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Formin-2 is required for spindle migration and for the late steps of cytokinesis in mouse oocytes

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Abstract

Female meiotic divisions in higher organisms are asymmetric and lead to the formation of a large oocyte and small polar bodies. These asymmetric divisions are due to eccentric spindle positioning which, in the mouse, requires actin filaments. Recently Formin-2, a straight actin filaments nucleator, has been proposed to control spindle positioning, chromosome segregation as well as first polar body extrusion in mouse oocytes. We reexamine here the possible role of Formin-2 during mouse meiotic maturation by live videomicroscopy. We show that Formin-2 controls first meiotic spindle migration to the cortex but not chromosome congression or segregation. We also show that the lack of first polar body extrusion in fmn2$^{-/-}$ oocytes is not due to a lack of cortical differentiation or central spindle formation but to a defect in the late steps of cytokinesis. Indeed, Survivin, a component of the passenger protein complex, is correctly localized on the central spindle at anaphase in fmn2$^{-/-}$ oocytes. We show here that attempts of cytokinesis in these oocytes abort due to phospho-myosin II mislocalization.

Keywords: Oocyte; Spindle migration; Formin 2; Actin; Mouse

Introduction

Spatial and temporal control of cell division is essential to ensure the equal segregation of genetic material between daughter cells. Female meiotic divisions are typically asymmetric, giving rise to two daughter cells of different size: a huge oocyte and tiny polar bodies. The asymmetric partitioning of the cytoplasm is due to the off-center positioning of the spindle and of the cleavage furrow. Cell division is accomplished during cytokinesis with the formation of an actomyosin contractile ring at anaphase which will contract during telophase, resulting in the formation of a cleavage furrow between the two daughter cells (Matsumura, 2005). Cytokinesis is a complex process which involves at least five classes of proteins controlling the successive events required for the eventual physical separation of the two daughter cells (Glotzer, 2005). A first set of proteins is recruited during anaphase to the central spindle (also called the spindle midzone). This includes the MKLP1-Cyk4 complex (or centralspindlin complex) and the Aurora B-Incenp-Survivin complex. Different studies have shown that the central spindle together with the asters at the spindle poles are involved in the positioning of the cleavage furrow (Bonaccorsi et al., 1998; Cao and Wang, 1996; Glotzer, 2005; Mishima et al., 2004; Rappaport, 1985). Thus if the spindle is off-center, the division is asymmetric with a mother cell giving rise to two cells of different sizes. This situation is encountered during female meiosis in the mouse. However, in oocytes the spindle does not have asters at poles (Szollosi et al., 1972), thus the cleavage furrow position is controlled only by the central spindle. The second set of proteins belongs to the RhoA pathway including the RhoGEF (ECT2) and a RhoGAP which remains to be identified. These proteins are involved in the activation of the third class of proteins which corresponds to

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myosin II and its regulators. Non-muscle myosin II is activated by phosphorylation and drives constriction of the contractile ring (Jordan and Karess, 1997; Straight et al., 2003). However, the contractile activity of myosin is latent until it interacts with parallel bundles of actin filaments. This is regulated by the fourth class of proteins corresponding to the Formin-Profilin machine. Formin belongs to a family of ubiquitous, highly conserved multidomain proteins (Faix and Grosse, 2006). Formins are defined by the presence of a formin homology 2 (FH2) domain which confers an actin nucleating activity to these proteins. However, unlike Arp2/3 or Spire which generate branched filaments and are involved in cell migration, Formins together with Profilin nucleate straight actin filaments and are essential for contractile ring assembly (Chang et al., 1997; Evangelista et al., 2002; Kovar et al., 2003; May, 2001; Pruyne et al., 2002; Quinlan et al., 2005). Finally, the completion of cytokinesis involves the membrane fusion machinery that bridges the space remaining after full ingression of the contractile ring (Fielding et al., 2005; Gromley et al., 2005; Low et al., 2003). In mouse oocytes, the asymmetric partitioning of the cytoplasm allows most of the maternal stores to be retained in the oocyte, a vital property for further embryo development. Immature mouse oocytes (oocytes at the Germinal Vesicle, GV, stage) are arrested in the ovaries in prophase I. After meiosis resumption, which is evidenced by Germinal Vesicle BreakDown (GVBD), the first meiotic spindle forms in the center of the oocyte and reaches the cortex at the end of the first M-phase (Verlhac et al., 2000). It has been reported that in mouse oocytes, different events of meiotic maturation are dependent on actin filaments. Indeed the first meiotic spindle migrates to the cortex in an actin-dependent manner. Moreover, when chromosomes come close to the cortex after spindle migration, they induce a cortical differentiation, which restricts the progression of the cleavage furrow and is materialized by enrichment of actin filaments and reduction of microvilli (Longo and Chen, 1985; Maro et al., 1984, 1986; Verlhac et al., 2000). Eventually, when oocytes are incubated in a medium containing cytochalasin D, a microfilament inhibitor, meiotic spindles are functional (they can segregate their chromosomes) but cytokinesis does not occur and the first polar body is not extruded (Kubiak et al., 1991; Verlhac et al., 2000). Therefore, the coordination of these different events (spindle migration, restriction of the cleavage furrow and cytokinesis) is essential for the success of the first meiotic division. Formin-2 is an FH2 containing protein that is expressed in the brain, the spinal cord and in oocytes. It has been reported that formin-2-deficient oocytes ($fmn2^{-/-}$) do not extrude a first polar body and harbor chromosomes which remain most of the time centrally located, suggesting that the first meiotic spindle does not migrate to the cortex in these oocytes (Leader et al., 2002). From observations on fixed material, it has been concluded that Formin-2 would control progression from meiosis I to meiosis II and spindle migration. In mouse oocytes, first meiotic spindle migration to the cortex is independent of microtubules and depends only on actin filaments (Verlhac et al., 2000). In yeast, the actin and microtubule network have intricate relationships. Formins influence the microtubule network as an indirect consequence of their action on actin cables (Wallar and Alberts, 2003). In mammalian cells, the Formin protein, mDia1, localizes on the mitotic spindle (Kato et al., 2001) and mDia1 and mDia2 can induce the stabilization of a subset of microtubules (Palazzo et al., 2001). Yet, the first analysis of the formin-2 knock-out is reminiscent of treatment of mouse oocytes with cytochalasin D. It was important to determine if Formin-2 is involved in segregation of homologous chromosomes in mouse oocytes and how an actin regulator could control cell cycle progression. Thus we decided to follow $fmn2^{-/-}$ oocytes by video microscopy. We show here that Formin-2 controls first meiotic spindle migration to the cortex but not chromosome congression or segregation. Moreover, $fmn2^{-/-}$ oocytes fail to extrude the first polar body. However, cortical differentiation and formation of the central spindle still occurs in these oocytes.

