probes for ATP measurements has led to the proposal that subcellular heterogeneity in ATP levels might be detectable, strong evidence of such findings is largely lacking at this time (Liemburg-Apers *et al.* 2011; Berg *et al.* 2009). Thus, whilst AT1.03 represents a novel way of measuring ATP in the live oocyte in real time, the long-standing question of whether mitochondrial accumulation in the spindle region results in enhanced supply of ATP supply to this area of presumably high energy demand remains unanswered.

ATP levels were next compared at different stages during oocyte maturation, and rates of consumption were examined to ascertain if a greater consumption of ATP was evident during formation and migration of the first meiotic spindle, perhaps reflecting a greater demand for ATP during this time. Although levels of ATP have been compared at GV and MII stages in some species, examination of ATP levels at

MI has not previously been reported. Additionally, rates of ATP consumption have not been described. Using the FRET probe it was possible to detect significantly higher ATP levels at GV stage than those at MI and MII, and significantly higher levels at MI than at MII. This is in contrast to levels reported for both pig and cattle, where ATP was found to be significantly higher at MII than at GV, as measured in single oocytes using a bioluminescence assay kit (Brevini *et al.* 2005; Stojkovic *et al.* 2001). However, in cat oocytes, ATP levels were higher at GV than at MII (Freistedt *et al.* 2001). In mouse oocytes where ATP levels were examined at 1-2 hour intervals using a bioluminescent assay kit with pooled groups of oocytes, no change was detected in ATP levels at different maturational stages, although measurements were made using pooled groups of oocytes which may have masked changes to ATP levels in individual oocytes (Van Blerkom *et al.* 1995). Moreover, although changes to ATP levels were detected during maturation by Yu and colleagues (Yu *et al.* 2010), no absolute difference in ATP levels at GV and MII stages was reported. These discrepancies may be accounted for both by species-specific differences in regulation of ATP levels, and by the different methods of ATP measurement employed. The differences in ATP level detected here at GV and MII by measuring levels at discrete time points were however confirmed by measuring ATP levels throughout maturation which yielded a consistent result. Furthermore, this indicates that the overall change in ATP levels is similar whether oocytes are matured *in vivo* or *in vitro* since ATP measurements at discrete time points were made using *in vivo* ovulated MII oocytes, whilst for continuous ATP measurements throughout maturation, oocytes were matured to MII *in vitro*.

Rates of ATP consumption have not previously been measured in the maturing oocyte and experiments revealed significant changes to the rate at which ATP was consumed at different maturational stages. ATP consumption was significantly higher in oocytes during MI and at MII than in those arrested at GV, likely reflecting increased energy demand for processes associated with maturational progress. A number of processes which occur during oocyte maturation might be expected to contribute to the increased rate of ATP consumption observed during MI and at MII arrest. Van Blerkom suggested that mitochondrial reorganisation during oocyte maturation may occur in order to provide ATP for energy consuming processes associated with spindle formation and function (Van Blerkom & Runner 1984). This proposal fits with an observed increase in energy usage during MI and MII when compared to oocytes at GV stage, and with the observed ATP dependence of the MI spindle (see 3.2.8).

However, disruption of microtubule dynamics with nocodazole did not result in either increased ATP levels or a decreased rate of consumption, suggesting that formation and function of the spindle is not having a significant effect on ATP usage. Likewise, inhibition of protein synthesis or SERCA pump activity did not alter ATP levels or the rate of ATP consumption. This was despite the reported high rate of protein synthesis (Schultz & Wassarman 1977) and up regulation of Ca^{2+} signalling machinery during oocyte maturation (Carroll *et al.* 1996). Similarly, although the Na^{+}/K^{+} ATPase has been reported to contribute significantly to ATP usage in a number of cell types (Mata *et al.* 1980; Silver & Erecinska 1997; McBride & Kelly 1990), including bovine and human blastocysts where 36% and 60% respectively of ATP is used by the Na^{+}/K^{+} ATPase to form the blastocoel cavity

(Houghton *et al.* 2003), inhibition of activity with ouabain did not result in any changes to ATP levels or rates of consumption. This suggests that the Na^+/K^+ ATPase does not make a substantial contribution to energy usage during oocyte maturation, consistent with a comparatively low level of activity detected in oocytes when compared to the blastocyst (Van Winkle & Campione 1991),.

