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# Changes in endoplasmic reticulum structure during mouse oocyte maturation are controlled by the cytoskeleton and cytoplasmic dynein

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**Abstract**

Oocyte maturation in mouse is associated with a dramatic reorganisation of the endoplasmic reticulum (ER) from a network of cytoplasmic accumulations in the germinal vesicle-stage oocyte (GV) to a network of distinctive cortical clusters in the metaphase II egg (MII). Multiple lines of evidence suggest that this redistribution of the ER is important to prepare the oocyte for the generation of repetitive $Ca^{2+}$ transients which trigger egg activation at fertilisation. The aim of the current study was therefore to investigate the timecourse and mechanism of ER reorganisation during oocyte maturation. The ER is first restructured at the time of GV-breakdown (GVBD) into a dense network of membranes which envelop and invade the developing meiotic spindle. GVBD is essential for the initiation of ER reorganisation, since ER structure does not change in GV-arrested oocytes. ER reorganisation is also prevented by the microtubule inhibitor nocodazole and by the inhibition of cytoplasmic dynein, a microtubule-associated motor protein. ER redistribution at GVBD is therefore dynein-driven and cell cycle-dependent. Following GVBD the dense network of ER surrounds the spindle during its migration to the oocyte cortex. Cortical clusters of ER are formed close to the time of, but independently of the metaphase I–metaphase II transition. Formation of the characteristic ER clusters is prevented by the depolymerisation of microfilaments, but not of microtubules. These experiments reveal that ER reorganisation during oocyte maturation is a complex multi-step process involving distinct microtubule- and microfilament-dependent phases and indicate a role for dynein in the cytoplasmic changes which prepare the oocyte for fertilisation.


*Keywords:* Oocyte maturation; Endoplasmic reticulum; $Ca^{2+}$ signalling; Microtubules; Microfilaments

## Introduction

In mammals the transition from egg to embryo is signalled by a series of transient increases in cytosolic [$Ca^{2+}$] elicited by the fertilising sperm (Cuthbertson and Cobbold, 1985; Stricker, 1999; Runft et al., 2002; Swann et al., 2004; Whitaker, 2006). The $Ca^{2+}$ oscillations are dependent upon production of inositol 1,4,5-trisphosphate ($InsP_3$) which promotes the opening of $InsP_3$-gated $Ca^{2+}$ channels ($InsP_3Rs$) (Miyazaki et al., 1992; Fissore and Robl, 1994; Jones and Nixon, 2000; Brind et al., 2000), permitting $Ca^{2+}$ stored within the endoplasmic reticulum (ER) to enter the cytosol (Eisen and Reynolds, 1985; Han and Nuccitelli, 1990). $Ca^{2+}$ is resequestered to the ER lumen by $Ca^{2+}$-ATPases whereupon it is bound by high capacity, low affinity chaperones (Meldolesi and Pozzan, 1998; Corbett and Michalak, 2000; Ashby and Tepikin, 2001). The ER therefore plays a central role at fertilisation as the source and sink for sperm-induced $Ca^{2+}$ oscillations.

Oocyte maturation describes the final stages of oogenesis during which the fully grown oocyte becomes a fertilisation-competent egg. In mammals this process entails the breakdown of the prophase nucleus (germinal vesicle breakdown, GVBD), formation and migration of the metaphase I spindle to the oocyte cortex, extrusion of the first polar body and establishment of the metaphase II spindle (MII). These nuclear changes are accompanied by various cytoplasmic modifications which render the oocyte capable of supporting the development of the

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fertilised egg (Eppig, 1996). One such alteration is a dramatic upregulation in the sensitivity of the $Ca^{2+}$-releasing machinery (Carroll et al., 1996 for review). This is best illustrated by the finding that metaphase II arrested eggs mount a significantly greater $Ca^{2+}$ response to sperm or $InsP_3$ than germinal vesicle-stage oocytes (Chiba et al., 1990; Fujiwara et al., 1993; Mehlmann and Kline, 1994; Jones et al., 1995). Studies of mouse oocytes indicate that the changes responsible for this upregulation may include increases in the amount and distribution of $InsP_3Rs$ (Parrington et al., 1998; Fissore et al., 1999; Xu et al., 2003), ryannodine receptors (Ayabe et al., 1995), content of $Ca^{2+}$ stores (Tombes et al., 1992; Jones et al., 1995), changes in plasma membrane $Ca^{2+}$ handling (Carroll, 2000), $InsP_3R$ phosphorylation state (Jellerette et al., 2004) and the three-dimensional structure of the ER (Kline, 2000). Experiments in *Xenopus* oocytes indicate that changes in the activity of $Ca^{2+}$ entry pathways may also be involved (Machaca and Haun, 2000, 2002). In mouse, a subsequent decrease in sensitivity of the $Ca^{2+}$-releasing machinery occurs following fertilisation by the time the second polar body is formed (FitzHarris et al., 2003; Tang et al., 2000), which may involve several of the same mechanisms (Brind et al., 2000; Jellerette et al., 2000; FitzHarris et al., 2003). Thus the $Ca^{2+}$-releasing machinery is specifically upregulated in the unfertilised egg, presumably reflecting the unique $Ca^{2+}$-mobilising demands of fertilisation.

As the principal source of calcium ions, the structure of the ER around the time of fertilisation has been the subject of considerable interest (Stricker, in press). Confocal imaging studies have revealed changes in the architecture of the ER during oocyte maturation in all species so far studied including *Xenopus* (Terasaki et al., 2001; Kume et al., 1997), sea urchin (Henson et al., 1990; Terasaki, 2004), the nemertean worm (Stricker et al., 1998), starfish (Jaffe and Terasaki, 1994), hamster (Shiraishi et al., 1995) and mouse (Mehlmann et al., 1995). In mouse, oocyte maturation evokes a reorganisation of the ER into a distinctive network of clusters which lie predominantly in the vegetal cortex of the mature unfertilised egg (Mehlmann et al., 1995). The ER clusters disperse shortly after fertilisation, and do not reform during mitosis (FitzHarris et al., 2003). Cortical ER clustering is therefore specific to metaphase II when the $Ca^{2+}$ stores are at their most sensitive. There is also a spatial correlation between ER clustering and the sensitivity of $Ca^{2+}$ release since fertilisation of $Ca^{2+}$ transients propagates from the vegetal cortex where the clusters reside (Kline et al., 1999; Oda et al., 1999; Sato et al., 1999; Deguchi et al., 2000). Therefore it is likely that ER clustering results in $InsP_3R$-rich microdomains which serve as cortical pacemaker sites for the initiation of $Ca^{2+}$ release at fertilisation (Dumollard et al., 2002). However, despite the perceived importance of cortical ER clusters in the mammalian egg, the timecourse and mechanism of their genesis are yet to be addressed.

The aim of the current study was to determine the spatiotemporal dynamics of ER reorganisation during oocyte maturation. Our experiments reveal a complex multi-step process in which the ER is first remodelled into a dense ring around the developing MI spindle, and then surrounds the spindle during its migration to the cortex. Further analyses of these reorganisations reveal roles both for microtubules and microfilaments in cytoplasmic reorganisation during oocyte maturation and provide the first evidence that the microtubule-based motor protein dynein plays a key role during oocyte maturation.

