Resolution of Chiasmata in Oocytes Requires Separase-Mediated Proteolysis

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SUMMARY

In yeast, resolution of chiasmata in meiosis I requires proteolytic cleavage along chromosome arms of cohesin's Rec8 subunit by separase. Since activation of separase by the anaphase-promoting complex (APC/C) is supposedly not required for meiosis I in *Xenopus* oocytes, it has been suggested that animal cells might resolve chiasmata by a separase-independent mechanism related to the so-called "prophase pathway" that removes cohesin from chromosome arms during mitosis. By expressing Cre recombinase from a zona pellucida promoter, we have deleted a floxed allele of separase specifically in mouse oocytes. This prevents removal of Rec8 from chromosome arms and resolution of chiasmata. It also hinders extrusion of the first polar body (PBE) and causes female sterility. mRNA encoding wild-type but not catalytically inactive separase restores chiasma resolution. Both types of mRNA restore PBE. Proteolytic activity of separase is therefore essential for Rec8's removal from chromosome arms and for chiasma resolution but not for PBE.

INTRODUCTION

During the first meiotic division, sister centromeres from homologous chromosomes are held together by chiasmata produced by reciprocal recombination between maternal and paternal chromatids (Petronczki et al., 2003). This process is essential for the traction of maternal and paternal kinetochores toward opposite poles of the meiosis I spindles (co-orientation) and hence for their segregation to opposite poles at the first meiotic division (see Figure 1). Since physical linkage of homologous centromeres due to chiasmata is in fact mediated by sister chromatid cohesion distal to the crossover site (marked by green arrowheads in Figure 1) (Miyazaki and Orr-Weaver, 1994), sister chromatid cohesion along chromosome arms is essential for meiosis I co-orientation.

Sister chromatid cohesion is mediated during both mitosis and meiosis by a multisubunit complex called cohesin (Nasmyth and Haering, 2005). In yeast, resolution of chiasmata is mediated by cleavage (exclusively along chromosome arms) of cohesin's α-kleisin (Rec8) subunit (Buonomo et al., 2000; Kitajima et al., 2003) by a site-specific protease called separase (Uhlmann et al., 2000; Waizenegger et al., 2000), whose activity is kept in check by the binding of an inhibitory chaperone called securin (Ciosk et al., 1998). Separase is activated at the onset of anaphase through the sudden destruction of securin (Cohen-Fix et al., 1996; Funabiki et al., 1996) by a ubiquitin protein ligase called the anaphase-promoting complex or cyclosome (APC/C) along with an accessory protein called Cdc20 (Peters, 2002). In mammalian cells, separase is also inhibited by the binding of Cdk1-cyclin B (Gorr et al., 2005), which is destroyed at the same time as securin by the APC/C. Kinetochores that have not attached to mitotic spindles delay the destruction of sister chromatid cohesion by sequestering Cdc20 in an inactive complex with the Mad2 protein, which prevents destruction of both securin and cyclin B (Nasmyth, 2005).

The finding that both separase (Siomos et al., 2001) and the APC/C (Furuta et al., 2000) are needed for meiosis I

