A centriole- and RanGTP-independent spindle assembly pathway in meiosis I of vertebrate oocytes

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pinde formation is essential for stable inheritance of genetic material. Experiments in various systems indicate that Ran GTPase is crucial for meiotic and mitotic spindle assembly. Such an important role for Ran in chromatin-induced spindle assembly was initially demonstrated in Xenopus laevis egg extracts. However, the requirement of RanGTP in living meiotic cells has not been shown. In this study, we used a fluorescence resonance energy transfer probe to measure RanGTP-regulated release of importin β. A RanGTP-regulated gradient was established during meiosis I and was centered on chromosomes throughout mouse meiotic maturation. Manipulating levels of RanGTP in mice and X. laevis oocytes did not inhibit assembly of functional meiosis I spindles. However, meiosis II spindle assembly did not tolerate changes in the level of RanGTP in both species. These findings suggest that a mechanism common to vertebrates promotes meiosis I spindle formation in the absence of chromatin-induced microtubule production and centriole-based microtubule organizing centers.

Introduction

Understanding how microtubules (MTs) reorganize during the cell cycle to assemble into a bipolar spindle is a classic problem of cell biology. Mitotic and meiotic spindles are highly dynamic structures, which assemble around chromosomes or sister chromatids and distribute them into each daughter cell. Errors in spindle assembly lead to severe DNA damage and aneuploidies, responsible for various forms of cancer. Therefore, it is essential that bipolar spindle assembly occurs correctly. Two pathways cooperate to assemble bipolar spindles. One pathway involves centrosomes, which generate astral MTs that continuously search for chromosomes. This is the “search-and-capture” model, which was postulated by Kirschner and Mitchison (1986).

Accumulating evidence suggests that the small GTPase Ran is also a key player in the spatial control of spindle formation during the M phase (for reviews see Gruss and Vernos, 2004; Zheng, 2004; Ciciarello and Lavia, 2005). Production of RanGTP depends on the activity of the regulator of chromosome condensation (RCC1), Ran’s nucleotide exchange factor. RCC1 remains bound to chromosomes during the M phase. Thus, it was originally proposed that a high concentration of RanGTP around chromosomes acts as a local switch for spindle assembly (Carazo-Salas et al., 1999; Kalab et al., 1999). This hypothesis has been validated for spindles assembled in vitro and for those assembled in somatic cells. In these systems, higher levels of RanGTP have been detected near chromosomes than in regions distant from chromatin, as indicated by fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy techniques (Kalab et al., 2002, 2006; Li and Zheng, 2004).

Experiments in the cell-free system of Xenopus laevis initially demonstrated a central role for RanGTP in centrosome-dependent MT production and in chromatin-induced, centrosome-independent spindle assembly (Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999; Zhang et al., 1999). High levels of RanGTP stimulate the nucleating capacity of centrosomes but are not essential for basic centrosome nucleation activity. In contrast, chromatin-mediated MT formation depends entirely on the presence of RanGTP in the cell-free system.

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Abbreviations used in this paper: CSF, cytostatic factor; dbcAMP, dibutiryl cAMP; FRET, fluorescence resonance energy transfer; GV, germinal vesicle; GVBD, GV breakdown; MT, microtubule; MTOC, MT organizing center; RCC1, regulator of chromosome condensation.

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(Carazo-Salas et al., 1999). More recently, siRNA experiments and microinjections in living cells of Caenorhabditis elegans, Drosophila melanogaster, and humans demonstrated that the presence of RanGTP is required for essential functions in spindle formation in centrosome-containing systems (Nachury et al., 2001; Askjaer et al., 2002; Bamba et al., 2002; Kalab et al., 2006; Silverman-Gavrila and Wilde, 2006; Tulu et al., 2006).

Ran induces MT formation by releasing various spindle assembly factors, including NuMA, TPX2, and HURP, from the inhibitory effect of importins in the vicinity of chromosomes (Gruss et al., 2001; Nachury et al., 2001; Wiese et al., 2001; Koffa et al., 2006; Sillje et al., 2006; Wong and Fang, 2006).