Thus is it possible that either none of the processes investigated contributes significantly to the changes in ATP consumption described here or alternatively, that the increase in consumption is a result of a cumulative effect of many maturation-associated processes all of which together contribute to increased energy requirements. These might be expected to be linked to the activation of MPF and MAPK, potentially triggering a cascade of energy consuming activities. However, inhibition of MPF did not result in changes to ATP levels or consumption. Surprisingly however, inhibition of MAPK had rapid and substantial effects on ATP levels, which appeared to occur as a result of dissipation of the mitochondrial membrane potential.

ERK/MAPK signalling has recently been shown to have an important role in maintaining mitochondrial integrity in some cell types, with inhibition of ERK/MAPK resulting in dissipation of mitochondrial membrane potential and depletion of cellular ATP (Monick *et al.* 2008; Rasola *et al.* 2010). Rasola and colleagues found that the effects of MAPK inhibition were mediated by opening of the mPTP (Rasola *et al.* 2010). However inhibition of pore opening with CsA did not prevent the effects of MAPK inhibition on ATP levels and mitochondrial membrane potential in the oocyte. This is however consistent with the findings of Monick and

colleagues who demonstrated that ATP depletion and loss of mitochondrial membrane potential upon inhibition of MAPK preceded the release of necrosis and apoptosis markers from the mitochondria suggesting that pore opening occurred after ATP depletion and dissipation of the mitochondrial membrane potential (Monick *et al.* 2008). Additionally, in one study it was reported that ERK inhibitors caused ATP depletion in glucose-deprived cells through an effect on the F_0/F_1 ATP synthase, although it was not clear how this effect was mediated (Yung *et al.* 2004).

ERK/MAPK may have a protective role in maintaining mitochondrial membrane potential and ATP production in oocytes, however the finding that mitochondrial membrane potential is dissipated and ATP levels depleted in GV stage oocytes is puzzling, since MAPK is not activated until after oocytes resume maturation (Verlhac *et al.* 1993; Verlhac *et al.* 1994). It is possible that a mitochondrial pool of ERK/MAPK exists which is active even at the GV stage, sufficient to maintain mitochondrial integrity but small in comparison to the dramatic activation of MAPK associated with maturation. Indeed, the targeting of ERK to distinct subcellular compartments can influence the downstream targets of the signalling cascade (Wortzel & Seger 2011) and a subset of ERK is localised to the mitochondria in some cell types (Alonso *et al.* 2004; Zhu *et al.* 2003). It has also been shown that this mitochondrial pool of ERK can be differentially activated to the cytosolic pool (Baines *et al.* 2002; Poderoso *et al.* 2008), and in tumorigenic cancer cell lines it is specifically this mitochondrial pool of ERK which is constitutively activated and provides protective effects via maintenance of mitochondrial integrity (Rasola *et al.* 2010), supporting the idea that specific activation of the mitochondrial pool is possible. However, in light of the difficulties encountered with alternative MAPK

inhibitors, it was not possible to exclude that the effect of U0126 on mitochondria may be non-specific and unrelated to MAPK activity.