## Materials and methods

### *Mice, chemicals and solutions*

Unless otherwise stated, oocytes were collected from MF1 mice (Harlan, UK). The experiment investigating LTXBO mice was performed in the laboratory of Dr. JJ Eppig, at the Jackson Laboratory, ME, using mice bred in that laboratory. All other experiments were performed at University College London. Chemicals and reagents were obtained from Sigma (UK) unless otherwise stated. Oocyte manipulations were performed in HEPES-buffered KSOM media (H-KSOM; Lawitts and Biggers, 1993; Summers et al., 2000) which contains (in mM) 95 NaCl, 2.5 KCl, 0.35 $KH_2PO_4$, 0.2 $MgSO_4$, 10 Na lactate, 0.2 glucose, 0.2 Na pyruvate, 25 $NaHCO_3$, 1.7 $CaCl_2$, 1 glutamine, 0.01 tetra sodium EDTA, 0.03 streptomycin $SO_4$ and 0.16 penicillin G and 1 mg/ml bovine serum albumin (BSA).

### *Oocyte handling and in vitro maturation*

Germinal vesicle-stage (GV) oocytes were recovered from the ovaries of 21–24-day-old mice that had been administered a 7 IU intraperitoneal injection of pregnant male serum gonadotrophin (PMSG; Intervet) 48 h earlier. Oocytes were released into H-KSOM medium by scraping the surface of the ovary with a 27-gauge needle. Only oocytes with an intact layer of cumulus cells were recovered, and cumulus cells were subsequently removed by repeated pipetting with a narrow pipette. Denuded oocytes were then placed in drops of media under oil to prevent evaporation (Mineral oil; embryo tested) and maintained at 37 °C. In experiments examining *in vivo* oocyte maturation, human Chorionic Gonadotrophin (5 IU) was administered 48 h after PMSG. Pre-ovulatory oocytes were recovered from the ovary as described above, with hyalauronidase added (300 μg/ml; embryo tested grade, Sigma UK) to disperse cumulus cells. Ovulated eggs were recovered from the oviduct as previously described (FitzHarris et al., 2003).

Experiments examining LTXBO and F1 oocytes were performed using two different *in vitro* maturation culture systems. Cumulus–oocyte complexes were placed in 1.5 ml drops of either Waymouths media with 10% foetal bovine serum (Sigma Chemicals, St. Louis, MO) with 100 IU/l human recombinant FSH (hFSH; National Hormone and Pituitary Program, NIDDK; O'Brien et al., 2003) or in minimum essential media (MEM; all components from Sigma, MO) with BSA (3 mg/ml; ICN Immunochemicals, Lisle, IL; Hirao and Eppig, 1999). Oocytes were cultured at 37 °C in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% $N_2$ for 16 h prior to transfer into M2 media (Fulton and Whittingham, 1978) containing 4 mg/ml BSA for examination of the ER.

### *Labelling the endoplasmic reticulum (ER)*

To label the endoplasmic reticulum, DiI (Molecular Probes) was microinjected as a solution in soyabean oil as previously described (FitzHarris et al., 2003). Imaging was performed using a Bio-Rad μ-radiance confocal scan head (Bio-Rad, Hemmel Hempstead, UK) mounted on a Zeiss Axiovert microscope using a 40× oil immersion objective lens. DiI was excited with 514 nm light and emitted fluorescence collected with a 600 nm longpass filter. Imaging was performed on a heated microscope stage maintained at 37 °C, 30 min after microinjection. Cortical confocal planes estimated to be 6 μm from the plasma membrane were selected using the focus motor as previously described (FitzHarris et al., 2003). The equatorial confocal plane was selected by focussing manually. To co-label the ER and chromatin, oocytes were micro-injected with DiI prior to a 5-minute incubation in 1 μg/ml Hoechst 33342. Imaging was performed with a Zeiss LSM-510 laser-scanning microscope (Carl Zeiss Inc) with a 40× oil immersion objective using the 'multitracking' mode.

DiI was illuminated with a 543 nm laser and emitted light collected with a 600 nm longpass filter. Hoechst 33342 was excited with a UV laser (360 nm) and emitted fluorescence collected using a 385/470 bandpass filter. LTXBO and F1 oocytes were examined at the Jackson Laboratory using a Leica TCS NT confocal microscope fitted with a 63× 1.2 NA Water immersion PlanApo objective. DiI was excited using 568 nm light, and emission over 590 nm was collected.

### *Manipulation of the cytoskeleton*

Stock solutions of nocodazole (10 mM) and Latrunculin A (6 mM) were prepared in DMSO and stored at −30 °C. 1 in 1000 dilutions was made in H-KSOM before use. Sodium orthovanadate (Sigma) was made daily in H-KSOM to a concentration of 500 μM (Carabatsos et al., 2000). Clone 70.1 (Sigma) was microinjected to an estimated final concentration of 1–2 mg/ml. To confirm delivery of the antibody, 70.1 was coinjected with fluorescein dextran (3 kDa; Sigma) and oocytes were examined using a fluorescence microscope. Controls were injected with fluorescein dextran only.

### *Western blots*

Western blots were performed on groups of 200 GV-arrested oocytes, using a 1:1000 dilution of the 70.1 antibody. Methods otherwise as previously (Marangos et al., 2007).

## Results

### *ER reorganisation during oocyte maturation*

In the current study we examined the structure of the ER in mouse oocytes using the dicarbocyanine dye $DiI_{18}$ (DiI) and confocal microscopy. This technique has been employed previously in a variety of cell types, including mammalian oocytes and early embryos, and has proven to be a reliable ER marker in all cases (see Kline, 2000 for review). To verify that the appearance of DiI-labelled ER was the same here as previously reported, we examined the structure of the ER in GV stage oocytes and MII eggs. At GV stage the ER is arranged as a network of small accumulations throughout the cytoplasm (Fig. 1). The germinal vesicle membrane is prominently labelled by DiI, indicating continuity between ER and nuclear membranes, though there is no labelling within the GV itself. At MII the ER extends throughout the cytoplasm in a more uniformly reticular manner, but there is no labelling in the area where the spindle resides in the animal cortex. Distinctive clusters of ER 1–2 μm in diameter populate the cortical confocal slice in eggs, but not in GV oocytes. The presence of ER clusters in the oocyte cortex causes the outer edge of the equatorial slice to appear more strongly labelled in MII oocytes. Therefore, as previously described, the ER undergoes a considerable reorganisation during oocyte maturation.

We next examined the ER structure of *in vivo* matured oocytes at various timepoints following hCG administration. The ER was examined at GV stage, and 3, 6 and 9 h after hCG, at which times GVBD had occurred. Following GVBD a distinctive ring of fluorescence was observed in the centre of the oocyte (Fig. 2A). Formation of this ER ring was associated with GVBD since oocytes which had been arrested at GV stage for 6 h (in the presence of 200 μM IBMX) displayed ER structure indistinguishable from freshly isolated oocytes (Fig. 2B). Nine hours after hCG the prominent ER ring was positioned in the oocyte cortex (Fig. 2A). Cortical clusters of ER were only ever evident in MII oocytes. Thus, cortical clusters of ER are formed in the latter stages of oocyte maturation, close to the time of Pb1 extrusion.