Many studies have focused on elucidating the function of RanGTP in spindle assembly in meiotic X. laevis egg extracts. However, there is no in vivo evidence demonstrating the role of RanGTP in meiotic spindle formation in vertebrates. Meiotic spindle assembly in developing vertebrate oocytes occurs in the absence of centrioles (Szollosi et al., 1972; Huchon et al., 1981; Gard et al., 1995). During meiosis, two successive M phases occur without an intermediate S phase to produce haploid gametes. The first meiotic division is reductional with the segregation of homologous chromosomes. The second meiotic division is equational and resembles mitotic division. Cytostatic factor (CSF) then arrests vertebrate oocytes in metaphase II for many hours, until fertilization. In mouse and X. laevis oocytes, MTs nucleate around condensing chromosomes and spindles self-organize in the presence of multiple MT organizing centers (MTOCs). In mouse oocytes, chromosomes gather quickly on a broad metaphase plate through interactions of their arms and MTs. Kinetochore–MT interactions are established at the end of the first meiotic M phase (MI) only (Brunet et al., 1999). Therefore, the oocyte model system is useful for the study of acentrosomal spindle assembly and for the assessment of the role of the Ran pathway in meiosis.

We detected the accumulation of RanGTP around the chromosomes during all stages of mouse meiotic maturation with a previously described FRET-based probe for RanGTP-regulated release of importin β cargo molecules (Kalab et al., 2006). The overexpression of Ran mutants in mouse oocytes and the knock down of RCC1 in X. laevis oocytes led to assembly of functional meiosis I spindles in the presence of excess or low RanGTP levels. In contrast, meiosis II spindle assembly was strictly dependent on RanGTP levels in both species. In mouse oocytes, we show that meiosis II progression also depended on RanGTP levels. We demonstrate that there is a mechanism that promotes spindle formation in the absence of both chromatin-induced MT production and centriole-based MTOCs.

**Results**

**The mouse first meiotic spindle is assembled from asters of MTs nucleated distantly from the chromosomes**

We followed spindle formation during meiotic maturation in mouse oocytes with high temporal resolution. Mouse oocytes are transparent and ideal for time-lapse microscopy. Meiosis resumes spontaneously in mouse oocytes when they are removed from the ovaries. We initially maintained the oocytes in prophase I at the germinal vesicle (GV) stage in dibutyryl cAMP (dbcAMP)–supplemented medium and injected them with RNA encoding GFP-tagged tubulin (tubulin-GFP; Fig. 1). GV breakdown (GVBD) occurred ~2 h after release from the dbcAMP-containing medium (Table I). MTs were nucleated from asters that were distant from the chromosomes (Fig. 1 A).
These randomly distributed asters reorganized around chromosomes into a bipolar barrel-shaped structure ~2 h after GVBD. The MI spindle migrated along its long axis to the cortex of the oocyte (Verlhac et al., 2000a) and then anaphase occurred, and the first polar body was extruded ~9 h after GVBD. A metaphase II (MII) spindle rapidly assembled from remaining MTs around sister chromatids and remained stable during arrest (Fig. 1 B).

Ran and its effectors are differentially regulated in mouse and X. laevis oocytes

We investigated the potential role of the Ran system during meiosis by assessing the levels of Ran and RCC1 by Western blot analysis. Endogenous Ran and RCC1 were present in immature (GV) and mature (MII) oocyte extracts from mouse and X. laevis (Fig. 2). Although RCC1 levels were approximately two times higher in mouse MII oocyte extracts than in GV oocyte extracts (Fig. 2 A, right), this difference was much greater in X. laevis oocytes. In these oocytes, the amount of Ran, RanGAP, and RanBP1 were similar in immature and mature eggs, but the amount of RCC1 increased during meiosis resumption (Fig. 2 B). We biochemically analyzed meiosis in X. laevis and assayed the amounts of RCC1 during meiotic maturation of mouse oocytes. 30 immature (GV) or mature (MII) mouse oocytes were collected and immunoblotted using a monoclonal anti-Ran antibody and an anti-tubulin antibody. (B, left) RCC1 levels increase during meiosis in X. laevis oocytes, but RanGAP, RanBP1, and Ran levels remain constant. Oocyte lysates (GV) or egg lysates (MII) were immunoblotted using anti-RanGAP, anti-RCC1, anti-RanBP1, and anti-Ran antibodies. (Top right) Histone H1 kinase activity (arbitrary units) during meiotic maturation induced by progesterone. (bottom right) Increase of RCC1 amount during meiosis resumption in X. laevis. Two oocytes were used for each time point.

