The Meiosis I-to-Meiosis II Transition in Mouse Oocytes Requires Separase Activity

M. Emilie Terret,[1,4] Katja Wassmann,[2,4]
Irene Waizenegger,[3,5] Bernard Maro,[2]
Jan-Michael Peters,[3] and Marie-Hélène Verlhac[1,*]
[1]Division Méiotiques
[2]Division Biologie Moléculaire et Cellulaire
   du Développement
UMR 7622
Centre National de la Recherche Scientifique
Université Pierre et Marie Curie
75252 Paris, cedex 05
France
[3]Research Institute of Molecular Pathology (IMP)
Dr. Bohr-Gasse 7
1030 Vienna
Austria

Summary

Faithful segregation of homologous chromosomes during the first meiotic division is essential for further embryo development. The question at issue is whether the same mechanisms ensuring correct separation of sister chromatids in mitosis are at work during the first meiotic division. In mitosis, sister chromatids are linked by a cohesin complex holding them together until their disjunction at anaphase [1–6]. Their disjunction is mediated by Separase, which cleaves the cohesin [7, 8]. The activation of Separase requires prior degradation of its associated inhibitor, called securin [9, 10]. Securin is a target of the APC/C (Anaphase Promoting Complex/Cyclosome), a cell cycle-regulated ubiquitin ligase that ubiquitinates securin at the metaphase-to-anaphase transition and thereby targets it for degradation by the 26S proteasome [11–14]. After securin degradation, Separase cleaves the cohesins and triggers chromatid separation, a prerequisite for anaphase. In yeast and worms, the segregation of homologous chromosomes in meiosis I depends on the APC/C and Separase activity. Yet, it is unclear if Separase is required for the first meiotic division in vertebrates because APC/C activity is thought to be dispensable in frog oocytes. We therefore investigated if Separase activity is required for correct chromosome segregation in meiosis I in mouse oocytes.

Results and Discussion

We show here for the first time that segregation of homologous chromosomes in meiosis I requires the 26S proteasome and Separase activities in mouse oocytes. In *X. laevis* oocytes, reduction of APC/C-mediated proteolysis by several means (treatment with the protea-

some inhibitor MG132, microinjection of antibodies against the APC/C activator fizzy, depletion of fizzy by antisense injection, microinjection of antibodies against the APC/C core subunit Cdc27, injection of the APC/C inhibitor Mad2, and injection of undegradable securin) has no effect on the metaphase-to-anaphase transition in meiosis I, and oocytes are able to segregate their homologous chromosomes normally without degradation of cyclin B and securin, whereas reduction of APC/C-mediated proteolysis in meiosis II prevents the metaphase-to-anaphase transition [15, 16]. On the other hand, previous observations suggested that the APC/C may be required for the metaphase I-to-anaphase I transition in mouse oocytes. In mitosis, ubiquitination by the APC/C and subsequent degradation of B-type cyclins causes MPF activity to disappear, a prerequisite for the exit of mitosis. During meiotic maturation, MPF activity is highest in metaphase I (MI), drops as oocytes exit meiosis I, increases again, and peaks in metaphase of meiosis II (Figure 1). In mouse oocytes, overexpression of cyclin B1 leads to an arrest in metaphase I [17], suggesting that degradation of cyclin B1 is required for the metaphase-to-anaphase transition in mouse oocytes and/or that the APC/C can be saturated by an excess of its substrate. Furthermore, overexpression of a component of the spindle checkpoint, Mad2, which interacts with and inhibits the APC/C in mitosis and therefore induces a metaphase arrest, causes a metaphase I arrest in meiosis (K.W., submitted).

Immature mouse oocytes can be induced to undergo meiosis I synchronously and then arrest in metaphase II in culture (Figure 1). To determine whether degradation of key substrates by the 26S proteasome was required for the first meiotic transition, immature oocytes were allowed to progress into prometaphase I (to avoid the observation of effects unrelated to the metaphase-to-anaphase transition) and were then treated for 5–6 hr with the 26S proteasome inhibitor MG132. Whereas control oocytes underwent the metaphase-to-anaphase transition of meiosis I normally and extruded their first polar body (Figures 2A and 2C), MG132-treated oocytes arrested in metaphase I (Figure 2C) with high levels of MPF activity (Figure 2D), a high cyclin B1 level (Figure 2E), a metaphase I spindle that has migrated to the cortex, and chromosomes aligned on the metaphase plate (Figure 2B). Therefore, these data suggest that 26S proteasome activity is required for the metaphase-to-anaphase transition in meiosis I (Figure 1).