Having found changes to ATP levels and consumption during oocyte maturation, a detailed examination of ATP levels was carried out throughout oocyte maturation. This revealed changes in ATP levels shortly after GVBD and at the time of polar body extrusion. During the course of this work, a report was published describing the measurement of ATP levels during oocyte maturation using a luciferase assay (Yu *et al.* 2010). Yu and colleagues described three phases of increased ATP levels, and found two decreases in ATP levels which were temporally correlated with the disruption of the actin filament network and a reduction in mitochondrial clustering. The results described here are largely consistent with these findings although only one decrease in ATP levels was observed using the FRET probe, shortly after GVBD, and whilst ATP levels then increased gradually, a definitive drop was not consistently seen prior to the polar body extrusion peak. The magnitude of the changes in ATP was also somewhat different here in that the change seen shortly after GVBD was substantially larger than that seen during polar body extrusion when measured with the FRET probe, whereas the first and last peaks described by Yu et al. were of a similar magnitude. Changes to ATP levels during maturation have also been described in bovine oocytes, with a drop in ATP observed around GVBD, followed by increasing levels of ATP towards MII (Nagano *et al.* 2006), suggesting that changes to ATP levels associated with specific maturational events may be a phenomenon which exists in a number of species. However, the functional significance of these changes and the mechanisms regulating them remain to be determined.

A moderate increase in ATP levels was also seen in oocytes arrested during maturation in the presence of IBMX for an extended period. This result is somewhat surprising since one would expect ATP levels to remain largely stable in an arrested oocyte. However, experiments conducted in our lab using oocytes which have been arrested overnight in IBMX prior to release from meiotic arrest have revealed that these oocytes undergo GVBD more quickly, and progress through maturation more rapidly, than do freshly isolated oocytes (unpublished observations). This suggests that the artificial GV arrest maintained by IBMX may exert effects on the oocyte which we do not fully understand. Indeed, this is corroborated by the increased level of mitochondrial accumulation in the spindle region at MI observed in control oocytes arrested overnight in IBMX prior to release compared to those that had been released from meiotic arrest within a few hours of collection, as described in chapter 3. Nonetheless, the gradual increase in ATP level observed in arrested oocytes was markedly different to the changes observed in maturing oocytes, indicating that these are likely to be associated with oocyte maturation.

The FRET probe was also used to ascertain whether cumulus enclosed oocytes displayed a similar pattern of ATP changes during maturation. The pattern of ATP changes was indeed maintained, however the cumulus enclosed oocytes exhibited a higher level of ATP, which could be abolished by the presence of a gap junction inhibitor during maturation. The oocyte metabolises glucose poorly and is dependent upon oxidative phosphorylation of pyruvate to sustain maturation (Biggers *et al.* 1967; Barbehenn *et al.* 1974; Brinster 1971; Eppig 1976; Leese & Barton 1984). In contrast, cumulus cells exhibit high glycolytic activity and it is thought that ATP and

energy substrates produced in the cumulus cells are supplied to the oocyte via gap junctions (Downs 1995; Johnson *et al.* 2007; Downs & Utecht 1999; Heller & Schultz 1980). Indeed, isolated cumulus cells produce pyruvate, supporting this notion (Leese & Barton 1985; Donahue & Stern 1968). Furthermore, maturation of oocytes from mice deficient in the pyruvate dehydrogenase enzyme, which catalyses the first step in oxidative metabolism of pyruvate, could be partially rescued if they were cultured as part of a cumulus-oocyte complex (Johnson *et al.* 2007), suggesting metabolic co-operation exists between the cumulus cells and the oocyte and is likely to account for the higher ATP levels observed here in the cumulus-enclosed oocytes.

Interestingly, the extent of cumulus-oocyte coupling has been reported to decrease progressively during oocyte maturation, and is greatly reduced at the end of maturation compared to that at the start. (Eppig 1982; Salustri & Siracusa 1983). Conversely, the higher ATP level in cumulus enclosed oocytes was not present at the start of maturation but, once established, persisted throughout. The effect was however blocked by inhibition of gap junctions, suggesting it is due to transfer of molecules from the cumulus cells to the oocyte. It is possible that the presence of gap junctions at the early stages of maturation therefore institutes a higher 'set point' for ATP in these oocytes which then persists for the remainder of the maturation time. Indeed, it is interesting that the difference in ATP level between cumulus enclosed and denuded oocytes seems to establish itself at the time of the drop in ATP around the time of GVBD. Thus it may be that cumulus-derived metabolic support reduces the fall in ATP which occurs at this stage and allows the oocyte to maintain a higher ATP level which continues, even in the absence of high levels of continuing cumulus-oocyte coupling. Further work will however be necessary to elucidate the

mechanisms at play in causing this effect. Nonetheless, the higher level of ATP in cumulus enclosed oocytes may have implications for continued development since higher ATP content in human and bovine oocytes has been shown to correlate with a greater capacity for continued development (Van Blerkom *et al.* 1995; Stojkovic *et al.* 2001).