Rango probe indirectly reports RanGTP levels: the presence of a free cargo gradient is indicative of a RanGTP gradient. The local increase in the I_{FRET}/I_{CFP} ratio (Fig. 3, A and B) indicated the accumulation of free Rango and, hence, RanGTP near the chromosomes. The I_{FRET}/I_{CFP} ratio was consistently high in the same region, confirming the increase in FRET (unpublished data). The distribution of the RanGTP gradient in oocytes shows that RanGTP accumulated around chromosomes and decreased linearly with distance from the metaphase plate (Fig. 3 C). Thus, there was a broad (the size of an oocyte, ~100 μm), shallow RanGTP gradient in mouse oocytes. We followed RanGTP-induced release of importin β in live oocytes and indirectly detected the accumulation of RanGTP around chromosomes during all steps of mouse meiotic maturation (Fig. 3 D). The kymograph shows that the local accumulation of RanGTP strictly followed the movement of chromosomes to the cortex during MI (Fig. 3 D). This is the first evidence of such a RanGTP gradient occurring during the time course of division in a living organism.

A broad RanGTP-regulated importin β release gradient is established around chromosomes during mouse meiotic maturation

A RanGTP gradient has been demonstrated to surround the chromatin in X. laevis egg extracts and mitotic cells (Kalab et al., 2002, 2006; Caudron et al., 2005). We used a mouse system to determine whether a RanGTP gradient was present in live living cells, for example, the mouse oocyte (~100 μm in diameter). For this purpose, we used the previously described FRET probe, Rango, which has a high FRET signal when it is liberated from importin β by RanGTP (Kalab et al., 2006). An increase in the FRET signal (increase in I_{FRET}/I_{CFP} ratio) emitted by the}

| Type of oocyte | GVBD (h) | PB1 (h) | Large PB1 |
|---------------|----------|---------|-----------|
| Control       | 1 h 30 min | 91.6%/BD + 9 h | —         |
| (n = 33)      | (± 3 min)  | (± 39 min) |           |
| Wild type     | 1 h 24 min | 87.5%/BD + 9 h | —         |
| (n = 34)      | (± 6 min)  | (± 31 min) |           |
| T24N          | 1 h 30 min | 77%/BD + 9 h  | 13.8      |
| (n = 75)      | (± 18 min) | (± 17 min)  |           |
| Q69L          | 1 h 29 min | 84%/BD + 8 h 33 min | 58 |
| (n = 26)      | (± 8 min)  | (± 15 min)  |           |

Percentage of first polar body extrusion (%PB1). Percentage of abnormally large first polar body estimated visually (large PB1).

![Figure 2. Characterization of Ran and its regulators in mouse and X. laevis oocytes.](image-url)

Table 1. Timing of GVBD and of PB1 after dbcAMP release of oocytes injected with RNA encoding Ran mutants

| Type of oocyte | GVBD (h) | PB1 (h) | Large PB1 |
|---------------|----------|---------|-----------|
| Control       | 1 h 30 min | 91.6%/BD + 9 h | —         |
| (n = 33)      | (± 3 min)  | (± 39 min) |           |
| Wild type     | 1 h 24 min | 87.5%/BD + 9 h | —         |
| (n = 34)      | (± 6 min)  | (± 31 min) |           |
| T24N          | 1 h 30 min | 77%/BD + 9 h  | 13.8      |
| (n = 75)      | (± 18 min) | (± 17 min)  |           |
| Q69L          | 1 h 29 min | 84%/BD + 8 h 33 min | 58 |
| (n = 26)      | (± 8 min)  | (± 15 min)  |           |

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β occurred during meiosis resumption. Next, we examined the role of Ran. For this purpose, we manipulated the levels of RanGTP by injecting two mutant forms of Ran into GV-stage oocytes. RanT24N has much weaker affinity for guanine nucleotide than does wild-type Ran, binds RCC1, and is a potent inhibitor of Ran’s GDP–GTP exchange activity (Dasso et al., 1994). Thus, it inhibits the generation of RanGTP. RanQ69L is unable to hydrolyze GTP and remains locked in the GTP-bound state (Bischoff et al., 1994).

We first checked that our mutants were expressed continuously and at similar levels during mouse meiotic maturation (Fig. S1, A, B, and C, available at http://www.jcb.org/cgi/content/full/jcb.200605199/DC1). We directly measured the efficiency of our mutants in ovo during meiotic maturation by injecting the Rango probe with each mutant form of Ran. The mutants blocked nuclear import in GV oocytes (Fig. 3 E), as described previously (Palacios et al., 1996). From GV to MII, there were lower levels of liberated Rango in oocytes receiving RanT24N than in controls, consistent with inhibition of RanGTP production (Fig. 3 E, compare 2 and 5 with 1 and 4). There were substantially higher levels of RanGTP in the cytoplasm of oocytes receiving RanQ69L than in controls (Fig. 3 E, compare 3 and 6 with 1 and 4). In both cases, there was no detectable gradient of RanGTP in oocytes.