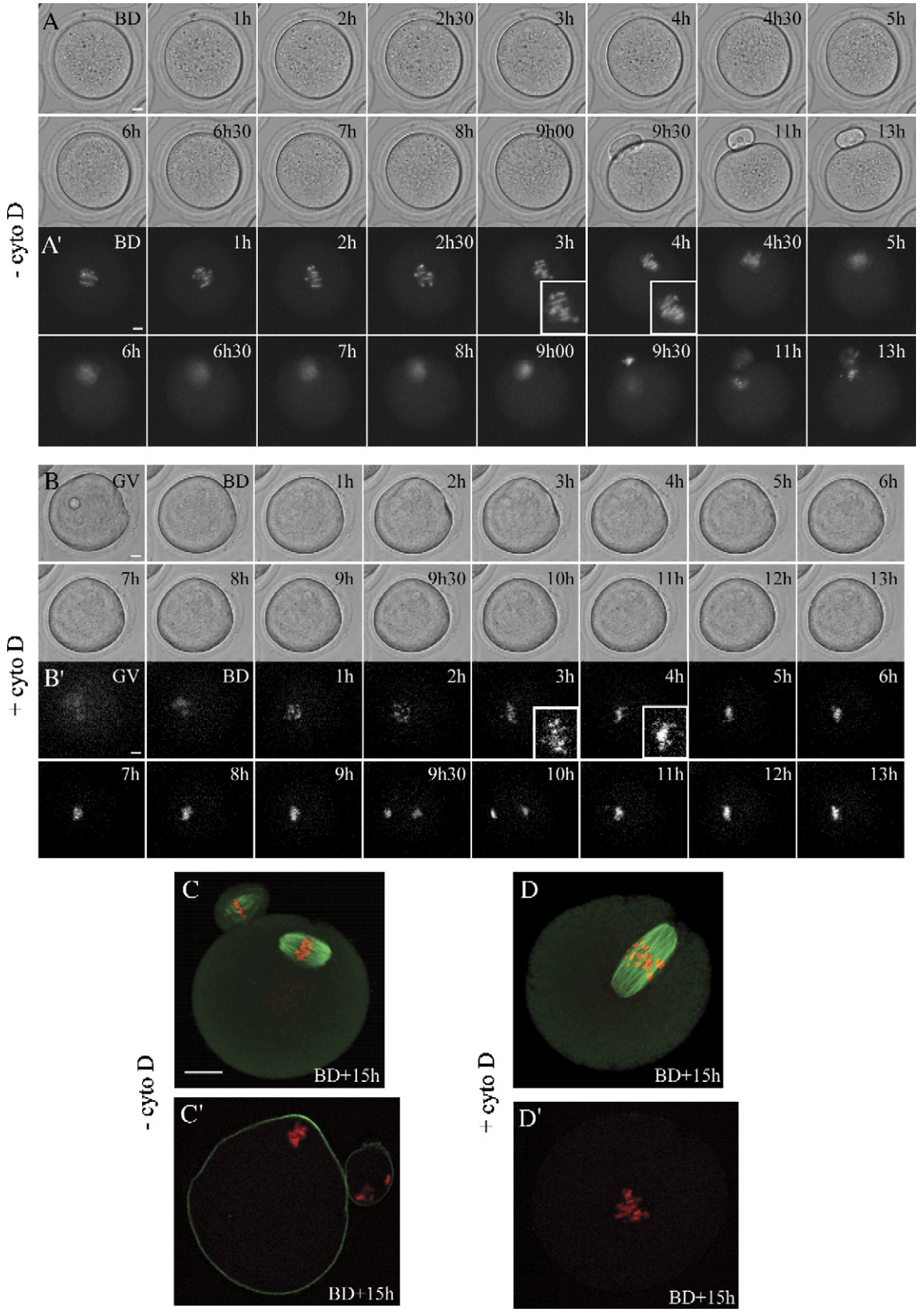
Results

Actin filaments are required for spindle migration and for cytokinesis, but not for cell cycle progression through anaphase

To examine more precisely the different actin-dependent events of meiotic maturation we followed cytochalasin-D-treated oocytes by video microscopy. We first checked that the drug efficiently disrupted cortical actin during the whole time course of meiotic maturation by staining oocytes that had undergone meiotic maturation in the drug with phalloidin–TRITC. As shown on Fig. 1C′, non-treated MII arrested oocytes harbor a cortical actin staining and a local actin enrichment surrounding chromosomes (i.e., cortical differentiation), while no signal is detected in cytochalasin-D-treated oocytes (Fig. 1D′). Thus, the drug efficiently disrupts actin filaments and is stable throughout meiotic maturation. As was observed previously, treated oocytes do not extrude a first polar body (Fig. 1B). By following chromosomes in real time, we show here that cytochalasin D does not prevent chromosomes congression or anaphase but prevents chromosomes migration to the cortex (Fig. 1B′). Moreover, anaphase precedes cytokinesis and first polar body extrusion in control oocytes, but in cytochalasin-D-treated oocytes, cytokinesis is inhibited (Figs. 1A, B). Thus, approximately 3 h after anaphase onset, chromosomes reaggregate and reorganize into a broad and equatorial metaphase plate. Control oocytes observed 15 h after GVBD (Fig. 1C) showed typical barrel-shaped MII spindles with chromosomes aligned on the metaphase plate while cytochalasin-D-treated oocytes showed longer barrel-shaped spindles (containing twice the amount of DNA) with a broad metaphase plate and sometimes mis-aligned chromosomes (Fig. 1D). Thus oocytes treated with cytochalasin D do not extrude a first polar body but are arrested in metaphase II as evidenced by homologous chromosome segregation and MII spindle formation. These data confirm that actin filaments are required for spindle migration to the cortex of the oocyte and for cytokinesis, but not for cell cycle progression through anaphase.

Formin-2 is required for cytokinesis completion

Next, we followed fmn2$^{-/-}$ and fmn2$^{+/-}$ oocytes by videomicroscopy during meiotic maturation. As shown in Fig. 2, 97% of fmn2$^{+/-}$ behaves exactly like wild type oocytes and cleave asymmetrically to extrude the first polar body. 78% of fmn2$^{-/-}$ oocytes show only a cortical contraction (arrow) and do not extrude the first polar body, 15% of fmn2$^{-/-}$ oocytes



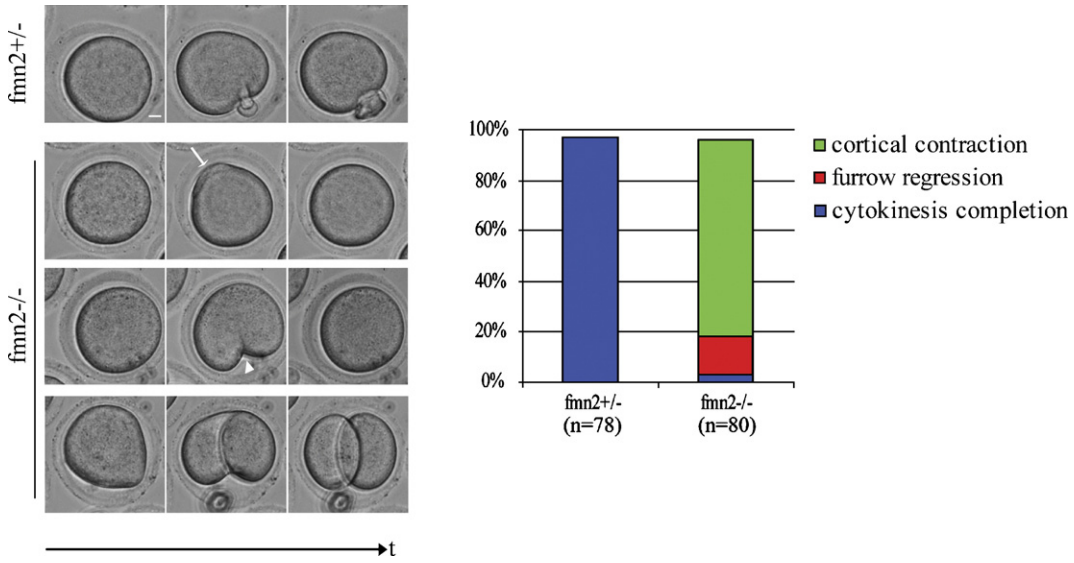


Fig. 2. Formin-2 is required for the completion of cytokinesis. Time-lapse microscopy of phase contrast before, during and after cytokinesis timing in control fmn2$^{+/-}$ oocytes ($n=78$) or fmn2$^{-/-}$ oocytes ($n=80$). The different populations (cortical contraction (arrow), furrow regression (arrowhead), cytokinesis completion) of oocytes are depicted (left) and quantified (right). The scale bar represents 10 μm.

display a clear furrow ingression (arrowhead) but eventually cytokinesis fails and the furrow regresses. Only 3% of fmn2$^{-/-}$ oocytes completed cytokinesis. However, these oocytes do not extrude a polar body, but rather cleave symmetrically. Thus, in most of the observed oocytes, Formin-2 is required for cytokinesis. However, in 18% of fmn2$^{-/-}$ oocytes, a cleavage furrow forms which most of the time regresses before cytokinesis completion.

Formin-2 is required for spindle migration but not for homologous chromosomes segregation and spindle formation

To figure out at what stage of the cell cycle the fmn2$^{-/-}$ oocytes are arrested, we followed Hoechst-treated oocytes by videomicroscopy. As shown on Fig. 3, the anaphase onset, which corresponds to homologous chromosome segregation, took place with the same timing both in fmn2$^{+/-}$ (8 h 25±54 min) and fmn2$^{-/-}$ (8 h 45±52 min) oocytes (Figs. 3A′, B′ and Movie 1). Moreover, we did not observe lost chromosomes in fmn2$^{-/-}$ oocytes, which suggests that chromosomes congression was not affected. However, while heterozygous oocytes extruded their first polar body, homozygous deficient oocytes did not (Figs. 3A, B). In these fmn2$^{-/-}$ oocytes, chromosomes reaggregated rapidly after anaphase onset and reorganized into a broad metaphase plate. We followed, *in vivo*, the movement of the chromosomes in these oocytes (Fig. 3C). While in fmn2$^{+/-}$ oocytes, migration of the chromosomes (and of the spindle) to the cortex of the oocyte begins around 3 h after GVBD, chromosomes remain centrally located in fmn2$^{-/-}$ oocytes (Fig. 3D).