The segregation of homologous chromosomes during the first meiotic division requires prior resolution of the chiasmata. In *S. cerevisiae* and *C. elegans*, resolution of chiasmata depends on the cleavage of a meiosis-specific cohesin, Rec8 [18, 19]. At the metaphase I-to-anaphase I transition, Rec8 is cleaved by Separase along chromosome arms but is resistant to proteolytic cleavage in the vicinity of centromeres. Rec8 located in the centromeric region will be further cleaved by Separase at the metaphase II-to-anaphase II transition [18, 20]. Similarly to mitosis, it is the activation of APC/C in metaphase I that

*Correspondence: marie-helene.verlhac@snv.jussieu.fr
[4]These authors contributed equally to this work.
[5]Present address: Boehringer Ingelheim Austria, Dr. Boehringer-Gasse 5-11, 1121 Vienna, Austria.
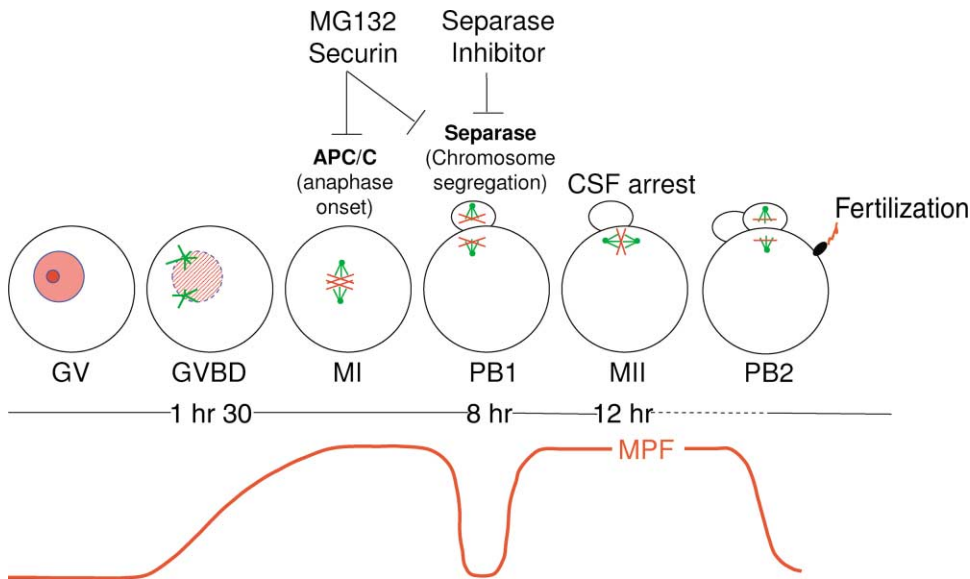
Figure 1. Meiotic Maturation of Mouse Oocyte

Mouse oocytes are arrested in prophase I in the ovaries and harbor a large nucleus (in pink) called the germinal vesicle. Meiosis resumption starts with the Germinal Vesicle BreakDown (GVBD), followed by a long prometaphase I in which chromosomes become condensed (red hatchings) and in which spindle formation occurs (green). After separation of homologous chromosomes and first polar body extrusion (PB1), meiosis II starts without DNA replication and oocytes arrest in metaphase II. This block is called the CSF (Cytostatic Factor) arrest and will be bypassed by fertilization, which allows separation of sister chromatids. MPF activity appears in red. The stages at which MG132, the Separase inhibitor, and securin will act are indicated on the scheme.

triggers securin degradation, Separase activation, and hence homologous chromosome segregation after Rec8 cleavage [21–23].