We then injected RNA encoding tubulin-GFP with each form of Ran into mouse oocytes and followed spindle formation by video microscopy and by visualization of immunofluorescence after oocyte fixation. Meiosis resumption and first polar body extrusion had similar kinetics in control and injected oocytes (Table I). This suggests that ectopic expression of the different forms of Ran did not affect the cell cycle per se. We observed the same succession of events with kinetics similar to those present in tubulin-GFP-injected oocytes (Fig. 1) in oocytes injected with wild-type Ran (RanWT; Fig. 4 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200605199/DC1). This shows that overexpression of RanWT did not affect meiotic...
maturation and spindle assembly, which is consistent with a previous report (Moore et al., 2002).

Asters appeared in the cytoplasm after GVBD, not exclusively around the chromosomes in mouse oocytes injected with RanQ69L, consistent with observations made in *X. laevis* egg extracts. These asters fused to form a bipolar spindle that was abnormally long (Fig. 4 B; Fig. 5, A and B; and Videos 2 and 3, available at http://www.jcb.org/cgi/content/full/jcb.200605199/DC1). 7 h after GVBD, spindles with a similar size as those in control oocytes (47% were within the range of the standard deviation for control spindles) or longer (averaging 30% longer) were present in 53% of the oocytes (Fig. 5, A, B, and C). The MI spindle that formed under these conditions migrated, and the pole closest to the cortex pushed into the cortex. Consistent with the formation of longer spindles in oocytes injected with RanQ69L, 58% of the oocytes extruded a large first polar body (Table I).

RanT24N injected into mouse oocytes delayed the establishment of MI spindle bipolarity by ~3 h in most oocytes (Fig. 4 C; Fig. 5, A, B, and C; and Video 4, available at http://www.jcb.org/cgi/content/full/jcb.200605199/DC1). During this time, MTs were unorganized around the chromosomes (Fig. 5, A, B, and C). Approximately 5 h after GVBD, two poles formed and a spindle with very small poles assembled around chromosomes. Despite a 3-h delay in spindle formation, anaphase took place normally and the first polar body was extruded.

Direct control of MTOC distribution by Ran GTPase did not cause the observed defects in MI spindle formation after overexpression of Ran variants. Oocytes injected with either mutant form of Ran had γ-tubulin foci randomly distributed.

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**Figure 4.** Excess or reduced levels of RanGTP induce spindle defects during meiosis I and II of mouse oocytes. Time-lapse microscopy of phase contrast (DIC) and tubulin-GFP (Tub-GFP) of oocytes injected with tubulin-GFP together with RanWT (A) or RanQ69L (B) or RanT24N (C) RNA. Images were taken every 15 min. Arrowheads indicate aster. Times after GVBD are indicated in the bottom right corner. *n* = 34 (RanWT), 26 (RanQ69L), and 75 (RanT24N). Bars, 10 μm.
around condensing chromosomes 1 h after GVBD, as in controls (Fig. S1 E). The distribution of γ-tubulin foci in oocytes injected with Ran mutants was different from the distribution in controls because it is MT dependent (Fig. S1 E, cont + Noco).

We analyzed chromosome spreads from control and injected oocytes that had undergone MI to assess whether meiosis I spindles that formed in the presence of RanQ69L or RanT24N were functional. MII-arrested oocytes had only 20 monovalent chromosomes in all cases (Fig. 5 D, compare 2, 3, and 4), showing that homologous chromosome segregation occurred normally. The Ran pathway is essential for meiosis II. MII spindles formed after RanQ69L and RanT24N expression in mouse oocytes were much more disorganized than MI spindles under these conditions (Fig. 4, B and C). The injection of RanWT did not affect MI spindle organization (Fig. 4 A and Fig. 6, A [2] and B), as observed by live imaging. The injection of RanQ69L and RanT24N induced similar spindle defects in MI. Oocytes either displayed an opened bipolar spindle connected to numerous cytoplasmic asters (50% of oocytes injected with RanQ69L and 78% of RanT24N; Fig. 6, A [3 and 4] and B) or no organized spindle (30% of oocytes injected with RanQ69L and 17% of oocytes injected with of RanT24N; Fig. 6, A [5 and 6] and B). The expression levels of our constructs were very similar in oocytes injected with RanWT, RanQ69L, and RanT24N (Fig. S1 D).