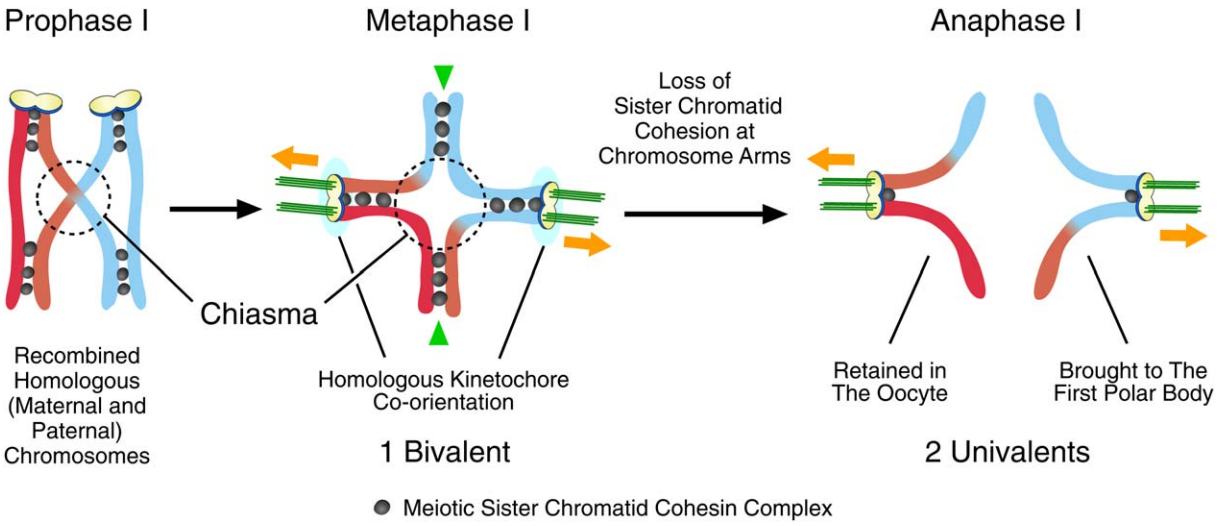

Figure 1. Chiasma Resolution Allows Chromosome Segregation in Meiosis I

Schematic diagrams of meiosis I chromosome segregation where only one chiasma is generated between a pair of homologous chromosomes. Red and sky-blue objects represent homologous chromosomes from maternal and paternal origins. Yellow and green objects represent kinetochores and microtubules, respectively. The meiotic sister chromatid cohesin complex (cohesin) is depicted by black disks. The physical connection between homologous centromeres due to chiasmata is mediated by cohesin molecules marked by green arrowheads distal (with regard to centromeres) to the crossover site. See main text for detail. See also Figure 5B for immunofluorescence images detecting centromeres and cohesin on chromosome spreads.

chromosome segregation in *Caenorhabditis elegans* suggested that chiasmata might be resolved by a common mechanism in all eukaryotic organisms. However, the finding that neither depletion of Cdc20 (Taieb et al., 2001) nor injection of antibodies against APC/C subunits (Peter et al., 2001) blocked meiosis I in *Xenopus laevis* oocytes raised the possibility that chiasmata in vertebrates might be resolved by a process that is independent of the APC/C-separase pathway. It has been suggested that they might instead be resolved by a mechanism related to the so-called "prophase pathway" (Losada et al., 1998; Sumara et al., 2000) that removes cohesin from chromosome arms during mitosis, not by kleisin cleavage but by phosphorylation of cohesin's Scc3-SA2 subunit (Hauf et al., 2005). This notion is controversial because microinjection of mRNA encoding nondegradable securin hinders both extrusion of the first polar body (PBE) and chromosome segregation at meiosis I in mouse oocytes (Herbert et al., 2003). The findings that a Mad2-dependent mechanism, which presumably inactivates the APC/C, blocks meiosis I (Wassmann et al., 2003) and that injection of an admittedly poorly characterized peptide-based separase inhibitor partially hinders chiasma resolution (Terret et al., 2003) also point to a role for the APC/C-separase pathway.

Since the resolution of chiasmata is such a fundamental process, it is essential to address the role of separase using a technique that eliminates the function of separase and no other protein specifically in oocytes. To do this, we used a transgene that expresses Cre recombinase from the *Zona pellucida 3* promoter (Lewandoski et al., 1997) active during growing oocytes to delete exons that encode separase's protease domain from a floxed allele of the gene (Wirth et al., 2006). We find that oocytes lacking active separase neither resolve chiasmata nor extrude permanently polar bodies. Crucially, mRNA encoding wild-type but not catalytically inactive separase restores chiasma resolution. Our data imply that proteolytic cleavage by separase is essential for Rec8's removal from chromosome arms and for chiasma resolution but not for PBE, which can be promoted by separase via a mechanism that does not involve proteolytic cleavage.