To determine whether chromosome segregation in meiosis I in mouse oocytes is regulated by a mechanism similar to that in *S. cerevisiae* and *C. elegans*, we injected mouse immature oocytes with a Separase inhibitor [24]. This inhibitor is a derivative of the human cohesin Scc1
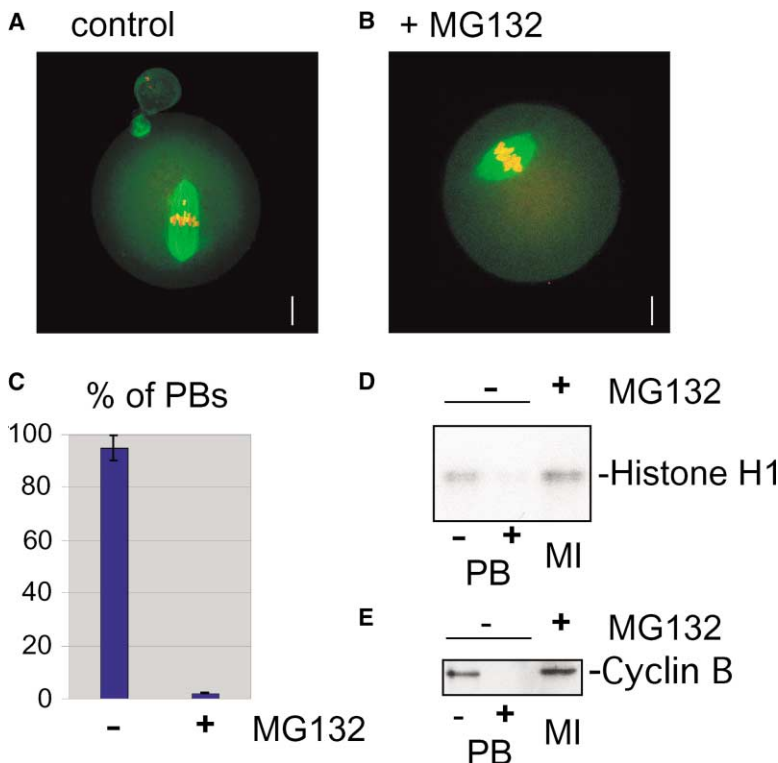

Figure 2. Mouse Oocytes Arrest in Metaphase I upon Treatment with MG132

Mouse oocytes were treated with 5 μM MG132 for 5–6 hr in prometaphase I where indicated.
(A and B) Oocytes were analyzed by confocal microscopy. Microtubules appear in green; chromosomes appear in red. The scale bar represents 10 μm.
(C) Percentage of polar body (PB) extrusion with or without MG132 treatment.
(D) In vitro kinase assay showing MPF activity in oocytes before and after polar body extrusion ($\pm$PB, 8 hr after GVBD) and MG132 treatment with Histone H1 as a substrate.
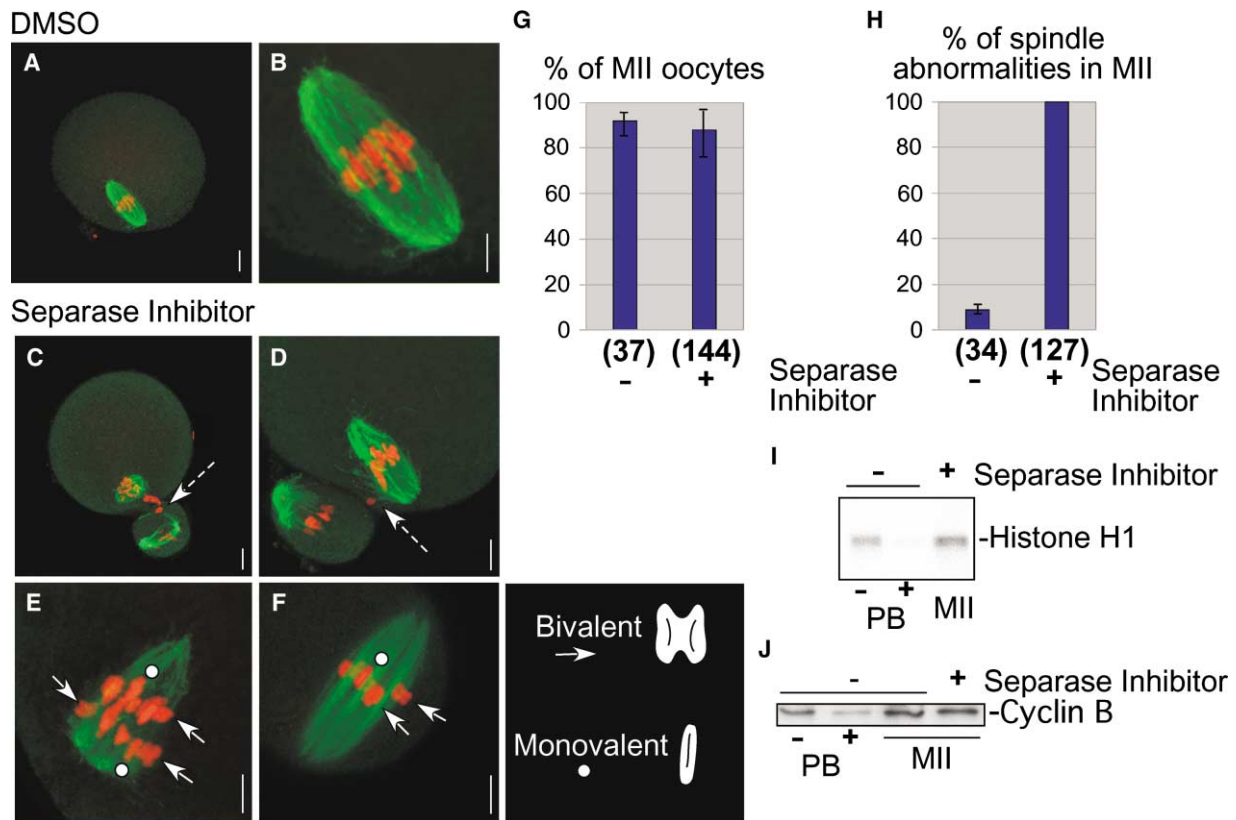(E) Western blot visualizing the levels of cyclin B1 in oocytes as in (D).