We activated MI oocytes that had been injected with RanWT and RanT24N with strontium, as described previously (Tsurumi et al., 2004) to determine whether spindles assembled in the presence of Ran mutants were functional. Controls (non-injected or RanWT-injected oocytes) and RanT24N-injected oocytes had similar levels of activation (Table II). Nevertheless, MII to anaphase II transition was much slower (with a 2-h delay) in oocytes injected with RanT24N than in controls. This suggests that the spindle checkpoint was activated in these oocytes, potentially slowing the exit from CSF arrest. This observation is consistent with the observed MII spindle defects after injection of RanT24N. Furthermore, all oocytes injected with RanT24N had severe cytokinesis defects; they cleaved instead of extruding a second polar body (Table II and Fig. 6 C), and membrane blebbing was often present in the cytokinetic furrow. We observed obvious segregation defects in 46% of these oocytes but did not see these defects in controls. Only one pronucleus was present in control oocytes, whereas there were lagging chromosomes in RanT24N-injected oocytes (Table II and Fig. 6 C). Thus, over-expression of RanT24N strongly impaired the progression of meiosis II.

The role of Ran in meiotic spindle assembly in X. laevis oocytes

Our findings in mouse oocytes suggest that inhibition of RanGTP production primarily perturbed spindle formation during meiosis II without completely inhibiting the establishment of meiosis I spindles and without affecting entry into the M phase. As shown in Fig. 2, RCC1 levels in immature and mature oocytes were very different in X. laevis oocytes. Increasing RCC1 levels throughout meiotic maturation suggest that RanGTP has an important function during MI. As RCC1 levels rise substantially during maturation of oocytes, preventing the synthesis of RCC1 during maturation kept RCC1 levels lower than in controls. Injection of antisense
deoxyoligonucleotides prevented the rise in RCC1 levels, which occurs concomitantly with meiosis resumption (Fig. 7, A and C). The knock down of RCC1 did not modify the kinetics of meiosis resumption (not depicted) and meiosis I spindle formation (Fig. 7, D and E). In control and RCC1-depleted oocytes, 75–90% of MI spindle structures looked normal (Fig. 7 E). Similar to our findings in mice, oocytes with low levels of RCC1 and, thus, low levels of RanGTP, progressed through MI, as observed by polar body extrusion (not depicted) and the drop in histone H1 kinase activity after GVBD in both control and RCC1-depleted oocytes (Fig. 7 B).

Similar to meiosis in mouse oocytes, MII spindle formation was sensitive to perturbation of the GTP–GDP cycle of Ran. Although 67% of the spindles in sham-injected X. laevis oocytes had normal bipolar organization and aligned chromosomes (76% in uninjected controls), only 14% looked normal after RCC1 knockdown, and 86% of the structures were aberrant (monopolar MT structures, spindle-like structures without a correct antiparallel array of MTs around the chromosomes and without chromosome alignment) or appeared as chromatin aggregates with no MTs (Fig. 7, F and G). These spindle defects were not caused by disruption of Cdk1 activities, as shown by histone H1 kinase assays (Fig. 7 B).

Our findings suggest that X. laevis oocyte maturation requires a large increase in RCC1 levels and, thus, RanGTP production. Inhibition of RCC1 activity led to defects in MII, as in mice. Thus, the mechanisms of meiotic spindle assembly in mice and X. laevis have similar principles: local RanGTP production is essential during MII but much more weakly influences spindle function during the reductive first meiotic cell division.

**Discussion**

In this study, we investigated the function of Ran during assembly of the first and second meiotic spindles in living mouse and X. laevis oocytes, two vertebrate models of acentrosomal spindle formation. We microinjected Ran mutants and directly monitored RanGTP levels by FRET in mouse oocytes, or we knocked down RCC1 in X. laevis. Ran regulated meiosis I spindle formation differently than meiosis II spindle formation in these two models.