RESULTS

Oocytes Lacking Separase Fail to Segregate Chromosomes or to Extrude Polar Bodies Permanently in Meiosis I

Since separase is essential for mitosis, its deletion causes embryonic lethality in mice (Kumada et al., 2006; Wirth et al., 2006). To investigate separase function specifically during oocyte maturation, we tested whether Cre recombinase expressed during the growing oocyte stage from the *Zona pellucida 3* promoter (*Zp3-cre*) (Lewandoski et al., 1997) can cause efficient deletion of a floxed version of the gene (*Separase*^{flox}) in which exons encoding its protease domain are flanked by *loxP* sites (Wirth et al., 2006). Genotyping of offspring showed that 22 out of 22 *Separase*^{flox} alleles were converted to *Separase*$^{\Delta}$ in the female germline of *Separase*$^{flox/+}$ *Zp3-cre* mice. *Separase*$^{flox/+}$ *Zp3-cre* and *Separase*$^{flox/flox}$ females are fertile, but *Separase*$^{flox/flox}$ *Zp3-cre* females are sterile (n = 6). This

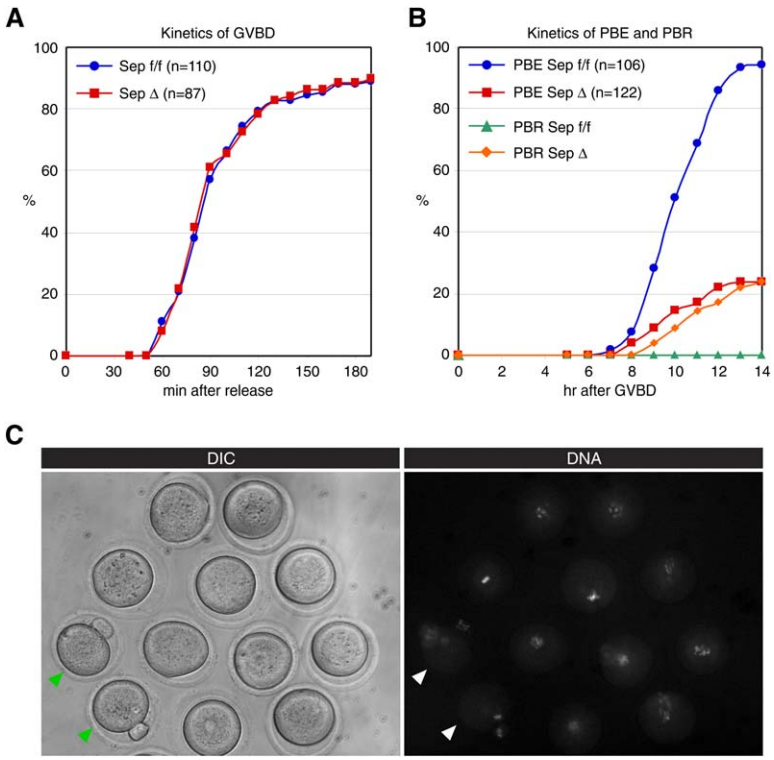

Figure 2. Oocytes Lacking Separase Fail to Extrude PBs Permanently in Meiosis I

In vitro maturation of *Separase*$^{flox/flox}$ *Zp3-cre* oocytes (Sep Δ) and control *Separase*$^{flox/flox}$ oocytes (Sep f/f) was characterized.

(A) Kinetics of germinal vesicle breakdown (GVBD). GV-stage oocytes were isolated in M2 medium containing dbcAMP that inhibits GVBD and released into inhibitor-free medium (at time = 0). Oocytes that had lost GVs were scored at 10 min intervals following release. The numbers of oocytes examined are indicated (n).

(B) Kinetics of polar body extrusion (PBE) and polar body retraction (PBR). Oocytes that had undergone GVBD within 1.5 hr after release into dbcAMP-free M16 medium were selected (at time = 0) and cultured further. PBE and PBR were scored at 1 hr intervals. The numbers of oocytes examined are indicated (n).