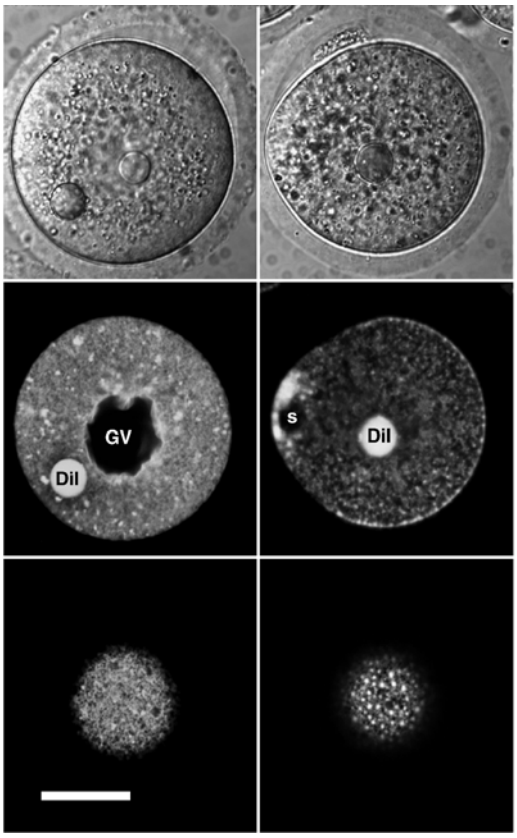


Fig. 1. DiI-labelled endoplasmic reticulum in GV and MII stage oocytes. Oocytes were microinjected with DiI as a solution in soyabean oil and examined using confocal microscopy. Typical examples of an oocyte at GV stage (left) and MII stage (right). Brightfield (top) and equatorial (middle) and cortical (bottom) fluorescence confocal slices are shown. Note that the DiI-containing oil droplet is visible in both the brightfield and fluorescence images (DiI). The germinal vesicle (GV) and region containing the MII spindle (s) are also indicated. More than thirty oocytes of each stage were examined with little variation in the appearance of the ER between similar stage oocytes. The appearance of the ER as labelled here using DiI is similar to as previously reported (Mehlmann et al., 1995). Scale bar 30 μm.

### *The relationship between the ER and chromosomes following GVBD*

The finding that ER accumulates as a dense ring in the centre of the oocyte implies an association with the meiotic apparatus, which forms centrally following GVBD. Therefore, to examine the spatial relationship between the MI chromosomes and the ER, MI oocytes were co-labelled with DiI and with Hoechst 33342. As expected, confocal imaging revealed that the dense ER ring forms around the chromosomes (Fig. 3). High magnification images revealed that within the dense ring the ER lies in close apposition with the chromosomes, apparently filling the spaces between individual chromosomes (Fig. 3). Thus, as in some mitotic cells (Harris, 1975), the ER is very closely associated with the spindle and chromosomes in MI oocytes.

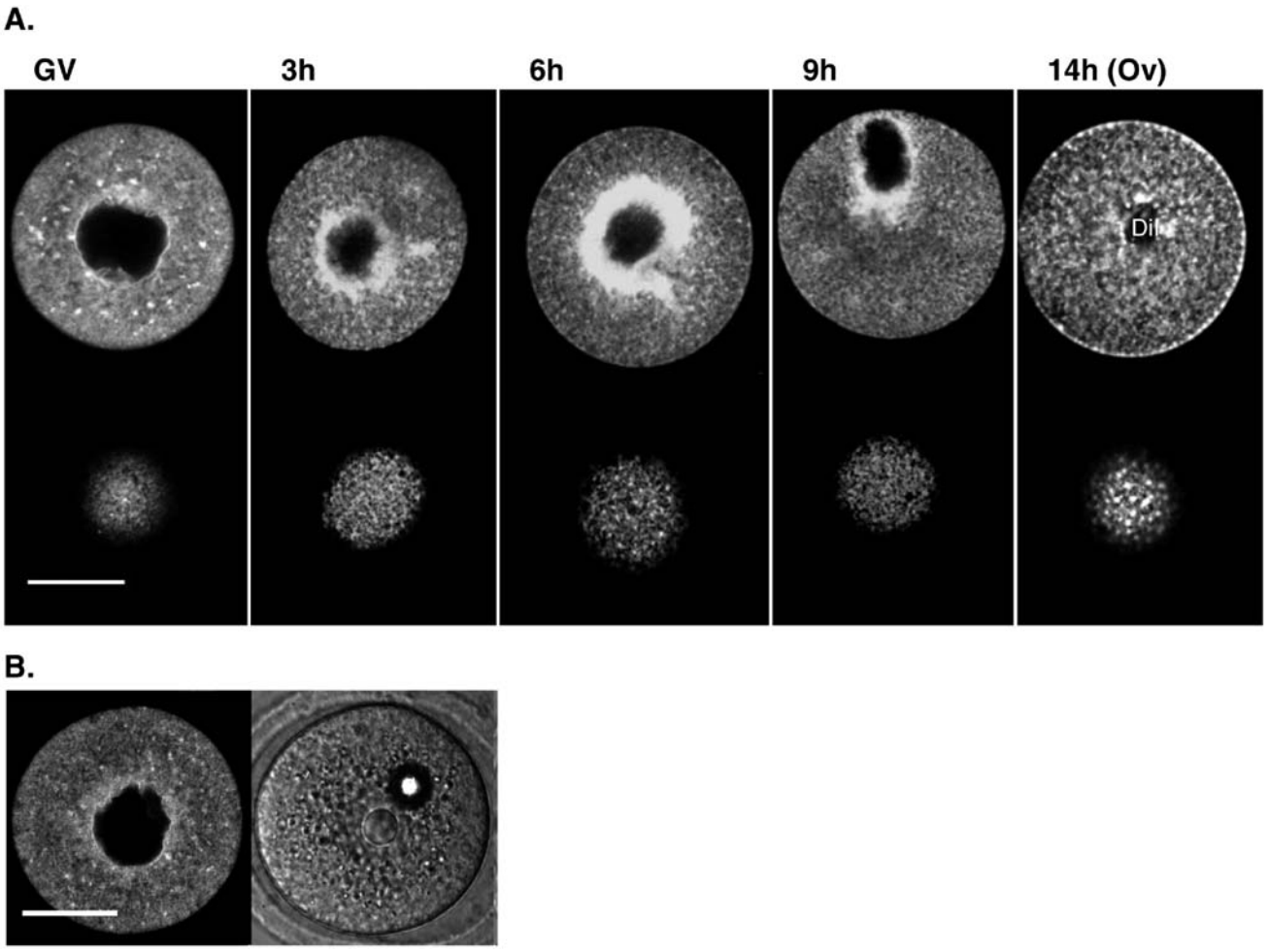


Fig. 2. Timecourse of ER reorganisation during oocyte maturation. (A) Oocytes were recovered and the structure of the ER examined using DiI and confocal microscopy at 3-hour intervals following hCG administration. Equatorial (top) and cortical (bottom) sections are shown. Note that oocytes which have undergone GVBD exhibit a prominent ring of ER within the cytoplasm (3, 6 and 9 h). Note also that cortical clusters of ER are only visible in oocytes 14 h after hCG administration (ovulated oocytes). A minimum of 7 oocytes were examined at each time-point over the course of at least 2 days (B) The ER was labelled in oocytes arrested for 6 h in the presence of IBMX. A typical equatorial confocal slice is shown. Notice that the prominent ring of ER which develops following GVBD is not observed in these GV-arrested oocytes ($n=11$).