Figure 3. Aberrant Homologous Chromosome Segregation due to Microinjection of a Separase Inhibitor in Mouse Oocytes

(A–F) Mouse oocytes were microinjected with 20 mM Separase inhibitor or DMSO (at the same dilution) as indicated. Oocytes injected with (A and B) DMSO or with the (C–F) Separase inhibitor were analyzed by confocal microscopy. (C) and (D) show examples of cut phenotype. (E) and (F) show a mix of homologous chromosomes and sister chromatids (arrow and spot). Microtubules appear in green; chromosomes appear in red. The scale bar represents 10 μm.

(G) Percentage of oocytes that went to metaphase II (MII) after injection of DMSO alone (−) or after injection of the Separase inhibitor (+).

(H) Percentage of spindle abnormalities in metaphase II oocytes after injection of DMSO alone (−) or after injection of the Separase inhibitor (+). For (G) and (H) the number in parentheses corresponds to the number of injected oocytes.

(I) In vitro kinase assay showing MPF activity in oocytes injected with DMSO before or after polar body extrusion (±PB; 8 hr after GVBD) and in oocytes injected with the Separase inhibitor collected 14 hr after GVBD (MII).

(J) Western blot visualizing the levels of cyclin B1 in oocytes as in (I). The white arrows point to bivalent chromosomes from metaphase I that have not been segregated during the MI-to-MII transition.

cleavage site peptide and covalently binds to the active site of Separase. Control oocytes injected with a control FLAG peptide at 20 mM (data not shown) or diluted DMSO, the solvent of the drug, progressed through meiotic maturation normally. They extruded their first polar body and arrested in metaphase II with chromosomes aligned on the metaphase plate and a barrel-shaped spindle (Figures 3A, 3B, and 3G). Oocytes injected with the Separase inhibitor also extruded their first polar body with a percentage close to that of control oocytes (Figure 3G). They formed normal first meiotic spindles (data not shown). However, chromosome segregation was completely aberrant, with a mix of homologous chromosomes and sister chromatids in metaphase II (Figures 3E and 3F). Furthermore, some of them (15%) harbored chromosomes lagging between the oocyte and the first polar body (Figures 3C and 3D); this finding suggests that segregation did not occur normally. This phenotype, evoking the cut phenotype of fission yeast (for a review, see [25]), is reported here for the first time

in mouse oocytes. Consistent with a missegregation event, oocytes injected with the Separase inhibitor showed a perturbed metaphase II spindle organization with misaligned chromosomes and spindles that were not barrel shaped (Figure 3, compare [B] and [E] or [F] and Figure 3H). These oocytes were indeed in metaphase II since they had extruded their first polar body and showed high MPF activity and high levels of cyclin B1 (Figures 3I and 3J). Our results demonstrate that inhibition of Separase perturbed the metaphase I-to-II transition and strongly suggest that Separase activity is required for correct chromosome segregation in meiosis I (Figure 1).