By following microtubules in tubulin–GFP injected fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes, we also observed directly that the first meiotic spindle does not migrate to the cortex in fmn2$^{-/-}$ oocytes as opposed to fmn2$^{+/-}$ oocytes. Indeed, in both cases a typically barrel-shaped MI spindle assembled in the center of the oocyte. In heterozygous oocytes, the spindle migrated towards the cortex, anaphase took place and the first polar body was extruded (Fig. 4A′and Movie 2). A metaphase II (MII) spindle rapidly reassembled around sister chromatids and remained stable during the MII arrest. Conversely in fmn2$^{-/-}$ oocytes, the spindle stayed more centrally located and anaphase took place without spindle migration. Approximately 1 h after anaphase onset, two bipolar structures assembled around each set of chromosomes, which then fused, into a unique MII spindle (Figs. 4B′, C′ and Movie 3). In fmn2$^{+/-}$ oocytes, fixed 15 h after GVBD, the MII spindle was barrel-shaped with chromosomes aligned on the metaphase plate (Fig. 4D). The spindle of fmn2$^{-/-}$ oocytes was generally longer (having twice the amount of DNA) with a broad metaphase plate and sometimes non-aligned chromosomes remaining at spindle poles (Fig. 4E, arrows). Thus oocytes from the fmn2$^{-/-}$ strain do not extrude a first polar body but are arrested in metaphase II

Fig. 1. Actin filaments are required for spindle migration and cytokinesis but not for homologous chromosome segregation. Time-lapse microscopy of phase contrast (A, B) and Hoechst stained chromosomes (A′, B′) of maturing oocytes from the OF1 strain either non-treated (−, $n=50$) or treated (+, $n=36$) with 1 μg/ml cytochalasin D. A magnification of the chromosomes area (white boxes) shows that chromosomes congression takes place normally in non-treated and treated oocytes. Times after GVBD (Germinal Vesicle BreakDown) are indicated in the upper right corner. The scale bars represent 10 μm. (C, D) Immunofluorescent images showing the microtubules in green and the chromosomes in red of a control non-treated oocyte (C, $n=30$) and oocyte treated with 1 μg/ml cytochalasin D (D, $n=30$). The same PMT settings were used for confocal acquisitions in panels C′ and D′. (C′, D′) Phalloidin–TRITC staining showing cortical actin in green and the chromosomes in red of a control non-treated oocyte (C′, $n=20$) and oocyte treated with 1 μg/ml cytochalasin D (D′, $n=20$). Oocytes were observed 15 h after GVBD. The scale bar represents 20 μm (GV, Germinal Vesicle; BD, Germinal Vesicle BreakDown).

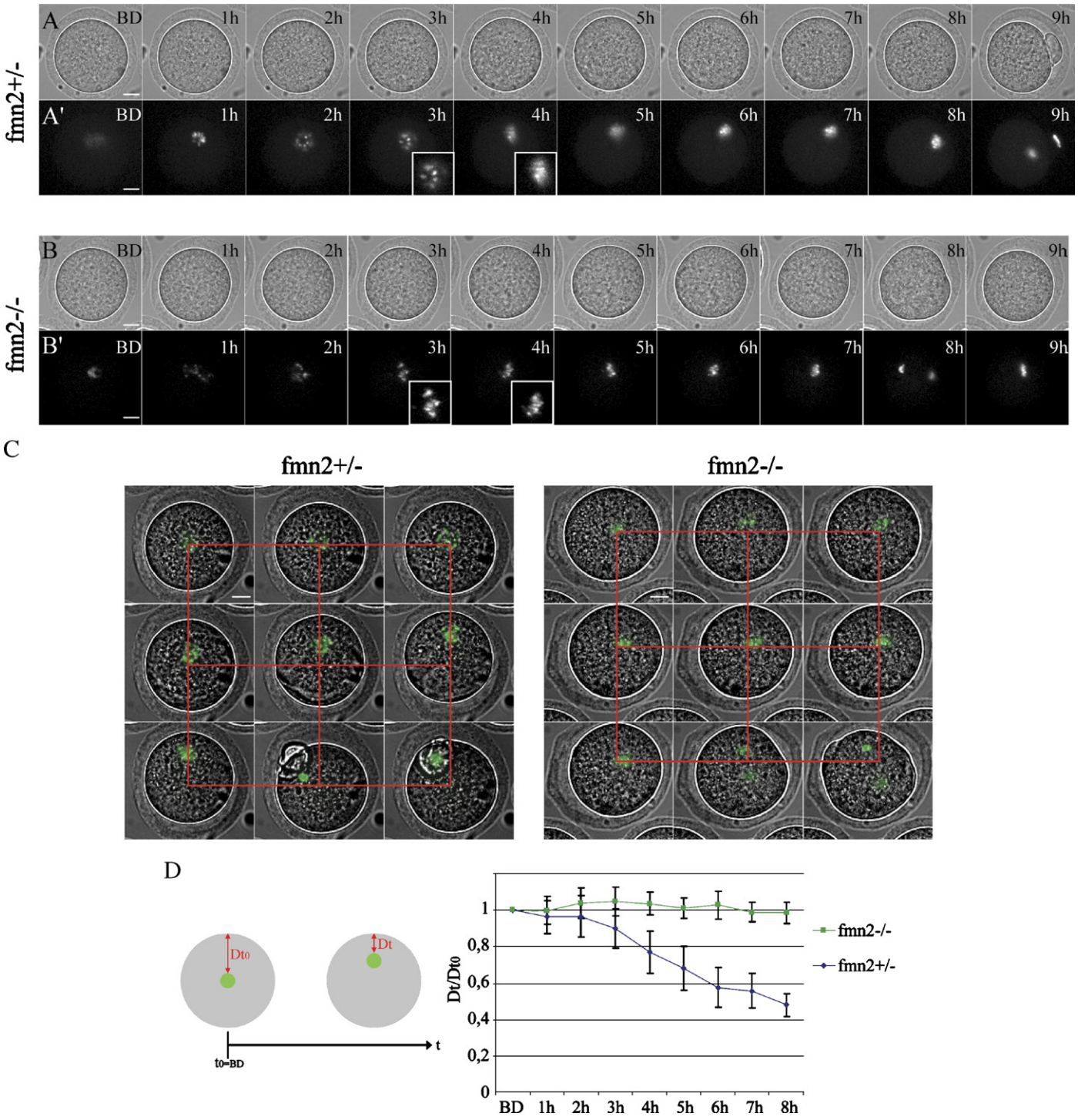


Fig. 3. Formin-2 is required for spindle migration but not for homologous chromosome segregation. Time-lapse microscopy of phase contrast (A, B) and Hoechst stained chromosomes (A′, B′) in control fmn2$^{+/-}$ oocytes (n=77) or fmn2$^{-/-}$ oocytes (n=77). Times after GVBD are indicated in the upper right corner. A magnification of the chromosomes area (white boxes) shows that chromosomes congression takes place normally in fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes. Chromosomes movement was followed during meiotic maturation (C, 1 frame/h). Red lines show the position of the chromosomes at the beginning of the experiment. The shorter distance between chromosomes and the cortex was measured in 15 fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes at each time point (Dt) and divided by the distance to the cortex at the beginning of the experiment (Dt0). The ratio Dt/Dt0 was plotted against time. The scale bars represent 10 μm (BD, Germinal Vesicle BreakDown).

as evidenced by homologous chromosome segregation and MII spindle formation. Our results show that both cytochalasin-D-treated oocytes and fmn2$^{-/-}$ oocytes display functional meiotic spindles, able to assemble, segregate chromosomes and reassemble with normal kinetics. Eventually, our data show that, differently from previous observations (Leader et al., 2002), Formin-2 is not required for homologous chromosome segregation.