5.4 Summary

Work in this chapter has described the validation of a novel FRET-based approach to measuring ATP in mouse oocytes. Using the FRET probe, changes in the level of ATP and the rate of ATP consumption were detected at different stages of oocyte maturation. Furthermore, dynamic changes to ATP levels throughout maturation were observed. Cumulus enclosed oocytes were found to have higher ATP levels, dependent on the presence of gap junctions, suggesting a role for metabolic co-operation between cumulus cells and the oocyte in determining ATP levels during maturation.

6. Conclusions

In recent years, the concept of mitochondria as important regulators of developmental competence has emerged (Van Blerkom 2011). Nonetheless, despite observations linking aberrant mitochondrial distribution or activity with compromised development, a detailed picture of mitochondrial function in the oocyte, and the mechanisms by which mitochondria contribute to successful development, remain unclear. The aim of the work presented in this thesis was to better understand the role of mitochondria during oocyte maturation. This was investigated both in terms of the spatial distribution of mitochondria, and with regard to the regulation of ATP levels and consumption during the transition from the germinal vesicle stage to metaphase II arrest.

Observations in a number of species indicate that mitochondrial redistributions occur during the course of oocyte maturation. Whilst the precise details of these translocations appear to differ somewhat between species, it is clear that they form part of the normal progression of the oocyte from GV stage to MII arrest. Indeed, it has been proposed that they may serve to localise mitochondria to areas of high energy demand, with the meiotic spindle identified as a primary target (Van Blerkom & Runner 1984; Eichenlaub-Ritter *et al.* 2004). The detailed examination of the position of mitochondria in relation to microtubules described in this thesis has indeed shown a strong association of mitochondria with the first meiotic spindle. Furthermore, of particular interest is the observation that, by carrying out live cell imaging of mitochondrial distribution throughout oocyte maturation, an accumulation of mitochondria can also be seen during formation of the second

meiotic spindle. This has not been reported previously and suggests that mitochondrial association with the spindle may well be a normal, and possibly necessary, feature of spindle formation.

Another notable observation was derived from live-cell imaging of mitochondrial redistributions during oocyte maturation in that, at the time of polar body extrusion, a mechanism for retention of mitochondria in the oocyte appears to exist. Mitochondria which have been trafficked towards the cortex with the spindle are retracted towards the centre of the oocyte as the first polar body is extruded. It seems likely that this occurs in order to limit loss of these organelles to the polar body, which may be crucial for oocyte and early embryo development in light of the fact that not only do mitochondria provide the primary source of ATP during these early developmental stages, but mitochondrial replication is inactivated until after implantation. As such the mitochondrial complement contained within the oocyte must be capable of meeting all the energy requirements until such a time as mitochondrial replication resumes and glycolysis is activated. It would therefore be of interest to elucidate how retention of mitochondria at the time of polar body extrusion is regulated, as failure to retract mitochondria away from the polar body may have negative consequences for continued successful development.

The large size of the oocyte, with its diameter of 80 µm, may present a unique challenge in terms of energy provision and correlations have been made between aberrant mitochondrial redistributions or disrupted mitochondrial function and the formation of an abnormal spindle (Zeng *et al.* 2007; Zhang *et al.* 2006; Johnson *et al.* 2007; Wang *et al.* 2009). Furthermore, the data presented in this thesis

demonstrating an acute sensitivity of the first meiotic spindle to ATP decline supports the notion of an ATP-dependent spindle, which may require localised mitochondria to meet ATP demand. Indeed, the concept of compartmentalisation of ATP supply and demand has been emerging in a number of cell types (van Horssen *et al.* 2009; Kuiper *et al.* 2009; de Groof *et al.* 2002; Kennedy *et al.* 1999; Weiss & Lamp 1989). Nonetheless, whilst the hypothesis that mitochondrial aggregation is necessary to provide sufficient ATP supply for spindle formation and function is an attractive one, direct experimental evidence to confirm this idea is lacking.