*The mechanism of ER reorganisation at GVBD*

*Endoplasmic reticulum reorganisation following GVBD is inhibited by nocodazole*

Germinal vesicle breakdown in mouse is not inhibited by manipulations which disturb the cytoskeleton. Taking advantage of this, we used cytoskeletal depolymerising agents to consider the possible role of microfilaments and microtubules in the substantial ER reorganisation which occurs at GVBD. To investigate the role of microfilaments we used Latrunculin A (LatA), which causes actin cytoskeleton depolymerisation by complexing with actin monomers (Deng et al., 2005; Spector et al., 1989). The role of microtubules was studied using nocodazole, a microtubule depolymerising agent which has been extensively employed and characterised in mouse oocytes (Wickramasinghe and Albertini, 1992; Kubiak et al., 1993; Winston et al., 1995). As expected, both drugs were capable of preventing second polar body extrusion after egg activation (data not shown), which confirms their effectiveness (Maro et al., 1984; Winston et al., 1995). GV stage oocytes were transferred to media containing either LatA or nocodazole shortly prior to GVBD. Neither treatment prevented GVBD. As before, untreated oocytes which progressed to metaphase I exhibited a dense ring-like accumulation of ER (Fig. 4). Development of the ER ring at GVBD was unaffected by LatA. In contrast, the ER ring was strikingly absent in nocodazole-treated oocytes (Fig. 4). Neither nocodazole nor LatA had any impact upon ER structure in GV-arrested oocytes (Fig. 4). Thus, nocodazole affects oocyte ER structure specifically at GVBD by preventing the formation of the dense ER ring.

*Inhibition of cytoplasmic dynein prevents ER reorganisation at GVBD*

The finding that ER reorgansation at GVBD is inhibited by nocodazole but not LatA indicates that microtubules, but not microfilaments, are essential for ER motility at GVBD.

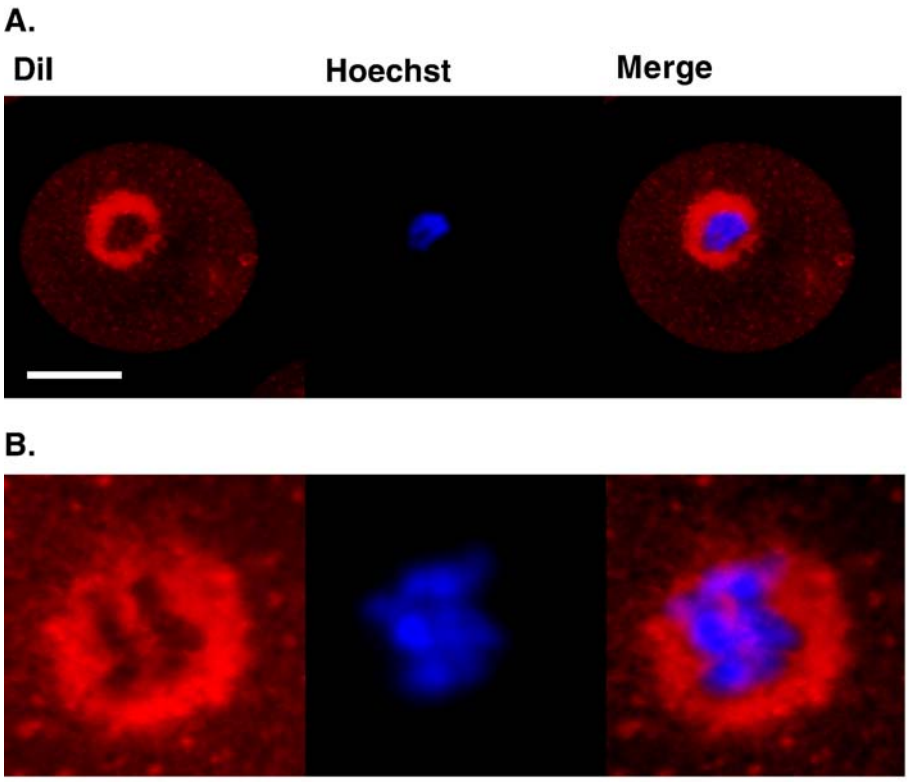


Fig. 3. The ER envelops and invades the meiotic machinery during prometaphase I. (A) Oocytes were co-labelled with DiI and Hoechst to visualise the spatial relationship between ER and chromatin in prometaphase I oocytes 6 h after release from the ovary. Note that the dense ring of ER surrounds the chromatin during prometaphase I. (B) Another example at higher magnification, illustrating that ER invades the spindle and lies closely apposed with the chromatin ($n=8$).

Microtubule networks of cultured cells are organised with their growing ends (plus-ends) in the cell periphery, and with their minus ends at the microtubule organising centre (MTOC). In interphase cultured cells the MTOC typically resides proximal to the nucleus. However, freshly dissected GV stage mouse oocytes possess one or more MTOCs which reside in the cortex. Around the time of GVBD these are replaced first by two MTOCs at opposing poles of the germinal vesicle, and then by a ring of MTOCs which surround the developing spindle at prometaphase I (Messenger and Albertini, 1991; Can et al., 2003). Motor proteins responsible for the transport of organelles along microtubules fall into two families: the kinesin family which mediates transport towards the plus-end, and dyneins which support negative end-directed transport (see Allan and Schroer, 1999 for review). Since at GVBD the ER is redistributed towards the centrally placed MTOCs and meiotic machinery, we hypothesised that this motility might reflect negative end-directed membrane transport by dynein. To test our hypothesis, GV stage oocytes were transferred to media containing sodium orthovanadate (vanadate). Vanadate is a phosphatase inhibitor which can be used to examine dynein-dependent transport virtue of its high level of selectivity for dynein over kinesins (Niclas et al., 1996). Vanadate has been employed in mouse oocytes previously and was found to disturb spindle structure (Carabatsos et al., 2000). Vanadate treatment did not prevent GVBD, but prevented formation of the prominent ER ring characteristic of MI oocytes (Fig. 5). Vanadate-treated MI oocytes displayed ER structure indistinguishable from that of nocodazole-treated oocytes.