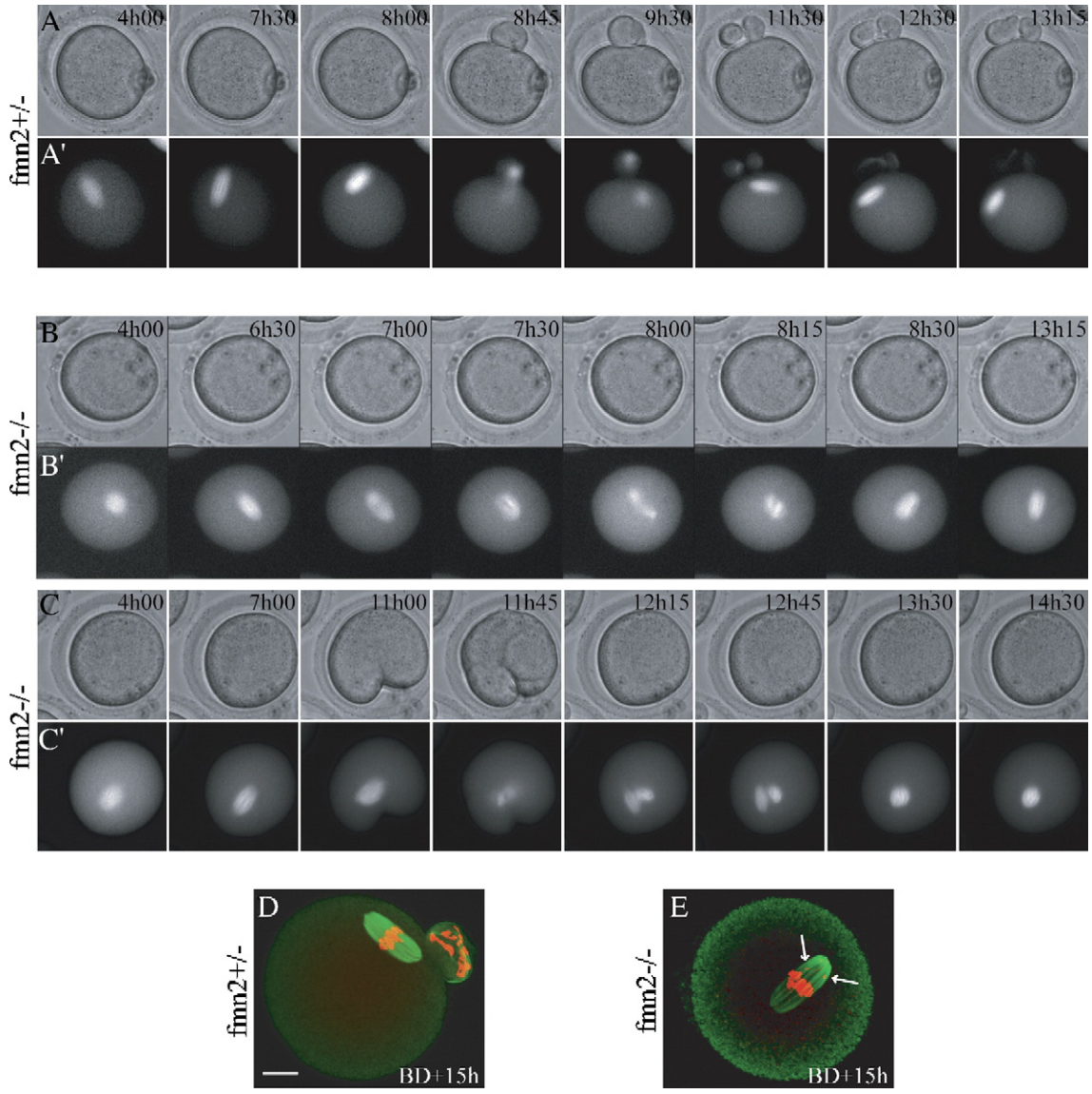


Fig. 4. Two bipolar structures fuse into a unique metaphase II spindle in fmn2$^{-/-}$ oocytes. Time-lapse microscopy of phase contrast (A, B, C) and tubulin–GFP RNA injected (A′, B′, C′) control fmn2$^{+/-}$ (A, A′, n=22) or fmn2$^{-/-}$ (B, B′, C, C′, n=23) oocytes. Times after GVBD are indicated in the upper right corner. (D, E) Immunofluorescent images showing the microtubules in green and the chromosomes in red of control fmn2$^{+/-}$ oocyte (D, n=30) and fmn2$^{-/-}$ oocytes (E, n=30), some chromosomes remain unaligned at the spindle pole (arrows). Oocytes were observed 15 h after GVBD. The scale bar represents 20 μm (BD, Germinal Vesicle Breakdown).

Since first meiotic spindle migration requires actin filaments and, as we show here, Formin-2, we could hypothesize that straight actin cables are being formed around the time of spindle migration connecting the chromosomes to the cortex (Maro and Verlhac, 2002). Using anti-actin antibodies or phalloidin coupled to FITC, such types of cables have never been observed in this model (Maro et al., 1984; Simerly et al., 1998). However, the cortex of mouse oocytes is so enriched in microfilaments that they could titrate out the anti-actin antibody or the phalloidin–FITC. Therefore, we attempted to visualize these potential cables by injecting actin–GFP encoding RNA into oocytes. We were not able to visualize such filaments (data not shown) suggesting that there may not exist real actin cables, as the ones present in yeast, but rather thin filaments connecting the chromosomes to the cortex.

Formin-2 is not required for chromosome induced cortical differentiation

Next, we decided to asses the role of Formin-2 in cytokinesis. When the chromosomes come close to the cortex after spindle migration, they induce a cortical differentiation, which would restrict the progression of the cleavage furrow, ensuring that the oocyte cytoplasm is not cleaved in halves (Maro et al., 1986). As we show before, chromosomes in fmn2$^{-/-}$ oocytes do not move to the cortex. Nevertheless, we observed that in 53% of fmn2$^{-/-}$ oocytes, chromosomes were naturally off-center, positioned close to the cortex of the oocyte. Indeed the position of chromosomes in oocytes is linked to the position of the germinal vesicle, which is not always centrally located at the time of GVBD (Verlhac et al., 2000). Thus we reasoned that

if cortical differentiation can occur in fmn2$^{-/-}$ oocytes, we should be able to detect it in these oocytes.

Staining with concanavalin A coupled to FITC (ConA-FITC) allows to follow the distribution of microvilli and can be used as a marker of cortical polarization. In fmn2$^{+/-}$ oocytes, a microvilli-free cortical domain surrounded chromosomes at the end of metaphase I and the polar body was totally devoid of microvilli just after anaphase (Figs. 5A, B). In fmn2$^{-/-}$ oocytes, when the chromosomes were in proximity to the cortex they were also surrounded by a microvilli-free domain (Fig. 5C). Moreover, at the time of anaphase, we could observe an enrichment of microvilli at the site of the attempted cleavage furrow in the fmn2$^{-/-}$ oocytes (Figs. 5B, D′, arrows). However, fmn2$^{-/-}$ oocytes with chromosomes more centrally located did not show signs of cortical polarization before anaphase (Fig. 5C′) as was also the case for oocytes without furrow ingression during anaphase (Fig. 5D). These results show that fmn2$^{-/-}$ oocytes can reorganize their cortex in response to the presence of chromosomes only if the chromosomes are already located at the periphery. Thus the lack of polar body extrusion in fmn2$^{-/-}$ oocytes is only due to a cytokinesis failure and not to a loss of cortical differentiation. These observations also show that cortical differentiation in mouse oocytes is a Formin-2-independent process.

Formin-2 is required for late steps of cytokinesis but not for central spindle formation

To determine more precisely the step of cytokinesis which is defective in fmn2$^{-/-}$ oocytes we tested if the central spindle is able to form. For this, we performed immunostaining of Survivin, a protein of the central spindle machinery. It is one of the chromosome passenger protein which localizes to inner centromeres from G2 until the metaphase-to-anaphase transition in mammalian cells and then translocates to the central spindle during anaphase and eventually localizes to the midbody during telophase (Adams et al., 2001; Vagnarelli and Earnshaw, 2004). In fmn2$^{+/-}$ oocytes, Survivin localizes on the chromosomes during late metaphase I, then it translocates to the central spindle during anaphase and to the midbody during telophase (Figs. 6A, B, C). Thus it behaves exactly like in somatic cells. We reasoned that if the central spindle is able to form in fmn2$^{-/-}$ oocytes, then we should be able to detect the corresponding Survivin staining. In these oocytes, Survivin localizes first on the chromosomes and then translocates to the central spindle during anaphase (Figs. 6D, E). Thus Survivin behaves similarly in fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes, except for the midbody staining due to the cytokinesis failure. This result strongly suggests that a lack of central spindle formation cannot account for the cytokinesis failure in fmn2$^{-/-}$ oocytes.