To further show that Separase activity is required for proper segregation of homologous chromosomes during the metaphase I-to-anaphase-I transition, we overexpressed its protein inhibitor, securin. 70% of oocytes microinjected with mRNA encoding securin arrested in metaphase I (Figures 4B and 4G) with high MPF activity (Figure 4I) and high cyclin B1 levels (Figure 4J). Chromo-
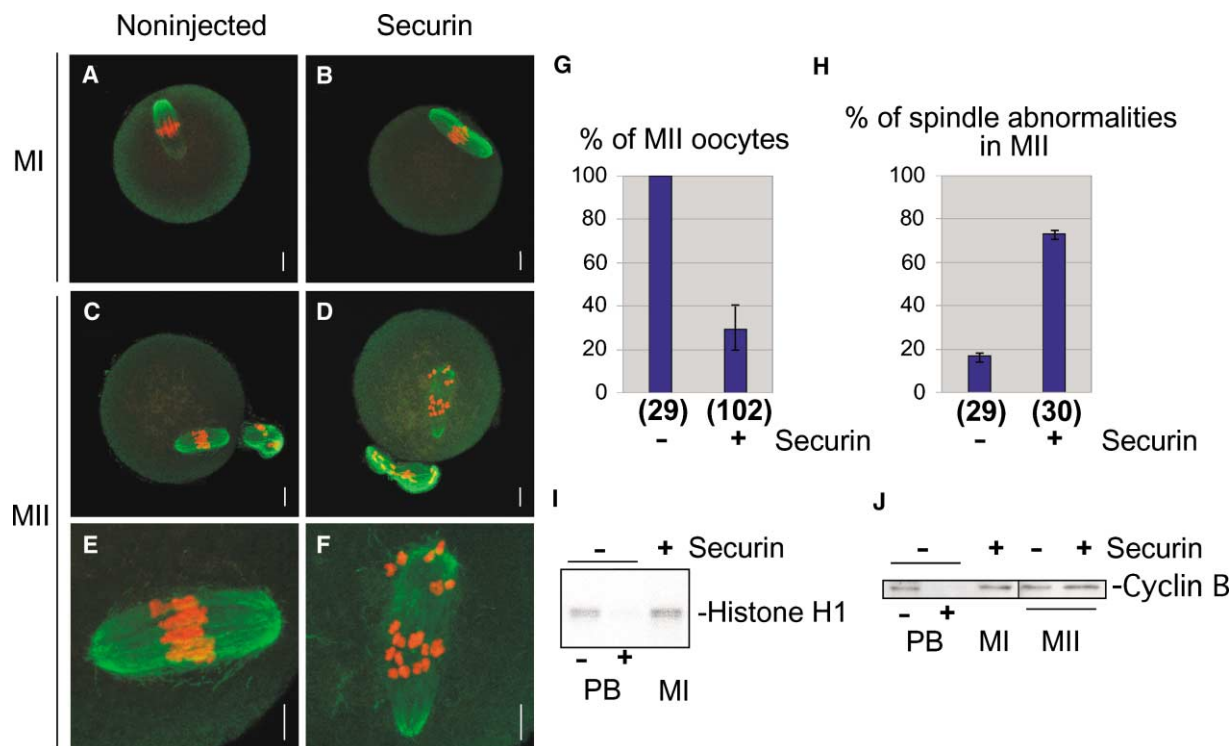
Figure 4. Mouse Oocytes Arrest in Metaphase I upon Microinjection of the RNA Encoding the Securin

(A–F) Mouse oocytes were microinjected or not with the RNA encoding the securin. Oocytes injected (B, D, and F) or not (A, C and E) with securin were analyzed by confocal microscopy; Microtubules appear in green; chromosomes appear in red. The scale bar represents 10 μm.
(G) Percentage of oocytes that went to metaphase II (MII) without (−) or with (+) injection of securin.
(H) Percentage of spindle abnormalities in oocytes that went to MII after injection (+) or not (−) of the securin. For (G) and (H) the number in parentheses corresponds to the number of injected oocytes.
(I) In vitro kinase assay showing MPF activity in noninjected oocytes before or after polar body extrusion (±PB, 8 hr after GVBD) and in oocytes injected with securin collected at the same time.
(J) Western blot visualizing the levels of cyclin B1 in oocytes as in (I) and in oocytes in MII (14 hr after GVBD) that were injected (+) or not (−) with the securin.