(C) Oocytes cultured in Hoechst-containing M2 medium for 15.3 hr after GVBD observed by time-lapse live microscopy. The DIC image and the corresponding DNA image of the identical field are shown. The two oocytes with PBs distinguished by an arrowhead are control Sep f/f; the other ten oocytes are Sep Δ oocytes. The images shown are selected from the original movie (Movie S1).

suggests that *Zp3-cre* causes efficient deletion of both *Separase*^{flox} alleles and that separase is essential for oocyte maturation. Despite their infertility, the ovaries of *Separase*$^{flox/flox}$ *Zp3-cre* females contain normal numbers of fully grown germinal vesicle (GV) stage oocytes surrounded by cumulus cells. Since *Separase*^{flox} deletion most likely occurs at the growing stage, when the *Zp3* promoter is active (Epifano et al., 1995), much oocyte growth must occur in the absence of any active separase gene.

When isolated from the follicle, fully grown mouse GV-stage oocytes resume meiosis when placed in culture medium. They break down germinal vesicles, form meiosis I spindles, and align bivalent chromosomes using their chiasmata (Figure 1; see also below). When all bivalents come under tension, chiasmata are resolved, and paternal and maternal centromeres (along with their associated parental and recombinant chromatids) segregate to opposite poles. One set of chromosomes is retained in the oocyte, while the other is segregated into the first PB. Oocytes subsequently enter meiosis II, form meiosis II spindles, biorient sister centromeres, and arrest at metaphase II awaiting fertilization. *Separase*$^{flox/flox}$ *Zp3-cre* (Sep Δ) oocytes containing GVs undergo germinal vesicle breakdown (GVBD) with the same efficiency and kinetics as *Separase*$^{flox/flox}$ (Sep f/f) oocytes (n = 87) (Figure 2A), which was confirmed by time-lapse live confocal microscopy (data not shown). Together, these results imply that separase is not required for the resumption of oocyte meiosis. In Sep f/f oocytes, PBE occurs between 8 and 12 hr after GVBD under our culture conditions (Figure 2B). Strikingly, only 20% of Sep Δ oocytes extruded PBs, and all PBs produced were retracted (PBR) within 1 hr (n = 122) (Figure 2B). The efficiency of transient PBE in Sep Δ oocytes was sensitive to culture conditions, occurring in only 3% of oocytes when M2 instead of M16 medium was used.

To visualize meiosis I chromosome segregation, we cultured Sep Δ and Sep f/f oocytes in medium containing Hoechst. Time-lapse live microscopy showed that chromosomes from Sep f/f oocytes aligned on metaphase plates 8–9 hr after GVBD and segregated to oocytes and PBs 9–10 hr after GVBD (see Figure S1 and Movie S1 in the Supplemental Data available with this article online). Under these culture conditions, few if any PBs were extruded in Sep Δ oocytes (n = 17). The chromosomes of Sep Δ oocytes failed to segregate and remained in a single group up to 15 hr after GVBD, by which time control oocytes would have arrested in metaphase II (Figure 2C and Movie S1).

We also used time-lapse confocal microscopy to follow chromosome movements in live oocytes that had been injected with mRNA encoding histone H2B fused to monomeric red fluorescent protein (mRFP). Separase depletion had little or no effect on the dynamics of formation of the metaphase plate, which started between 5 and 8 hr, or on the movement of chromosomes (Figure 3 and Movie S2). Strikingly, Sep Δ oocytes failed to segregate chromosomes and, under these culture conditions, also failed to extrude the first PB (n = 28). Control Sep f/f oocytes segregated chromosomes into the first PBs between 8 and 10 hr of the culture. Prolonged confocal microscopy showed