Next, we decided to check if active myosin II is properly localized in fmn2$^{-/-}$ oocytes. Myosin II is composed of a heavy and light chain and is activated by phosphorylation of its regulatory light chain (MLC) at Ser19/Thr18 (Moussavi et al., 1993). MLC phosphorylation controls both the assembly of the actomyosin contractile ring and its contractility (Jordan and Karess, 1997; Matsumura, 2005; Straight et al., 2003). Thus, we performed immunostaining of phospho-myosin II (Ser19). In fmn2$^{+/-}$ oocytes, phospho-myosin II is not localized during metaphase I, then during telophase it is enriched at the base of the forming polar body, where the cleavage furrow progresses (Figs. 7A, B). In fmn2$^{-/-}$ oocytes, phospho-myosin II is also not localized during metaphase I (Fig. 7C). As shown in Fig. 2, 18% of fmn2$^{-/-}$ oocytes are able to form a furrow at anaphase. Consistently, we found that in these oocytes, phospho-myosin II is enriched at the site of the forming furrow (Fig. 7E). However, 78% of fmn2$^{-/-}$ oocytes never form a furrow. In these oocytes, phospho-myosin II is mislocalized and enriched between the two sets of chromosomes at anaphase, at a structure that could correspond to the central spindle (Fig. 7D). This result shows that cytokinesis failure in fmn2$^{-/-}$ oocytes is due to phospho-myosin II mislocalization.

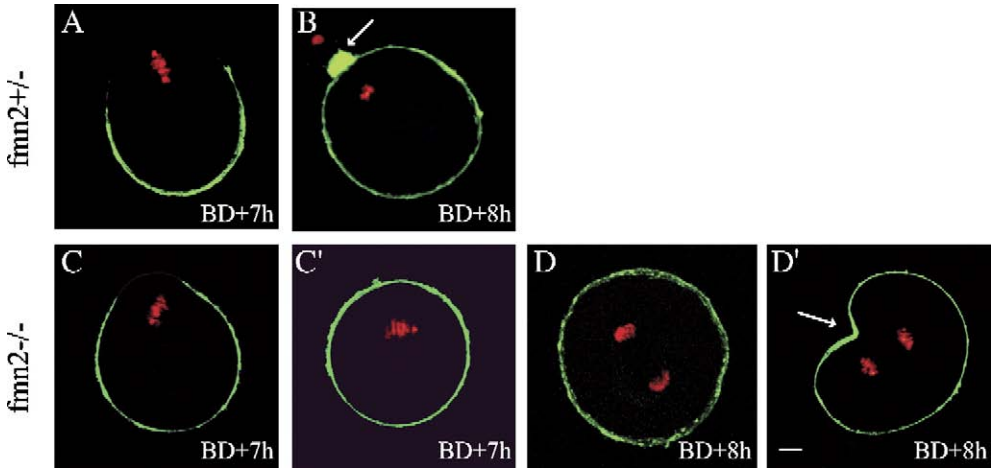


Fig. 5. Cortical differentiation in fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes. (A, B) fmn2$^{+/-}$ oocytes (n=46) and (C, C′, D, D′) fmn2$^{-/-}$ oocytes (n=70). (A, C, C′) oocytes fixed 7 h after GVBD showing either a microvilli-free domain surrounding chromosomes (A, C) or evenly distributed microvilli with centrally located chromosomes (C′). (B, D, D′) oocytes fixed 8 h after GVBD (at anaphase) showing the lack of microvilli on the polar body and the enrichment (arrow) at the base of it (B); the same enrichment is seen on the attempted cleavage furrow (arrow) of fmn2$^{-/-}$ oocyte (D′). The microvilli were stained with ConA-FITC (green) and the chromosomes with propidium iodide (red). The scale bar represents 10 μm (BD, Germinal Vesicle BreakDown).

Discussion

Formins (or FH proteins) are a family of ubiquitous, highly conserved multidomain proteins involved in a growing range of actin-based processes. Formin-2 was identified as an FH protein that is expressed in the brain, the spinal cord and in oocytes. Formin-2 knock-out mice suffer from reduced fertility, owing to defective meiotic maturation of the oocyte. fmn2$^{-/-}$ oocytes do not extrude a first polar body and harbor chromosomes which remain most of the time centrally located, suggesting that the first meiotic spindle does not migrate to the cortex in these oocytes. From these observations on fixed material, it has been concluded that Formin-2 would control progression from meiosis I to meiosis II and spindle migration (Leader et al., 2002). Thus it was important to determine if Formin-2 is involved in segregation of homologous chromosomes in mouse oocytes and how an actin regulator could control cell cycle progression. By following fmn2$^{-/-}$ oocytes by video microscopy, we show here that Formin-2 controls first meiotic spindle migration to the cortex and cytokinesis but not chromosome segregation. Thus, in 97% of the cases, fmn2$^{-/-}$ oocytes fail to extrude the first polar body but arrest in metaphase II.

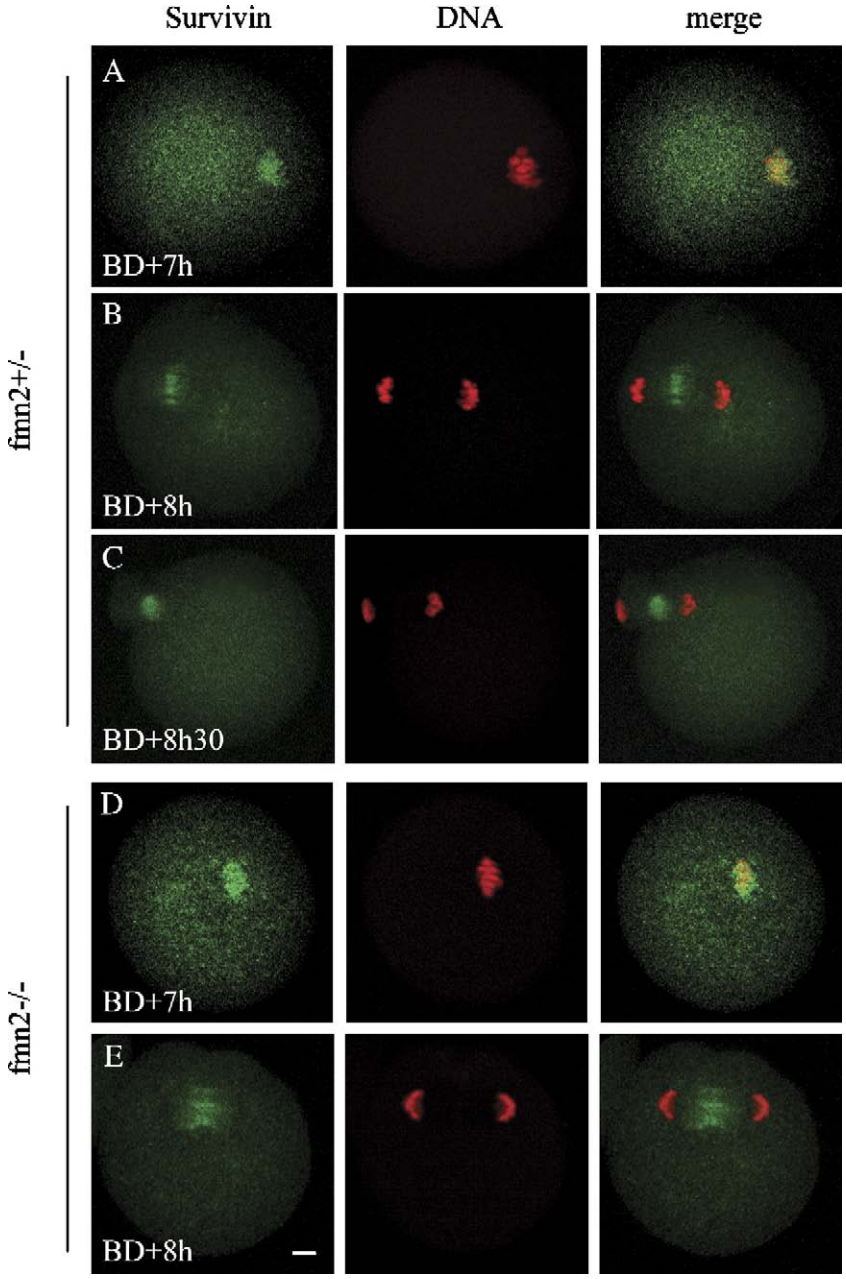


Fig. 6. Survivin localizes to the central spindle in fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes during anaphase. (A, B, C) fmn2$^{+/-}$ oocytes (n=20) and (D, E) fmn2$^{-/-}$ oocytes (n=16). (A, D) Oocytes fixed 7 h after GVBD showing the chromosomal localization of Survivin during late metaphase I. (B, E) oocytes fixed 8 h after GVBD showing that Survivin localizes on the central spindle during anaphase. (C) Oocytes fixed 8 h 30 min after GVBD showing the midbody localization of Survivin during telophase in fmn2$^{+/-}$ oocytes. Survivin staining in green, DNA in red. The scale bar represents 10 μm (BD, Germinal Vesicle BreakDown).