**Evidence for a broad RanGTP-regulated gradient**

We are confident that the FRET probe we used for the indirect detection of the RanGTP-regulated gradient functions normally in our model system for two reasons. First, the probe behaved as expected in GV oocytes. It was imported as a cargo into the nucleus. Also, its FRET emission was highest where RanGTP-induced release of importin β and, therefore, RanGTP concentration should have been highest, i.e., in the GV (Kalab et al., 2002, 2006). Second, the Rango probe responded as expected to overexpression of the two well-characterized Ran mutants.
of RanGTP. If we expressed RanQ69L during mouse MI, ectopic
the overall level of Ran that is important but the concentration
and the spindle assembled normally. This indicates that it is not
type Ran during MI in mouse oocytes, meiosis I was not altered
X. laevis
and mouse oocytes. If we overexpressed wild-
tion in
The Ran protein level was constant throughout meiotic matura-
β cargos throughout MI (Fig. 3 E). The analysis of the distribution of RanGTP in oocytes
showed that it accumulated around chromosomes and decreased
linearly with distance from the metaphase plate. This suggests
that the gradient of RanGTP in mouse oocytes was shallower
than that observed in X. laevis egg extracts and HeLa cells
(Kalab et al., 2002, 2006). It resembles to some extent the broad
gradient observed by Caudron et al. (2005). We also followed
changes in the gradient in real time. We consistently observed
a huge drop in RanGTP levels relative to the GV content at the
periphery of MI chromatin immediately after GVBD. The drop
in free Rango concentration (corresponding with RanGTP lev-
als) suggests that meiotic cytoplasm contains relatively higher
levels of RanGAP activity than cytoplasm in mitotic HeLa cells
(Kalab et al., 2002), resulting in low overall levels of RanGTP.
Despite the substantial drop in the free Rango concentration
around the chromatin, the chromosomes remained surrounded
by a localized gradient of free import β cargos throughout MI
and MII. This appears to be particularly important in matur-
ing mammalian oocytes, in which chromosomes migrate to the
cortex before cytokinesis in MI (Verlhac et al., 2000a). The po-
tional information given by local RanGTP accumulation and
carried during chromosome migration in MI might be important
for the asymmetry of the division. The cleavage phenotype ob-
served in RanT24N-injected oocytes undergoing parthenogenetic
activation suggests that local accumulation of RanGTP is essen-
tial for the asymmetry of the second meiotic division.

Ran mediates spindle length and timing of bipolarity establishment but not
recruitment of MTOCs in meiosis I
The Ran protein level was constant throughout meiotic matura-
tion in X. laevis and mouse oocytes. If we overexpressed wild-
type Ran during MI in mouse oocytes, meiosis I was not altered
and the spindle assembled normally. This indicates that it is not
the overall level of Ran that is important but the concentration
of RanGTP. If we expressed RanQ69L during mouse MI, ectopic
asters assembled in the cytoplasm and were rapidly incorpo-
rated into the forming spindle. Thus, in vivo, if active Ran
was ectopically expressed in the cytoplasm, spindle formation
occurred despite the promotion of ectopic aster assembly. This
finding is consistent with previously described effects of the
RanQ69L mutant on spindle formation in X. laevis egg extracts
(Carazo-Salas et al., 2001). The mechanism of aster incorpora-
tion into the spindle is unknown but could be associated with
the capacity of MTs to “self-organize” and assemble into bi-
polar spindles (Karsenti and Vernos, 2001). After MI, 53% of the
spindles formed in the presence of RanQ69L were considerably
longer (30% longer) than those found in control oocytes.
Recently, it was shown in D. melanogaster S2 cells that the
length of the spindle is sensitive to alterations in MT dynamics
(Goshima et al., 2005). We hypothesize that RanQ69L expression
leads to an increase in MT stabilization and, thus, to lengthening
of the spindle. As the central spindle defines the position of the
cleavage furrow and the MI spindle is perpendicular to the
oocyte cortex, a longer spindle should give a more symmetric
division. We observed that 58% of RanQ69L-injected oocytes
extruded a much larger polar body than controls.

| Type of oocyte | PB2 | Large PB2 | Obvious missegregations |
|---------------|-----|-----------|------------------------|
| Control (n = 53) | 96.2 | 0 | 0 |
| Wild type (n = 29) | 79.3 | 0 | 0 |
| T24N (n = 32) | 81.2 | 100 | 46.2 |

Percentage of second polar body extrusion (PB2). Percentage of abnormally large second polar body estimated visually (large PB2). Percentage of obvious missegregation after activation (more than one pronucleus in one cell, lagging chromosomes).

![Figure 7](image-url)
Despite the early ectopic asters and long length, all MI spindles in RanQ69L-injected mouse oocytes that we analyzed had normal chromosome segregation. Therefore, the increase in RanGTP did not compromise homologous chromosome segregation but did compromise the asymmetry of the first division in ~50% of the oocytes. This asymmetry is essential for the preservation of maternal stores for the future embryo. Thus, it is important for the gamete to regulate its RanGTP levels to remain fertile.