The development of a novel FRET-based recombinant probe for measurement of ATP levels could present a method with which to address this question in the oocyte. Unlike other methods for measuring ATP which can only give information on ATP levels in bulk cytosol, imaging of ATP levels with a FRET-based approach should allow subcellular heterogeneity in ATP levels to be detected. In the maturing oocyte however, regional differences in either ATP level or rates of ATP consumption were not detected. The absence of measureable subcellular heterogeneity does not necessarily preclude its existence. Indeed, it is worth noting that whilst the FRET probe employed here has been used in a number of studies, there has been only one very preliminary indication of any subcellular difference in ATP regulation (Liemburg-Apers *et al.* 2011). It will be important to try to establish to what degree any subcellular heterogeneity in ATP levels is detectable using this FRET based approach. Photorelease of controlled amounts of caged ATP may provide a reasonable indication of the extent to which differences in ATP levels in different regions of the cell can be measured. Moreover, photoreleasing caged ATP in different regions of the oocyte and monitoring the rate of disappearance may also

give an indication as to the rates of ATP diffusion and consumption in different areas. Furthermore, it may be of interest to examine whether other measures of mitochondrial function such as $\Delta\Psi_m$ and NADH display any subcellular heterogeneity.

It would also be of value to perturb the normal patterns of mitochondrial redistribution in order to shed light on their physiological role, and on whether they have any functional interaction with the ER, which undergoes a similar dramatic reorganisation during oocyte maturation. Mitochondrial redistributions have been correlated temporally and spatially with the organization of the microtubule network (Van Blerkom 1991; Brevini *et al.* 2005). Experiments described in this thesis support a role for the microtubule network in distributing mitochondria since nocodazole, a microtubule depolymerizing drug, prevents the normal translocations of mitochondria. In addition to an association with the meiotic spindle, mitochondria were also found clustered around MTOCs in the cytoplasm during maturation. The oocyte does not contain any centrioles so the meiotic spindle is formed via the action of several acentriolar cytosolic MTOCs (Messinger & Albertini 1991; Schuh & Ellenberg 2007). MTOCs are recruited to the centre of the oocyte shortly after GVBD, and exhibit a dramatic increase in their microtubule nucleating capacity (Schuh & Ellenberg 2007). Clustering of MTOCs then occurs to form the two opposing poles of the spindle and microtubules are organised into spindle structure (Brunet & Maro 2005; Schuh & Ellenberg 2007). An extensive cytoplasmic network of microtubules is not believed to exist at most stages of oocyte maturation (Combelles & Albertini 2001), thus mitochondrial association with cytoplasmic MTOCs may contribute to their trafficking to the spindle region. In the absence of

long microtubule tracks along which mitochondria can be transported, it is plausible that mitochondria are recruited to the central region of the oocyte concomitantly with MTOCs. Indeed, experiments presented here demonstrating an aggregation of mitochondria around cytoplasmic MTOCs support this model. The finding that dynein inhibits mitochondrial aggregation around the spindle is also in line with this notion, since the minus ends of microtubules are located at the MTOCs.