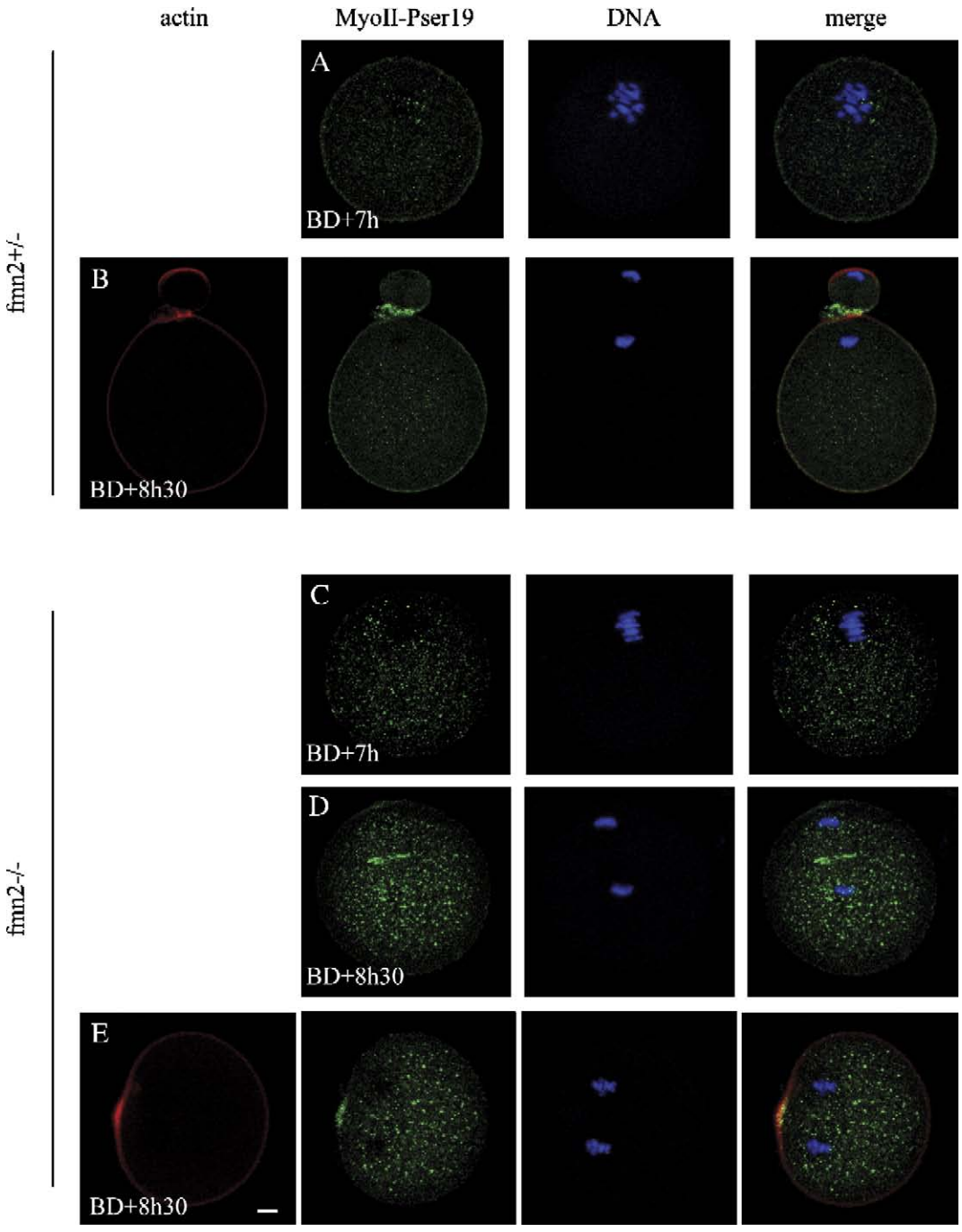


Fig. 7. Phospho-myosin II localization in fmn2$^{+/-}$ and fmn2$^{-/-}$ oocytes during anaphase. (A, B) fmn2$^{+/-}$ oocytes (n=30) and (C, D, E) fmn2$^{-/-}$ oocytes (n=30). (A, C) Oocytes fixed 7 h after GVBD showing that phospho-myosin II is not localized during metaphase I. (B, E) Oocytes fixed 8 h 30 min after GVBD showing that phospho-myosin II localizes at the cleavage furrow during anaphase in fmn2$^{+/-}$ oocytes and in fmn2$^{-/-}$ oocytes that initiate a furrow. (D) Oocytes fixed 8 h 30 min after GVBD showing that phospho-myosin II is mislocalized and enriched between the two sets of chromosomes in fmn2$^{-/-}$ oocytes that do not form a furrow. Phospho-myosin II staining in green, DNA in blue, actin in red. The scale bar represents 10 μm (BD, Germinal Vesicle BreakDown).

F-actin during meiotic maturation

By following cytochalasin-D-treated oocytes by video microscopy, we show here that F-actin is not required for chromosome congression at the metaphase plate in mouse oocytes (Fig. 1B). A recent study has shown that the actin network drives chromosome congression in starfish oocytes (Lenart et al., 2005). In these oocytes, the nucleus is 80 μm in diameter and the capture range of chromosomes by microtubule asters is around 30 μm. Thus in this model, microtubule asters are too short to capture chromosomes and another cytoskeleton component is involved in delivering chromosomes to the microtubule spindle. However, in mouse oocytes, the nucleus is 30 μm in diameter which is in the capture range of microtubules. This could explain the difference in F-actin requirement for chromosome congression between these two

experimental models. Moreover, cytochalasin-D-treated oocytes are able to segregate chromosomes but they are defective for spindle migration to the cortex and for cytokinesis. Thus, they do not extrude a first polar body and remain arrested at metaphase II with twice the amount of DNA in the oocyte.

Formin-2 before cytokinesis in mouse oocytes

By following fmn2$^{-/-}$ oocytes by video microscopy, we confirm that they are defective for meiosis I spindle migration under the cortex of the oocyte (Leader et al., 2002) (Fig. 3). Formins are straight actin filaments nucleator, thus it is tempting to speculate that spindle migration in mouse oocytes would entail Formin-2 nucleated straight actin filaments. Moreover, it has been shown, by antibody injection, in mouse oocytes that myosin IIA is involved in the first meiotic spindle migration to the cortex (Simerly et al., 1998). Myosin II is known to interact with straight actin filaments during cytokinesis, thus it could also allow the spindle movement along these potentially straight filaments. However, by using anti-actin antibodies or phalloidin coupled to FITC, such types of filaments have never been observed in this model (Maro et al., 1984; Simerly et al., 1998). Moreover, we were not able to visualize such filaments (data not shown) by injecting actin–GFP encoding RNA into oocytes, thus we can only hypothesize their existence.

However, in contradiction to what was previously assumed, based on observations of fixed cells, fmn2$^{-/-}$ oocytes perform anaphase and are able to segregate homologous chromosomes which reaggregate rapidly after anaphase onset and reorganized into a broad metaphase plate. Thus these oocytes are arrested in metaphase II demonstrating that Formin-2 is not involved in cell cycle progression in mouse oocytes.