RanT24N expression during MI induced a delay in spindle bipolarity establishment of ~2–3 h. This could be explained by low MT assembly activity in these oocytes, consistent with findings from X. laevis egg extracts. During MI in mouse oocytes, spindle poles are defined by the distribution of MTOCs, which aggregate progressively at the two opposite poles of the forming spindle. This distribution seems to be dependent on MTs, which we demonstrate assemble at low RanGTP levels. We hypothesize that if MT assembly is inefficient, it is difficult to distribute MTOCs and to establish two poles. However, despite substantially low RanGTP production because of RanT24N expression, a functional bipolar spindle can assemble in time. Similar principles are probably in place in X. laevis, because RCC1 levels continuously increased during meiotic maturation. Thus, levels of RCC1 are lower in MI than in MII. This is consistent with RanGTP production having an important function during MII in X. laevis. Inhibition of RCC1 expression did not prevent MI spindle assembly or extrusion of the first polar body but led to large defects only during MII in X. laevis oocytes. Although the decrease in RCC1 levels after injection of antisense oligos was smaller in MI than in MII, MI occurred in the presence of the same absolute levels of RCC1. However, these levels were insufficient for MII. Thus, our findings suggest that there are similar principles controlling meiotic spindle assembly in mice and X. laevis: local RanGTP production is crucial during MII but is less important during MI.

Our findings indicate that in mouse oocyte meiosis I, the Ran pathway controls spindle length and the timing of bipolarity establishment. However, pathways acting in parallel can compensate for defects induced by disruption of Ran function, even in the absence of centrosomes, which is characteristic of this system.

RanGTP is essential for spindle formation and correct progression in meiosis II

RanQ69L is locked in the GTP-bound form and, thus, constitutively activates its targets. If active Ran was ectopically expressed in mouse oocytes by RanQ69L expression, Ran targets were probably activated in the cytoplasm and not only around chromosomes. These compete for additional MT assembly factors and uncouple MT formation from chromosomes. We obtained unexpected results with RanT24N expression during MII. This mutant has been shown to inhibit spindle assembly in X. laevis egg extracts (Carazo-Salas et al., 1999; Kalab et al., 1999; Ohba et al., 1999; Wilde and Zheng, 1999). Expressing this mutant during MII in mouse oocytes led to defects very similar to those obtained with RanQ69L. At this point, we can only speculate how manipulating RanGTP levels induces apparently identical phenotypes with multiple ectopic asters in MI oocytes. Possibly, RanGTP activates a spindle-stabilizing factor specific to MII and its activation by RanT24N induces ectopic MT assembly. In RanT24N-injected oocytes, this factor would not be activated and the spindle would no longer be stabilized. Spindle fragmentation may lead to the formation of ectopic structures.

Although metaphase is a transient state of MI or mitosis, it can last for many hours or even days (until fertilization occurs) in MII-arrested oocytes. This suggests that alternative mechanisms to those present in systems that have been used for analysis of mitotic Ran function are acting in the mouse meiosis II model. We have shown previously that MII spindle organization must be maintained by meiosis II–specific mechanisms, which involve at least two proteins, MISS (MAPK-interacting and spindle stabilizing) and DOC1R (deleted in oral cancer 1 related; Lefebvre et al., 2002; Terret et al., 2003). The depletion of MISS in mouse oocytes leads to phenotypes that resemble that observed in RanT24N-expressing MI oocytes. We are now testing the hypothesis that MISS may in fact act as a RanGTP-regulated spindle-stabilizing factor.

In this study, we show that manipulating RanGTP levels in mouse oocytes (with the injection of the RanT24N mutant) strongly compromises the fidelity of sister chromatid segregation and the asymmetry of the second meiotic division. We did not observe obvious defects after manipulation of RanGTP levels in meiosis I similar to those observed in meiosis II. This strongly suggests that meiosis II spindle assembly is more sensitive to changes in the RanGTP gradient than meiosis I.

Different mechanisms for meiosis I and II spindle formation

In mouse oocytes, the first meiotic M phase is very long, lasting from 6 to 11 h, depending on the genetic background (Polanski, 1986, 1997). In X. laevis oocytes, the first meiotic spindle also assembles progressively from a large monopolar aster (Gard, 1992). In these two models, the second meiotic M phase is also very long, lasting hours or even days until fertilization occurs. However, the MII spindle assembles rapidly, and 1 h after the first polar body emission in mouse oocytes a stable bipolar spindle has already formed. It has been reported recently that during mitosis, the classical search-and-capture mechanism is not fast enough to account for observed rates of spindle assembly (Wollman et al., 2005). The RanGTP gradient around chromosomes would introduce a bias, improving the efficiency of search-and-capture and speeding up spindle assembly. However, we speculate that, even if the gradient is present in mouse oocytes, this bias is not required when the M phase lasts several hours, as is the case in MI. MII spindle formation in X. laevis oocytes is faster than MI spindle formation (Gard, 1992). This may explain why the Ran pathway mediates the timing of bipolarity establishment during MI in mouse and in X. laevis but is dispensable for the formation of a functional bipolar spindle. Consistent with this, when the spindle forms rapidly during MII, the presence of a RanGTP gradient around chromosomes is necessary for the establishment of a bipolar spindle.
Materials and methods