Regrettably, although mitochondrial redistributions are disrupted by perturbing the microtubule network and inhibiting dynein function, these experiments cannot be used to infer the function of mitochondrial translocations since both approaches also affect other cellular processes. As such, a method for specifically inhibiting the movement of mitochondria, whilst not disrupting the cytoskeletal network or the activity of motor proteins, is required. Mitochondrial adapter proteins have the potential to provide such an approach (Frederick & Shaw 2007). Indeed, attempts were made to disrupt mitochondrial trafficking in the oocyte by altering the expression or activity of Miro and Trak proteins, which have been shown to have a role in establishing mitochondrial distribution in a number of cell types, particularly in neurons (Hollenbeck & Saxton 2005). Whilst it was not possible to knock-down endogenous Miro and Trak using a morpholino approach, over-expression of wild-type and mutant versions of the proteins appeared to indicate that the Miro/Trak complex is not required for mitochondrial transport in the oocyte. Over-expression of Trak2 resulted in an increase in mitochondrial aggregation, but none of the experiments conducted were able to prevent mitochondrial aggregation. Thus, both the precise mechanisms controlling and regulating mitochondrial redistribution, and the functional role of those redistributions, remains unclear.

In conjunction with redistribution of mitochondria during oocyte maturation, dynamic changes to ATP levels also occur during the transition from GV stage to MII arrest. Experiments presented in this thesis address these changes by measurement of ATP levels using the FRET probe. However, during the course of this work, a report was published describing alterations to ATP levels during maturation measured using a luciferase luminescence approach (Yu *et al.* 2010). The overall pattern of changes observed using these two methods is broadly similar in that a decrease in ATP is observed shortly after GVBD and a peak of ATP occurs at the time of polar body extrusion. It was found here that this peak does not occur in oocytes which fail to extrude a polar body and no deviations were observed in oocytes either arrested at GV stage or expressing a mutant version of the probe which does not bind ATP. This suggests that the changes in FRET ratio observed are firstly specifically attributable to changes in ATP level and that secondly, these changes are associated with the progression of oocyte maturation. It may be of interest to measure NADH, $\Delta\Psi_m$ and oxygen consumption to further examine the changes to mitochondrial function during oocyte maturation.

In the report by Yu and colleagues (Yu *et al.* 2010), alterations in ATP level were correlated with the formation and dissolution of the actin cytoskeletal network, which was found to influence the extent of mitochondrial clustering. It is not clear however how clustering may contribute to the regulation of ATP levels. It would also be of interest to establish the reasons for and consequences of these changes in ATP levels. They are of a substantial magnitude when compared to the depletion of ATP observed on addition of oligomycin and are likely to reflect energy requirements associated with specific events of oocyte maturation. The finding that

cumulus enclosed oocytes have higher ATP levels through most of oocyte maturation, achieved by gap-junction dependent communication with surrounding cumulus cells, is also of note in light of the correlation between higher oocyte ATP levels and increased developmental competence in some species (Van Blerkom *et al.* 1995; Stojkovic *et al.* 2001; Zeng *et al.* 2007).

Finally, it will be of interest to understand the reasons for higher ATP consumption during MI and at MII arrest compared to GV stage which has been reported here. Increased consumption at MI and MII correlates with the presence of a spindle, aligning with the idea of the spindle as a high energy consumer. However, disassembly of the spindle with nocodazole does not impact upon ATP levels or consumption. Indeed, inhibition of a number of processes which might have been expected to contribute to increased energy use during the transition from GV stage to MII arrest did not affect ATP levels or consumption. That differing rates of consumption can be measured is demonstrated by the fact that they were detected at different stages of oocyte maturation. Nonetheless, it would appear that none of the processes investigated here are alone responsible for the altered rate of consumption observed, and the causes of differing rates of ATP consumption remain to be elucidated.

In conclusion, it is clear that mitochondrial distribution and regulation of oocyte ATP level undergo dynamic changes as oocytes progress through maturation from GV stage to MII arrest. The emerging role for mitochondria as determinants of developmental competence indicates that it will be of value to increase our understanding of these processes and of how they impinge on continued

developmental progression. Indeed, emerging evidence for a mitochondrial role in the decline of fertility with increasing maternal age provides a strong impetus for gaining a clear understanding mitochondrial dynamics in the oocyte. These studies will have important implications for the optimisation of *in vitro* maturation and *in vitro* fertilisation techniques, where regulation of mitochondrial ATP production is known to be important. Furthermore, this work could contribute to a rational approach for the design of cell culture media, by providing a method for testing the effects of exogenous factors on intracellular ATP.

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