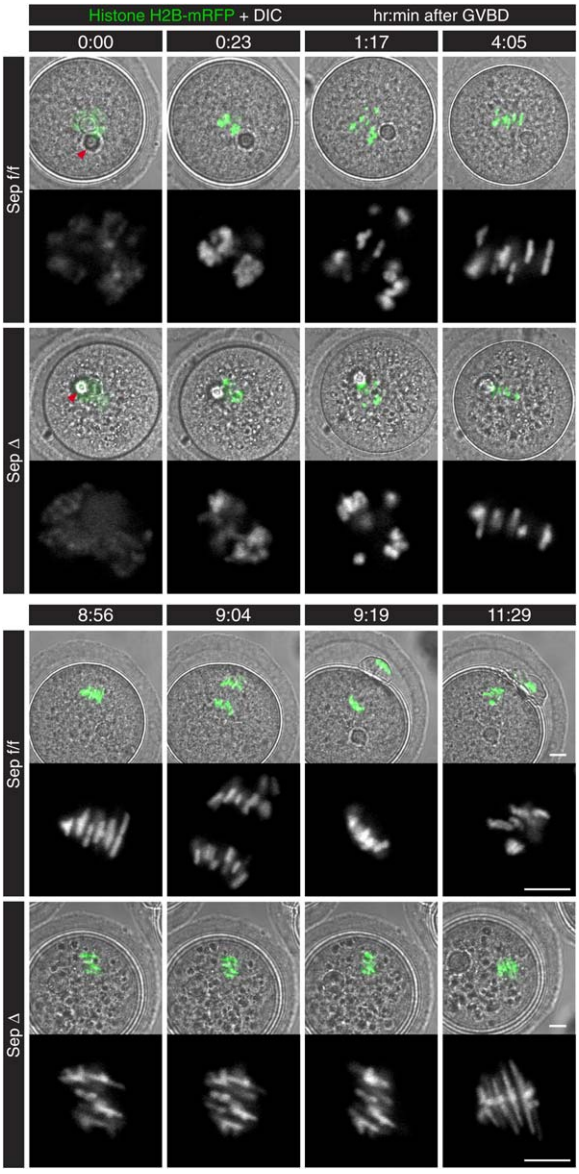

Figure 3. Oocytes Lacking Separase Fail to Segregate Chromosomes in Meiosis I

Chromosome movement and segregation of Sep Δ and Sep f/f oocytes expressing histone H2B-mRFP were observed by time-lapse live confocal microscopy. Upper panels show DIC images merged with images of the RFP channel pseudocolored in green; lower panels show magnified images of the RFP channel only in grayscale at higher magnification. Frames at the indicated times after GVBD were selected from the original time series (Movie S2), where images were acquired every 7 min 46 s with a resolution (xyz) of 160 nm × 160 nm × 5 μm. Time is shown in hr:min relative to GVBD (time = 0:00). An oil droplet resulting from the microinjection procedure is visible in the DIC images (arrowhead at 0:00). Scale bar = 10 μm.

that the chromosomes of Sep Δ oocytes remained in a single group up to 17 hr, by which time control oocytes would have arrested in metaphase II (data not shown). By the time Sep f/f oocytes had extruded PBs, chromosomes from Sep Δ oocytes were stretched, which presumably reflects persistent tractive forces exerted on unresolved bivalents (Figure 3, Sep Δ 11:29).

To observe spindle morphology and chromosomes simultaneously, we performed immunofluorescence confocal microscopy on oocytes fixed at various time points following GVBD (Figure 4A). Sep Δ oocytes established bipolar spindles and aligned most chromosomes on metaphase plates with kinetics similar to those of Sep f/f oocytes. Both reached this stage approximately 8 hr after GVBD. However, we noticed that Sep Δ oocytes had a greater tendency to form astral microtubules emanating from the bipolar spindle between 2 and 6.5 hr after GVBD (Figure 4A). The majority of Sep f/f oocytes underwent anaphase I between 8 and 10 hr after GVBD and reformed bipolar (meiosis II) spindles 12 hr after GVBD. In contrast, at 12 hr after GVBD, 7 out of 8 Sep Δ oocytes that had not extruded PBs did not possess bipolar spindles, while 13 out of 13 did by 17 hr (post-GVBD). This suggests that separase-deleted oocytes destroy their meiosis I bipolar spindles around the time that control oocytes would normally extrude PBs but may be slower than wild-type in re-forming meiosis II bipolar spindles. Most Sep Δ oocytes that transiently extruded PBs distributed chromosomal DNA unequally between oocytes and their PBs (n = 8; Figure 4B).