Mouse oocyte collection, culture, and microinjection
Oocytes were collected from 11-wk-old OF1 female mice, cultured, and microinjected as described previously (Verlhac et al., 2000b). Oocytes were maintained at the GV stage in M2 medium supplemented with 50 μg/ml dbcAMP (Sigma-Aldrich; Whittingham, 1971). In vitro synthesized RNAs were microinjected into the cytoplasm of GV oocytes. Injected oocytes were kept in M2 + BSA medium supplemented with dbcAMP for 3 h to allow overexpression of the corresponding protein. The resumption of meiotic maturation (GVBD) was triggered upon release of the oocytes into a drug-free medium. Mil oocytes were released from CSF arrest using 10 mM strontium in Ca²⁺/free M2 medium as described by Tsurumi et al. (2004).

In vitro maturation of *X. laevis* oocytes
Collegenase-treated stage VI oocytes were incubated in 5 μg/ml progesterone (Sigma-Aldrich) containing Barth buffer. Oocytes were checked for GVBD every 10 min for GVBD spot appearance. Oocytes with appearing GVBD spot were frozen in 20% DMSO for 3 h to allow overexpression of the corresponding protein. The resumption of meiotic maturation (GVBD) was triggered upon release of the oocytes into a drug-free medium. Mill oocytes were released from CSF arrest using 10 mM strontium in Ca²⁺/free M2 medium as described by Tsurumi et al. (2004).

Immunoblotting
Immunoblotting of mouse oocytes was performed as described previously (Terret et al., 2003). *X. laevis* oocytes were lyzed in H1 kinase buffer and centrifuged at 12,000 g for 15 min. Amounts corresponding to two thirds of the total lysate of one oocyte were used for immunoblotting. The superantigen was analyzed by immunoblotting.

Antibodies
For mouse oocytes, Ran was recognized using a monoclonal antibody from BD Biosciences. For *X. laevis* oocytes, rabbit polyclonal anti-human RCC1 and Ran were described previously (Hetzer et al., 2000), polyclonal antibody against RanGAP was a gift from T. Walther and I. Mattaj (European Molecular Biology Laboratory, Heidelberg, Germany), and rabbit polyclonal antibody against RanBP1 was a gift from M. Dasso (National Institutes of Health, Bethesda, MD). A rabbit polyclonal antibody against PPIG was used as control in Fig. 6 A. It was generated using recombinant full-length *X. laevis* PPIG as antigen in accordance with standard procedures. For detection and quantification of secondary antibody signals on Western blots, an Odyssey system (Li-Cor) was used.

Chromosome preparations from mouse oocytes
Chromosome preparations were performed as described previously (Hodges and Hunt, 2002). Chromosomes were stained using 5 μg/ml propidium iodide in distilled water, and slides were mounted in Citifluor (UKC Chemlab).

Histone H1 kinase assay
*X. laevis* oocytes lysates prepared as described for immunoblotting were incubated with histone H1 (Sigma-Aldrich) and γ[32P]ATP as described previously (Felix et al., 1989). Amounts corresponding to one third of the total lysate of one oocyte were used for H1 kinase assays.

Antisense experiments
Antisense oligonucleotides were designed against the two most common alleles of RCC1 mRNA found in the *X. laevis* EST database (Sanger Institute). An oligonucleotide pair against RCC1 mRNA regions –18 to +6, CCTTACA-AGTGGCCGCTGCAC, and –18 to +7, CCTTATAGCTGCTGTCCTCA, was used to inhibit RCC1 expression. As a control, an oligonucleotide pair against RCC1 mRNA region –44 to –20 was used: GATACAAAAAA-AACCCGGGCTGCCG and GATACAAATAATTACCCGGCTACAG, which led only to a very mild reduction of RCC1 levels (RCC1 amount between 80 and 100% of untreated oocytes). 75 ng of an oligo pair in PBS (37.5 ng/μl) were injected per oocyte before progesterone addition. Efficiency of RCC1 expression inhibition was routinely tested by immunoblotting.

Online supplemental material
Fig. S1 shows that Ran mutants are stably expressed during meiotic maturation. Video 1 shows control mouse oocytes expressing tubulin-GFP together with RanWT during meiotic maturation. Videos 2 and 3 show mouse oocytes expressing tubulin-GFP together with RanαGTP during meiotic maturation. Video 4 shows mouse oocytes expressing tubulin-GFP together with RanQ69L during meiotic maturation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200605199/DC1.

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