In order to confirm that the effect of vanadate upon ER structure at GVBD can be attributed to dynein inhibition, we performed a separate series of experiments, in which GV stage oocytes were microinjected with 70.1, an antibody raised against the intermediate chain of dynein, which exerts a functional inhibitory effect upon dynein function by preventing its association with the accessory complex dynactin (Steuer et al., 1990; Heald et al., 1997; Rusan et al., 2002) and has been used previously in mammalian oocytes to investigate the role of dynein in pronucleus migration (Payne et al., 2003). 70.1 prevented ER accumulation at GVBD in a similar manner to nocodazole and vanadate (Fig. 5E). Typical ER rings were observed in control-injected oocytes (Fig. 5D). Thus the accumulation of ER at GVBD is similarly prevented by two independent means of inhibiting dynein. To confirm that 70.1 interacts with dynein, western blots were performed on GV stage oocytes. As expected, the antibody binds to a band of approximately 70–75 kDa, similar to the band seen in previous studies (Steuer et al., 1990; Gaglio et al., 1997), thought to be dynein intermediate chains (Fig. 5F). Unexpectedly, the antibody also interacted with unidentified bands of sizes ~85 and ~95 kDa. Although we cannot definitively rule out the possibility that the antibody has dynein-independent effects, this is unlikely to interfere with our experiments here as similar phenotypes were observed with vanadate and nocodazole, with

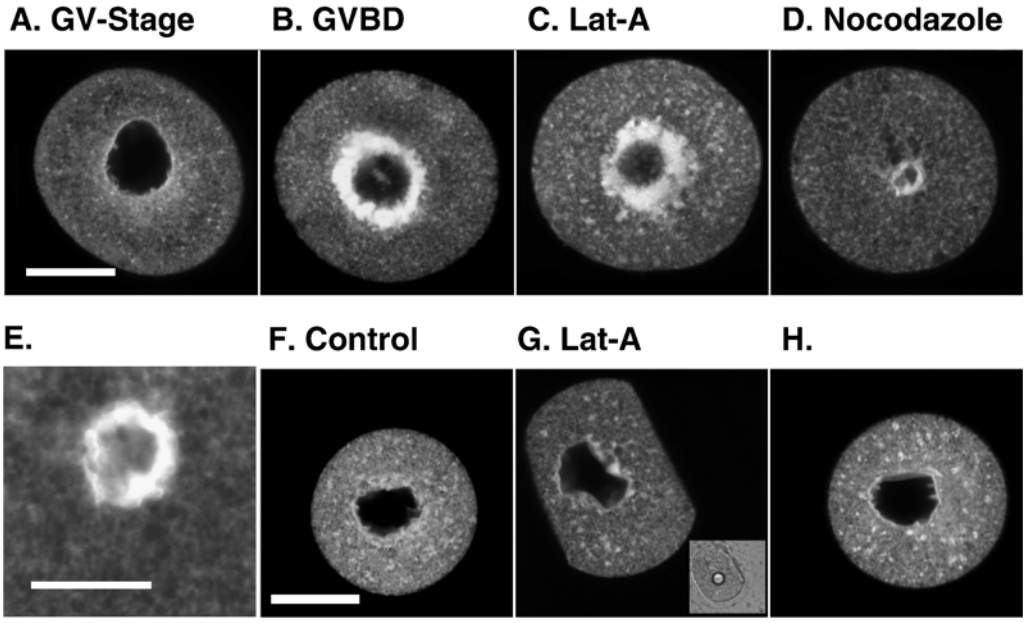


Fig. 4. Nocodazole inhibits ER accumulation at GVBD. DiI-labelled ER was examined in GV stage oocytes (A), in oocytes cultured for 4 h in control conditions (B; $n=5$) and the presence of 6 μM LatA (C; $n=10$) or 10 μM nocodazole (D; $n=8$). Neither drug treatment prevented GVBD. Notice that the thick ER ring characteristic of post-GVBD oocytes forms in the presence of LatA, but not in the presence of nocodazole. A zoomed image of a nocodazole-treated oocyte is shown in E. Images shown are typical of each treatment group and were taken on the same day. DiI-labelled ER was also examined in arrested GV stage oocytes following 1.5 h in the absence (F; $n=11$) or presence of LatA (G; $n=15$) or nocodazole (H; $n=11$). Note that nocodazole and LatA have no effect upon the appearance of the ER in GV-arrested oocytes, though LatA caused most oocytes of this developmental stage to lose shape (see inset brightfield, G). Scale bar 30 μm.

all three treatments producing phenotypes consistent with the classical action of dynein. We conclude that accumulation of the ER around the chromosomes at GVBD is dependent upon microtubules, and upon the microtubule-based motor protein dynein.

### *Cortical clusters of ER are generated independently of the MI–MII transition*

The finding that cortical ER clusters are formed close to the time of Pb1 extrusion raises the possibility that ER reorganisation may occur as a result of the MI–MII transition. Thus, to determine whether formation of ER clusters is related to nuclear progression we sought to examine ER structure in oocytes which have spontaneously arrested at MI. Since an unusually high proportion of oocytes of mouse strain LTXBO arrest at metaphase I, failing to extrude the first polar body (O'Neill and Kaufman, 1987; Hirao and Eppig, 1997), this mouse model provides an excellent opportunity to address this question. GV stage oocytes from LTXBO mice exhibited ER structure indistinguishable from that of control mice, and no cortical clusters of ER were detectable in the cortical slice. Following 18 h of in vitro maturation, MI-arrested LTXBO oocytes displayed cortical ER clusters similar to those observed in ovulated eggs (Fig. 6). Cortical ER clusters were similarly generated in a small number of F1 oocytes which were examined that had spontaneously arrested at MI (data not shown). Thus, although cortical ER clusters are formed close to the time of Pb1 extrusion, their generation occurs independently of the MI–MII transition.

### *Microfilaments are necessary for forming cortical ER clusters at MII*

We next sought to determine which cytoskeletal components participate in the formation of the cortical ER clusters at the MI–MII transition. Oocytes were matured *in vitro* for 8 h before being transferred to media containing either LatA or nocodazole. At this time all oocytes had undergone GVBD, but none had extruded the first polar body. Spindle migration had occurred in the majority of cases (over 80%), but the ER had not yet been remodelled into cortical clusters (Fig. 7A). The oocytes were then cultured for a further 8–10 h at which time Pb1 formation had occurred and cortical ER clusters had formed in controls (no drug; Fig. 7B). Nocodazole prevented Pb1 extrusion in all cases, but cortical clusters indistinguishable from those of controls were formed in all cases (Fig. 7C). Thus, intact microtubules are not necessary for the formation of cortical clusters of ER. In contrast, oocytes transferred to LatA failed to display cortical cluster of ER suggesting that microfilaments are necessary for the formation of ER clusters (Fig. 7D). However, an unexpected effect of LatA treatment was that a subpopulation of oocytes (5 of 20 oocytes in two separate experiments) had undergone exit from metaphase and formed two pronuclei as evidenced by DiI-labelled pronuclear membranes and Hoechst labelled nuclei (Figs. 7F, F′). In previous studies we have shown that exit from M-phase at MII results in dispersal of ER clusters (FitzHarris et al., 2003). Therefore, to confirm that the effects of LatA were a result of disrupting microfilaments rather than changes in the cell cycle we used nocodazole to induce the spindle assembly checkpoint (Kubiak et al., 1993) and prevent