Formin-2 during cytokinesis in mouse oocytes

Fmn2$^{-/-}$ oocytes are arrested in metaphase II but only 3% of these oocytes completed cytokinesis while 93% of oocytes harbored cytokinesis failure. This is reminiscent of cytochalasin-D-treated oocytes. However, as shown on Figs. 5 and 6, this is not due to a lack of cortical differentiation nor to a defect in central spindle formation. This is more probably due to a defect in the very late steps of cytokinesis rather than to a signaling problem. Indeed in 53% of fmn2$^{-/-}$ oocytes, the spindle forms relatively close to the cortex allowing cortical differentiation, yet only 3% of oocytes succeed in full cytokinesis. This suggests that Formin-2 is directly involved in cytokinesis. While in cytochalasin-D-treated oocytes the cortex displays no contraction at the time of anaphase, in 78% of fmn2$^{-/-}$ oocytes, a cortical contraction or protrusion is clearly visible at this time, and in 15% of oocytes, a furrow ingresses but eventually cytokinesis fails (Fig. 2). As shown on Fig. 1D′, in cytochalasin-D-treated oocytes, the cortical actin is not detectable after phalloidin–TRITC staining. This is not the case in fmn2$^{-/-}$ oocytes (Fig. 7E). We can therefore hypothesize that, in mouse oocytes the cortical actin is composed of Arp2/3 nucleated branched filaments (Evangelista et al., 2003). Moreover, Arp2/3 is known to be involved in cell protrusion where a filament pushes the cell surface as it elongates (Zigmond, 2004). Thus Arp2/3 nucleated filaments could account for the cortical contraction or protrusion which occurs at anaphase in 78% of fmn2$^{-/-}$ oocytes.

Another striking observation is that in 18% of fmn2$^{-/-}$ oocytes a division furrow forms but most of the time regresses before cytokinesis completion. Indeed, the division furrow depends on the presence of Formin nucleated straight actin filaments and on myosin II contractile activity. It is known that once polymerized, actin filaments produced by Arp2/3 or Formins can be identical. Indeed, Formin nucleated filaments *in vivo* are often bundled, but because branches created by Arp2/3 do not persist, Arp2/3 nucleated filaments can also make bundles (Zigmond, 2004). Therefore, their function may shift and merge. Thus, even in the absence of Formin-2 in the oocyte, straight actin filaments could be produced by unbranching Arp2/3 nucleated filaments, allowing myosin II to exert its contractile activity and to form a furrow. This could explain why 18% of fmn2$^{-/-}$ oocytes display a clear furrow ingression.

However, only 3% of fmn2$^{-/-}$ oocytes completed cytokinesis. It was recently shown that distinct pathways control the recruitment and maintenance of myosin II at the division furrow in somatic cells (Dean et al., 2005). Indeed, the initial localization of myosin II does not require F-actin, while the maintenance of myosin II and the contraction of the furrow require F-actin and Formin. In this study, it was suggested that the assembly of parallel, unbranched actin filaments, generated by Formin nucleation, is required for maintaining myosin II at the cleavage furrow. This could explain why in 18% of fmn2$^{-/-}$ oocytes, we observed active myosin II recruitment at the initiating furrow. However, in most of the cases, active myosin II was not localized on the cleavage furrow. Therefore, we can hypothesize that the cytokinesis defect in fmn2$^{-/-}$ oocytes is not due to a lack of myosin II activation but to the absence of Formin-2 nucleated filaments allowing to maintain it on the cleavage furrow to permit full cytokinesis. The mislocalization of active myosin II in most of fmn2$^{-/-}$ oocytes would be an indirect consequence of the lack of straight actin filaments at the cleavage furrow in the absence of Formin-2.

Formin-2 controls the spatial and temporal coordination of spindle migration and cytokinesis

By reinvestigating the phenotype of fmn2$^{-/-}$ oocytes we establish that these oocytes are defective for spindle migration to the cortex and for cytokinesis but not for cell cycle progression. Meiotic maturation in mouse oocytes is the succession of two asymmetric divisions. The asymmetric partitioning of the cytoplasm allows most of the maternal stores to be retained in the oocyte, a prerequisite of successful embryo development. This is ensured by positioning the meiosis I spindle under the cortex of the oocyte before cytokinesis. Spindle migration as well as cytokinesis is an actin dependant mechanism, and we show here that Formin-2 is also required. Thus, a single Formin (Formin-2) required for spindle migration and cytokinesis

ensures the spatial and temporal coordination of these two processes.

Materials and methods

Oocyte collection, culture and microinjection

Oocytes were collected from 11-week-old OF1, fmn2$^{+/-}$ and fmn2$^{-/-}$ female mice. The ovaries were placed directly into warmed (37°C) M2 medium supplemented with 4 mg/ml bovine serum albumin (sigma) and 50 μg/ml dibutiryl cyclic AMP (dbcAMP; sigma) (Whittingham, 1971) which prevent the resumption of meiotic maturation (GVBD, Germinal Vesicle BreakDown). Ovarian follicles were punctured to release the enclosed oocytes. Immature oocytes (GV, Germinal Vesicle) were collected and cultured further in M2+ BSA medium supplemented with 50 μg/ml dbcAMP under liquid paraffin oil at 37°C in an atmosphere of 5% CO2 in air. *In vitro* synthesized mRNAs were microinjected into the cytoplasm of GV oocytes using an Eppendorf Femtojet microinjector and borosilicate glass capillaries (Harvard apparatus). About 10 pl of pure mRNA solution was injected per oocyte. Injected oocytes were kept in M2+BSA medium supplemented with dbcAMP during 3 h to allow overexpression of the corresponding protein. The resumption of meiotic maturation (GVBD) was triggered by releasing the oocytes in a drug-free medium. Cytochalasin D (Sigma) is conserved as a 10 mg/ml stock solution in DMSO at −20°C and used at 1 μg/ml in M2+BSA medium.

Plasmid construction and in vitro transcription of synthetic RNA

The pRN3-β5-tubulin–GFP plasmid has been described (Verlhac et al., 2000) and was linearized with sfiI (Qbiogen). Capped mRNA were synthesized by using the mMessage mMachine T3 kit (Ambion) and extracted with the RNeasy Kit (Qiagen). mRNA were then resuspended in 40 μl DEPC water, aliquoted in 4 μl and stored at −80°C.

Video microscopy

Tubulin–GFP and Hoechst 33248 (5 ng/ml, Sigma) were visualized using a PL Fluotar 20×/0.5 objective lenses or a HC PL APO 20×/0.7 CS objective lenses (Leica) and a CCD camera (Micromax, Roper Scientific) under a computer-controlled video microscope (Leica DM IRBE) enclosed in a thermostatic chamber (Life Imaging Services). Exposure time was 300 ms every 15 min for tubulin–GFP and 100 ms every 15 min for Hoechst. The Metamorph software (Universal Imaging) and ImageJ (NIH) were used for movies analysis.

Immunofluorescence

The zona pellucida was removed using acidified (pH 2.5) Tyrode's medium. Oocytes were then centrifuged onto a glass coverslip coated with concanavalin A and fixed in 0.1% glutaraldehyde, 1% Triton X-100 in phosphate-buffered saline (PBS) for 10 min at 30°C. They were then permeabilized for 30 min at room temperature (RT) with 2% Triton X-100 in PBS and incubated 1 h at RT with a 1/100 solution of rat monoclonal antibody against tyrosinated α-tubulin (YL1/2). We used an FITC-conjugated anti-rat antibody (Miles) as a second layer. For Survivin and phospho-myosin II staining, oocytes were fixed in 3.7% formaldehyde for 30 min at 30°C and permeabilized in 0.25% Triton X-100 in PBS for 10 min at RT. They were then incubated 1 h at RT with a 1/150 solution of rabbit polyclonal antibody against Survivin (Novus, NB 500-201) or with a 1/250 solution of rabbit polyclonal antibody against Ser19-phospho myosin II light chain-2 (NEB, 3671). We used a cy3-conjugated anti-rabbit antibody (Jackson) as a second layer. For microvilli staining, oocytes were fixed in 1% paraformaldehyde in PBS for 5 min at 30°C and incubated 30 s at RT in a 7 mg/ml solution of concanavalin A-FITC (Sigma). They were then post-fixed 30 min at 30°C in 3% paraformaldehyde in PBS. Chromatin was visualized using propidium iodide (Molecular Probes, 5 mg/ml in PBS). Samples were observed with a Leica TCS-SP confocal microscope using a Plan APO 40×/1.25. Z-series were performed with one image per micrometer and a maximum projection of fifteen Z planes is shown for microtubule and Survivin staining, while six planes were projected for phospho-myosin II and microvilli staining. The Metamorph software (Universal Imaging) and ImageJ (NIH) were used for image analysis and treatment.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.08.044.