somes were aligned on the metaphase plate, and the spindle had migrated to the cortex (Figure 4B). Most (73%) of the remaining 30% that extruded the first polar body and progressed into metaphase II harbored abnormal metaphase II spindles with misaligned chromosomes (Figures 4D, 4F, and 4H). Therefore, overexpression of securin was more efficient than injection of a synthetic Separase inhibitor and blocked the metaphase I-to-anaphase I transition. Indeed, securin is a very potent molecule since it can bind and inhibit Separase molecules that have already been activated [24]. The Separase inhibitor may not be efficient at 100% on all homologous chromosomes, and it is possible that the force of the meiosis I spindle is strong enough to tear apart chromosomes that are still held together by cohesins; this tearing may result in the missegregation events observed. Moreover, the arrest of mouse oocytes in metaphase I by overexpression of securin is the result of both efficient Separase inhibition and saturation of the APC/C, which normally triggers securin and cyclin B degradation at the metaphase-to-anaphase transition (Figure 1). Differently, in X. laevis oocytes, the injection of a nondegradable securin efficiently blocks cyclin B degradation but does not prevent first polar body extrusion [15]. As mentioned above, a metaphase I arrest is also observed in mouse oocytes after overexpression of cyclin B1 and of the APC/C inhibitor Mad2 ([17]; K.W., submitted). Very recently it has been shown that Rec8 localization is regulated similarly to yeast Rec8 during male meiosis in the mouse [26]. Altogether, previous observations and the work presented here suggest that segregation of homologous chromosomes during the first meiotic division in mouse oocytes depends on a mechanism similar to the one acting during meiosis in S. cerevisiae and C. elegans: an APC/C-dependent degradation of securin that triggers Separase activation and subsequent cohesin cleavage.

How can we reconcile the requirement for APC/C during the first meiotic division in yeast, C. elegans, and now mouse and its apparent nonrequirement in X. laevis? The X. laevis oocyte is giant compared to a mouse oocyte (1000 times bigger), but the spindle is approximately the same size in both. One possibility seems that, for technical reasons, experiments that have been performed in X. laevis oocytes do not affect the localized active pool of APC/C in meiosis I. This would be similar to the situation previously encountered in which the early syncytial divisions of D. melanogaster embryos occurred without detectable oscillations in the total cyclin levels or Cdk1 activity [27]. However, it is now

established that local Cdk1 inactivation takes place near the spindle poles of syncytial embryos [28]. Alternatively, *X. laevis* may have evolved differently from other organisms and may have built a specific pathway, independent of the APC/C, to control homologous chromosome segregation in meiosis I.

Conclusions

Our results are in accordance with studies in *S. cerevisiae* and *C. elegans* but are contrary to what has been shown in another vertebrate species, *X. laevis*. The APC/C is required for the activation of Separase by targetting its inhibitor, securin, for degradation by the 26S proteasome. We propose here that the meiosis I-to-II transition in mouse oocytes also depends on APC/C activity, as has been shown in *S. cerevisiae* and *C. elegans*. In human oocytes, missegregation events in meiosis I are responsible for the generation of aneuploidies, which may lead to trisomies, malformations of the embryo, and spontaneous abortion. Therefore, it is of great importance to know the mechanisms controlling meiosis I. This study gives important insights into our understanding of the regulation of correct chromosome segregation in meiosis I in mammalian oocytes.

Experimental Procedures

Immature oocytes arrested in prophase I of meiosis were obtained by removing ovaries from 11-week-old OF1 female mice. Oocytes were removed and cultured as previously described [29]. For experiments with MG132, oocytes were treated with 5 μM MG132 (stock solution at 50 mM in DMSO) 5 hr after Germinal Vesicle Breakdown for 5–6 hr. Control oocytes were treated with DMSO diluted 1:10,000 in culture medium.

The pRN3Myc2securin was constructed by PCR amplification of mSecurin and subcloning at EcoR1/Not1 sites. In vitro synthesis of capped RNA was performed by using linearized pRN3Myc2securin with the mMessage mMachine kit (Ambion). The capped RNA were then purified on RNeasy columns (Qiagen) and eluted in water at a final concentration of 0.5 μg/μl. Aliquots were then stored at −80°C. The Separase inhibitor (stock solution 100 mM in DMSO) was diluted at a concentration of 20 mM in the injection buffer (10 mM Tris, 0.1 mM EDTA [pH 7.4]). As a control, we injected DMSO at the same dilution (1:5 in the injection buffer) and a FLAG peptide (stock solution 100 mM in DMSO) diluted at a concentration of 20 mM in the injection buffer (10 mM Tris, 0.1 mM EDTA [pH 7.4]). Microinjection into mouse oocytes was performed as described [30]. Immunofluorescent staining of microtubule and chromosomes was performed as described in [31]. The Histone H1 kinase assays were performed as described in [32], and cyclin B1 immunoblotting was performed as described in [31].

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Note Added in Proof

The manuscript by K.W. that is cited as submitted on the first page of this manuscript is now in press: K. Wassmann, T. Niault, and B. Maro (2003). Metaphase I arrest upon activation of the Mad2-dependent spindle checkpoint in mouse oocytes. Curr. Biol. *13*, 1596–1608.