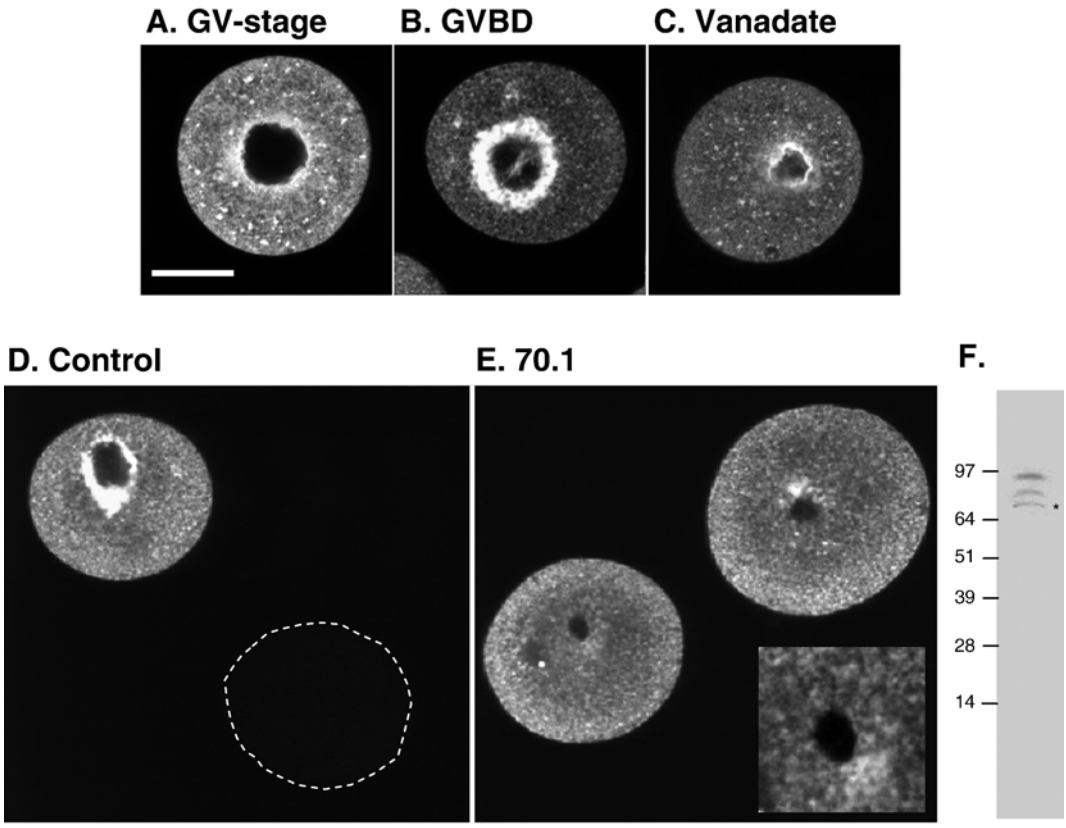


Fig. 5. Inhibition of dynein prevents formation of the ER ring at GVBD. DiI-labelled ER was studied in GV stage oocytes ($n=5$; A), and in oocytes which had been cultured for 4 h in the absence ($n=12$; B) or presence ($n=9$; C) of sodium orthovanadate (500 μM), during which time GVBD occurred. Notice that vanadate treatment prevents formation of the ER ring. Scale bar 30 μm. In a separate series of experiments, oocytes were microinjected with 70.1 and fluorescein dextran, or fluorescein dextran only (control). Fluorescein dextran was injected in both groups to confirm delivery of the injection solution. Oocytes were subsequently cultured for 4 h to allow GVBD to take place, and ER was then examined using DiI and confocal microscopy. (D) Control (fluorescein microinjection) oocytes displayed the usual ring of ER at prometaphase I ($n=5$). Note the dotted line indicates an oocyte which received a fluorescein injection but not a DiI injection, and which cannot be seen in the fluorescence micrograph, confirming that fluorescein does not interfere with the DiI image. (E) Oocytes which received a 70.1 injection prior to GVBD lack the ER ring characteristic of prometaphase I ($n=10$). A zoomed image of a post-GVBD 70.1-injected oocyte is shown in the inset. (F) A western blot of GV oocytes revealed that 70.1 clearly recognises a group of proteins ~70–75 kDa (see asterisk), as well as bands of molecular weight ~85 kDa and ~95 kDa.

exit from M-phase, while exposing oocytes to LatA as before. Hoechst labelling confirmed that all LatA/nocodazole co-treated oocytes were arrested in M-phase (not shown) and, in agreement with results obtained with LatA alone, the formation of cortical clusters was inhibited (Fig. 8E). These experiments reveal a role for microfilaments in ER cluster formation at the MI–MII transition.

Finally, in order to determine whether the cytoskeleton is necessary for maintaining the distinctive structure of the ER at MII, ER was examined in ovulated unfertilised eggs following brief (1.5–2 h) incubation in either LatA or nocodazole. As before, the effectiveness of both drugs was confirmed by the prevention of Pb2 extrusion following oocyte activation (not shown). Nocodazole had no effect upon the structure of the ER in unfertilised eggs (Fig. 8). In contrast, LatA had a profound effect upon ER structure, causing the appearance of ER clusters similar to those in the cortex throughout the cytoplasm (17 of 20 oocytes; Fig. 8). Thus microfilaments, which are known to be enriched in the oocyte cortex, are necessary for forming cortical ER clusters and then play a role in maintaining the characteristic ER formation in MII oocytes by restricting the clusters to the cortex.

## Discussion

### *ER motility driven by cytoplasmic dynein at GVBD in mouse oocytes*

Cytoplasmic dynein is a massive multi-subunit complex which, in association with dynactin, transports cargoes towards the negative ends of microtubules. Cytoplasmic dynein is extensively expressed and involved in a wide variety of cell functions including chromosome movements, spindle organisation and positioning, nuclear migration and organelle structure (Vallee et al., 2004; Karki and Holzbaur, 1999; Mountain and Compton, 2000). Despite the obvious relevance of these processes in meiosis, little is known about the role of dynein in mammalian oogenesis. In the course of our investigation into the structure of the endoplasmic reticulum we identified a dramatic reorganisation of the ER into a dense network surrounding the centrally placed prometaphase I spindle which occurs at the time of GVBD. This reorganisation was inhibited by disruption of the microtubule network, by an inhibitor of dynein, and also by a functionally inhibitory antibody against the dynein intermediate