References

Adams, R.R., Carmena, M., Earnshaw, W.C., 2001. Chromosomal passengers and the (aurora) ABCs of mitosis. Trends Cell Biol. 11, 49–54.

Bonaccorsi, S., Giansanti, M.G., Gatti, M., 1998. Spindle self-organization and cytokinesis during male meiosis in asterless mutants of *Drosophila melanogaster*. J. Cell Biol. 142, 751–761.

Cao, L.G., Wang, Y.L., 1996. Signals from the spindle midzone are required for the stimulation of cytokinesis in cultured epithelial cells. Mol. Biol. Cell 7, 225–232.

Chang, F., Drubin, D., Nurse, P., 1997. cdc12p, a protein required for cytokinesis in fission yeast, is a component of the cell division ring and interacts with profilin. J. Cell Biol. 137, 169–182.

Dean, S.O., Rogers, S.L., Stuurman, N., Vale, R.D., Spudich, J.A., 2005. Distinct pathways control recruitment and maintenance of myosin II at the cleavage furrow during cytokinesis. Proc. Natl. Acad. Sci. U. S. A. 102, 13473–13478.

Evangelista, M., Pruyne, D., Amberg, D.C., Boone, C., Bretscher, A., 2002. Formins direct Arp2/3-independent actin filament assembly to polarize cell growth in yeast. Nat. Cell Biol. 4.

Evangelista, M., Zigmond, S., Boone, C., 2003. Formins: signaling effectors for assembly and polarization of actin filaments. J. Cell Sci. 116, 2603–2611.

Faix, J., Grosse, R., 2006. Staying in shape with formins. Dev. Cell 10, 693–706.

Fielding, A.B., Schonteich, E., Matheson, J., Wilson, G., Yu, X., Hickson, G.R., Srivastava, S., Baldwin, S.A., Prekeris, R., Gould, G.W., 2005. Rab11-FIP3 and FIP4 interact with Arf6 and the exocyst to control membrane traffic in cytokinesis. EMBO J. 24, 3389–3399.

Glotzer, M., 2005. The molecular requirements for cytokinesis. Science 307, 1735–1739.

Gromley, A., Yeaman, C., Rosa, J., Redick, S., Chen, C.T., Mirabelle, S., Guha, M., Sillibourne, J., Doxsey, S.J., 2005. Centriolin anchoring of exocyst and SNARE complexes at the midbody is required for secretory-vesicle-mediated abscission. Cell 123, 75–87.

Jordan, P., Karess, R., 1997. Myosin light chain-activating phosphorylation sites are required for oogenesis in *Drosophila*. J. Cell Biol. 139, 1805–1809.

Kato, T., Watanabe, N., Morishima, Y., Fujita, A., Ishizaki, T., Narumiya, S., 2001. Localization of a mammalian homolog of diaphanous, mDia1, to the mitotic spindle in HeLa cells. J. Cell Sci. 114, 775–784.

Kovar, D.R., Kuhn, J.R., Tichy, A.L., Pollard, T.D., 2003. The fission yeast cytokinesis formin Cdc12p is a barbed end actin filament capping protein gated by profilin. J. Cell Biol. 161, 875–887.

Kubiak, J., Paldi, A., Weber, M., Maro, B., 1991. Genetically identical parthenogenetic mouse embryos produced by inhibition of the first meiotic cleavage with cytochalasin D. Development 111, 763–769.

Leader, B., Lim, H., Carabatsos, M.J., Harrington, A., Ecsedy, J., Pellman, D., Maas, R., Leder, P., 2002. Formin-2, polyploidy, hypofertility and positioning of the meiotic spindle in mouse oocytes. Nat. Cell Biol. 4, 921–928.

Lenart, P., Bacher, C.P., Daigle, N., Hand, A.R., Eils, R., Terasaki, M., Ellenberg, J., 2005. A contractile nuclear actin network drives chromosome congression in oocytes. Nature 436, 812–818.

Longo, F.J., Chen, D.Y., 1985. Development of cortical polarity in mouse eggs: involvement of the meiotic apparatus. Dev. Biol. 107, 382–394.

Low, S.H., Li, X., Miura, M., Kudo, N., Quinones, B., Weimbs, T., 2003. Syntaxin 2 and endobrevin are required for the terminal step of cytokinesis in mammalian cells. Dev. Cell 4, 753–759.

Maro, B., Verlhac, M.H., 2002. Polar body formation: new rules for asymmetric divisions. Nat. Cell Biol. 4, E281–E283.

Maro, B., Johnson, M.H., Pickering, S.J., Flach, G., 1984. Changes in actin distribution during fertilization of the mouse egg. J. Embryol. Exp. Morphol. 81, 211–237.

Maro, B., Johnson, M.H., Webb, M., Flach, G., 1986. Mechanism of polar body formation in the mouse oocyte: an interaction between the chromosomes, the cytoskeleton and the plasma membrane. J. Embryol. Exp. Morphol. 92, 11–32.

Matsumura, F., 2005. Regulation of myosin II during cytokinesis in higher eukaryotes. Trends Cell Biol. 15, 371–377.

May, R.C., 2001. The Arp2/3 complex: a central regulator of the actin cytoskeleton. Cell. Mol. Life Sci. 58, 1607–1626.

Mishima, M., Pavicic, V., Gruneberg, U., Nigg, E.A., Glotzer, M., 2004. Cell cycle regulation of central spindle assembly. Nature 430, 908–913.

Moussavi, R.S., Kelley, C.A., Adelstein, R.S., 1993. Phosphorylation of vertebrate nonmuscle and smooth muscle myosin heavy chains and light chains. Mol. Cell. Biochem. 127–128, 219–227.

Palazzo, A.F., Cook, T.A., Alberts, A.S., Gundersen, G.G., 2001. mDia mediates Rho-regulated formation and orientation of stable microtubules. Nat. Cell Biol. 3, 723–729.

Pruyne, D., Evangelista, M., Yang, C., Bi, E., Zigmond, S., Bretscher, A., Boone, C., 2002. Role of formins in actin assembly: nucleation and barbed-end association. Science 297, 612–615.

Quinlan, M.E., Heuser, J.E., Kerkhoff, E., Mullins, R.D., 2005. Drosophila Spire is an actin nucleation factor. Nature 433, 382–388.

Rappaport, R., 1985. Repeated furrow formation from a single mitotic apparatus in cylindrical sand dollar eggs. J. Exp. Zool. 234, 167–171.

Simerly, C., Nowak, G., de Lanerolle, P., Schatten, G., 1998. Differential expression and functions of cortical myosin IIA and IIB isotypes during meiotic maturation, fertilization and mitosis in mouse oocytes and embryos. Mol. Biol. Cell 9, 2509–2525.

Straight, A.F., Cheung, A., Limouze, J., Chen, I., Westwood, N.J., Sellers, J.R., Mitchison, T.J., 2003. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. Science 299, 1743–1747.

Szollosi, D., Calarco, P., Donahue, R.P., 1972. Absence of centrioles in the first and second meiotic spindles of mouse oocytes. J. Cell Sci. 11, 521–541.

Vagnarelli, P., Earnshaw, W.C., 2004. Chromosomal passengers: the four-dimensional regulation of mitotic events. Chromosoma 113, 211–222.

Verlhac, M.H., Lefebvre, C., Guillaud, P., Rassinier, P., Maro, B., 2000. Asymmetric division in mouse oocytes: with or without Mos. Curr. Biol. 10, 1303–1306.

Wallar, B.J., Alberts, A.S., 2003. The formins: active scaffolds that remodel the cytoskeleton. Trends Cell Biol. 13, 435–446.

Whittingham, D.G., 1971. Culture of mouse ova. J. Reprod. Fertil. Suppl. 14, 7–21.

Zigmond, S.H., 2004. Formin-induced nucleation of actin filaments. Curr. Opin. Cell Biol. 16, 99–105.