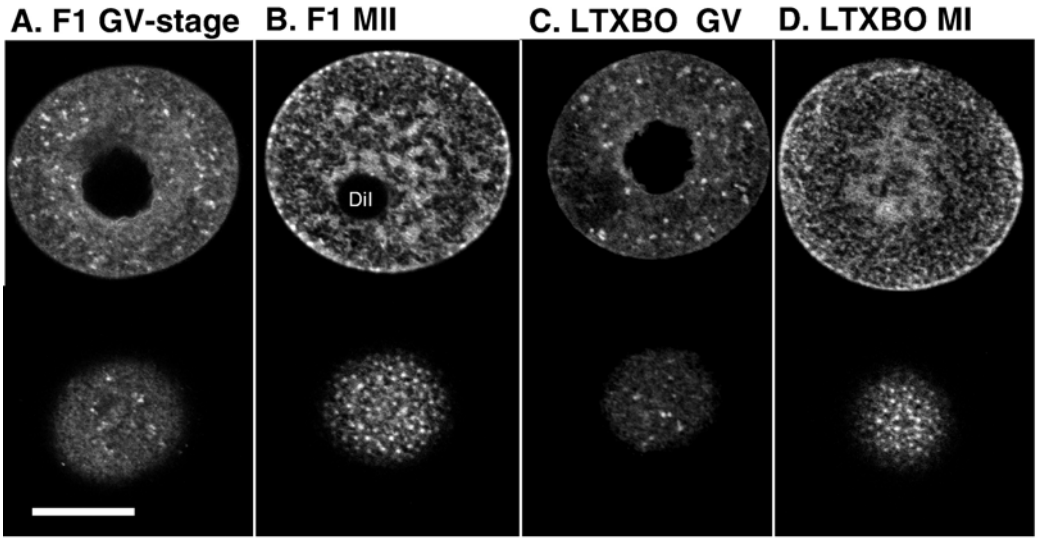


Fig. 6. Cortical ER clustering occurs independently of the MI–MII transition in LTXBO oocytes. The ER was examined in oocytes of strain LTXBO and F1 according to the usual protocol. Exemplar equatorial (top) and cortical (bottom) confocal planes are shown. Note that GV stage oocytes of mouse strain LTXBO (C) display ER structure similar to F1 (control mice; A), and MF1 mice (see Fig. 1). Note that, as before, GV oocytes do not possess cortical clusters of ER. Oocytes were cultured for 18 h prior to microinjection of DiI and examination of the ER. The ER was examined in MII-arrested F1 oocytes (B) and in MI-arrested LTXBO oocytes (D). Note the presence of punctuate ER clusters in the cortical confocal slice (bottom) in both cases. The oocytes shown were cultured in Waymouths medium with FSH, though identical results were also obtained after culture in MEM (see Materials and methods). Scale bar 30 μm. $n = 10–15$ for all groups.

chain. The direction of the ER motility towards the centrally placed meiotic spindle is also consistent with negative end-directed transport. These experiments provide a simple yet clear indication that dynein supports ER motility in mouse oocytes, thereby introducing dynein as a participant in the cytoplasmic modifications which prepare the oocyte for fertilisation.

It has long been established that MTs play a key role in determining ER structure. In somatic cells ER tubules frequently align with the MT network, and it is known that the structure of the ER in interphase is primarily determined by +ve end-directed kinesins which extend the ER along the MTs towards the cortex since inhibition of kinesin can cause the ER to retract towards the centrosomes (Feiguin et al., 1994; Allan, 1996). In contrast, although in vitro assays clearly demonstrate that dynein has the capacity to transport ER (Allan and Vale, 1991; Allan, 1995, 1996), evidence from intact cells that dynein affords ER motility in vivo has been scant. Good evidence comes from recent experiments in the fungus *Ustilago mayadis*, in which depletion of dynein was found to severely restrict cortical ER tubule movement (Wedlich-Soldner et al., 2002). The ER reorganisation which we describe at GVBD in mouse oocytes is, to our knowledge, the first demonstration of dynein-dependent ER motility in an intact animal cell. It remains to be determined whether accumulation of ER around the spindle which is characteristic of mitosis in several cell types is similarly dynein-dependent.

Dynein-driven ER reorganisation at GVBD is the first in an apparently complex series of steps which culminate in the generation of cortical ER clusters in the fertilisation-competent egg. Following its formation, the ER ring accompanies the enclosed spindle as it migrates to the oocyte cortex and was observed next to the plasma membrane shortly prior to the formation of the first polar body. Around the time of first polar body extrusion the dense ER ring disappears, and ER cortical clusters are generated in the vegetal hemisphere. Since LatA, but not nocodazole, was capable of preventing ER cluster formation, it would appear that the cortical clusters are formed from ER which is conveyed away from the spindle and throughout the cortex along the dense actin network which exists in the mouse oocyte cortex (Sun and Schatten, 2006). Although it is not yet clear whether this step normally takes place prior to or following Pb1 extrusion, the MI cortex is clearly capable of forming ER clusters as they were detected in MI-arrested oocytes. Given the acute sensitivity of mammalian oocytes and embryos to photo-damage (Daniel, 1964), more precise determination of the timecourse of these events may require real-time imaging using multi-photon microscopy (Squirrell et al., 1999; FitzHarris et al., 2005). Our examination of ovulated eggs indicated that microfilaments are also important for maintaining the characteristic ER structure during the metaphase II arrest since Latrunculin A caused the appearance of pan-cytoplasmic ER clusters. It may be that the cortex provides the site of ER clustering and that, in mouse, the dense actin network normally serves to anchor the clusters. The distinct phenotypes of LatA treatment in mature and maturing oocytes support the assertion that actin plays separate roles first in cluster formation at the MI–MII transition, and thereafter in maintaining the characteristic ER structure at MII.

An interesting question is whether the dense ring of ER which forms at the time of GVBD impacts $Ca^{2+}$ homeostasis in the vicinity of the enclosed spindle. Recent work demonstrated that ER accumulation around the spindle in *Drosophila* embryos facilitates the generation of localised [$Ca^{2+}$] increases which are associated with M-phase entry and exit (Parry et al., 2005). Though a careful search for localised $Ca^{2+}$ transients during mammalian oocyte maturation has not yet been carried

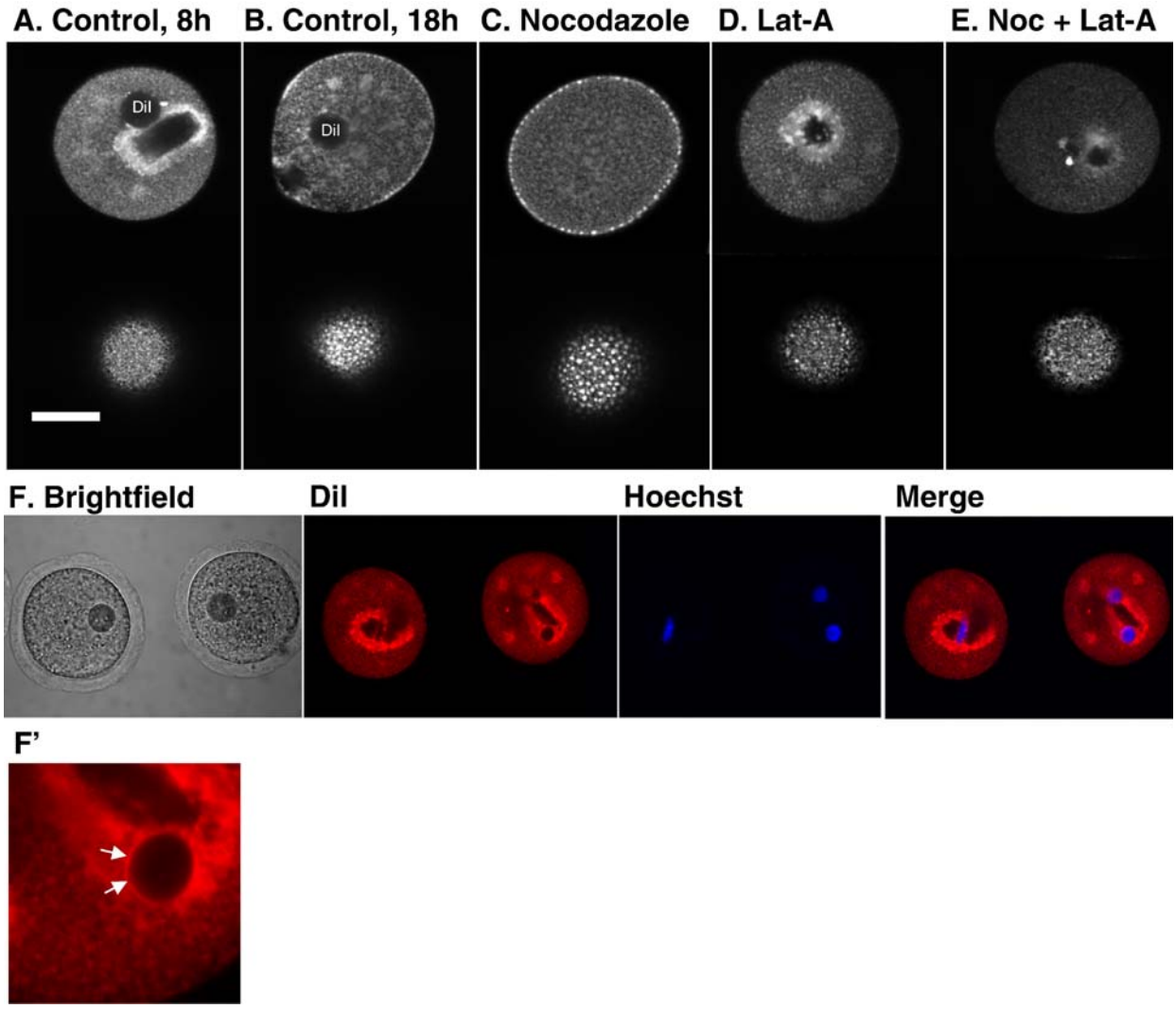


Fig. 7. Latrunculin A prevents the formation of cortical ER clusters. Cumulus–oocyte complexes were matured *in vitro* in MEM. (A) DiI-labelled ER in *in vitro* cultured oocyte 8 h after release from the ovary. Note that, as is the case for *in vivo* matured oocytes of this stage (see Fig. 1), there are no ER clusters in the cortical slice (bottom) and the ER ring lies in the cortex (top). (B–E) Oocytes were transferred to media containing nocodazole, LatA or both together, 8 h after release from the ovary, and ER was examined 8–10 h later. No polar bodies were extruded in the drug treatment groups. Note that distinctive cortical ER clusters are formed in control (no drug) and nocodazole-treated oocytes, but not in the presence of LatA. The LatA-treated oocyte presented in panel D was determined using Hoechst to be in interphase (not shown). A minimum of 14 oocytes were examined for each group, over the course of two separate experiments. Scale bars 30 μm. (F) LatA-treated oocytes were co-labelled with DiI and Hoechst. Note the condensed chromatin forming a metaphase plate in the left oocyte, whereas the chromatin has decondensed and been surrounded by nuclear membranes in the oocyte on the right. A zoomed image of the righthand oocyte is shown in panel F′; note the DiI-labelled nuclear membranes (arrowheads).

out, no such transients were detected in our recent study of one-cell mouse embryos, in which the relationship between the ER and spindle is similar (FitzHarris et al., 2003, 2005).

### *ER reorganisation, $Ca^{2+}$ release and cytoplasmic maturation*

It has long been realised that the two aspects of oocyte maturation, progression of the GV oocyte to metaphase II (nuclear maturation) and the generation of a cytoplasm capable of successful pre-implantation development (cytoplasmic maturation), are experimentally separable. For example, in mice, some oocytes which complete nuclear maturation are subsequently incapable of developing to blastocyst when fertilised. Conversely, some aspects of cytoplasmic maturation occur even in oocytes which have arrested at MI. These include changes in the pattern of protein synthesis which normally occur during oocyte maturation, the ability to commence development in response to sperm or $Ca^{2+}$ ionophore (Eppig et al., 1994) and the ability to mount a proper $Ca^{2+}$ signal in response to the fertilising sperm (Jones et al., 1995). In the current study, we have shown that generation of cortical ER clusters, which normally occurs at the time of the MI–MII transition, takes place in oocytes which arrest at MI. ER reorganisation is thus a structural feature of cytoplasmic maturation which can be uncoupled from the nuclear cycle. The finding of MII-like ER clusters in MI-arrested oocytes, which have been shown to produce a $Ca^{2+}$ signalling regimen in response to sperm which is similar to MII oocytes (Jones et al.,

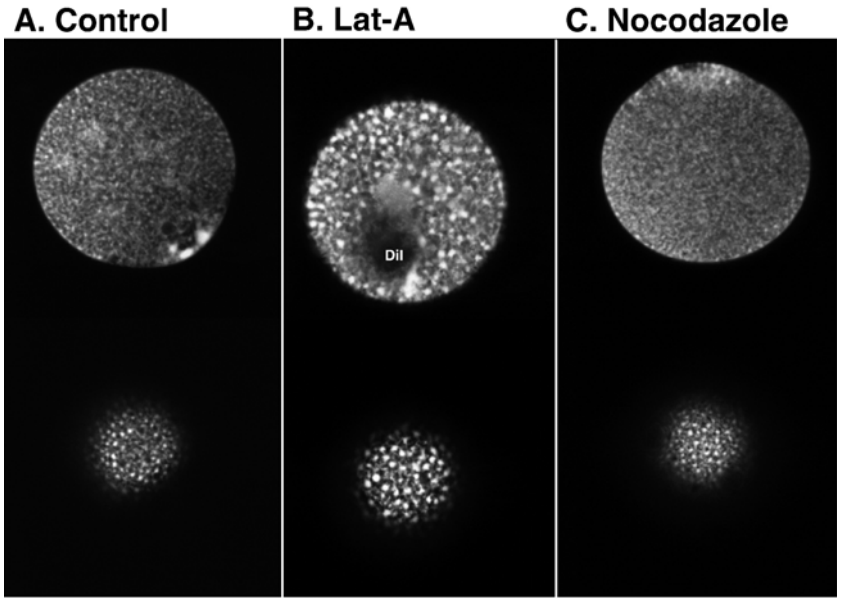


Fig. 8. Latrunculin A causes the appearance of cytoplasmic ER clusters in MII eggs. The ER was examined in ovulated MII oocytes following 1.5–2 h incubations in control media (A), LatA (B) or nocodazole (C). Equatorial (top) and cortical (bottom) slices of typical oocytes are shown. Note that the ER in nocodazole-treated oocytes is indistinguishable from control oocytes, whereas LatA treatment caused the appearance of ER clusters throughout the cytoplasm in 17/20 cases. The ER continued to resemble that of control oocytes in the other three cases (not shown). 20–24 oocytes were examined for each group. Scale bar 30 μm.

1995), further expands the corollary between ER clustering and the sensitivity of $Ca^{2+}$ release. Moreover the apparent capacity of some MI-arrested oocytes to develop when inseminated may owe to the development of $Ca^{2+}$ signalling components such as the endoplasmic reticulum, even in the absence of nuclear maturation.

## Conclusion

The experiments presented herein reveal that ER reorganisation during oocyte maturation is a complex process involving several phases of organelle motility which are underpinned by distinct components of the cytoskeleton. An initial accumulation of the ER around the spindle occurs at GVBD and is dependent upon microtubules and cytoplasmic dynein. Later, remodelling of the ER into the characteristic cortical clusters at MII is dependent not upon microtubules, but rather upon microfilaments. Precise coordination of the changing relationship between ER and the cytoskeletal components during oocyte maturation is thus essential for accurate reorganisation of the ooplasm prior to fertilisation.

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