To address whether Sep Δ oocytes can resolve chiasmata, we examined chromosome spreads prepared at different stages after GVBD (Figures 4C and 4D). Between GVBD and metaphase I, homologous centromeres of different parental origin are connected by chiasmata that join all four homologous chromatids together, forming bivalent chromosomes. Resolution of chiasmata at anaphase I produces univalent chromosomes containing a pair of homologous chromatids connected solely by cohesion between sister centromeres (see Figure 1). Oocytes from Sep f/f mice invariably contained 20 bivalents before PBE and 20 univalents after PBE (Figure 4C). In contrast, Sep Δ oocytes contained only bivalent chromosomes with unresolved chiasmata at all stages after GVBD and irrespective of whether they had extruded PBs (Figures 4C and 4D). Even when PBs had been extruded, all bivalents remained in the oocyte in 60% of cases, while some bivalents were lost—presumably into the PBs—in 40% of cases (Figure 4D). These data imply that separase is necessary for the resolution of chiasmata as well as for proper PBE.

Separase Is Necessary for Removing Cohesin from Chromosome Arms at Anaphase I

In yeast, cleavage of cohesin's α-kleisin Rec8 subunit by separase is accompanied by and necessary for cohesin's disappearance from chromosome arms at anaphase I (Buonomo et al., 2000; Kitajima et al., 2003). This event destroys sister chromatid cohesion along chromosome arms, which presumably resolves chiasmata. To address whether a similar process occurs during oogenesis, we needed first to investigate whether the meiosis-specific α-kleisin presumed to be orthologous to yeast Rec8 (also known as Rec8 in mammals) (Xu et al., 2005) is

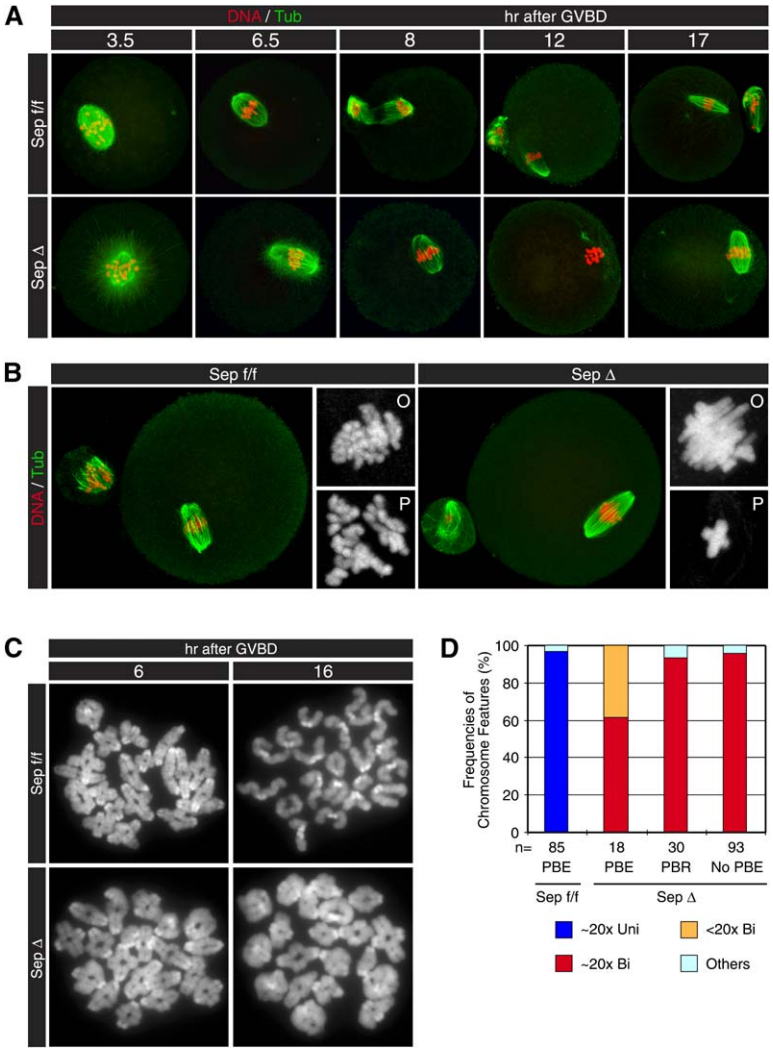

Figure 4. Oocytes Lacking Separase Fail to Segregate Bivalents into Univalents in Meiosis I

Sep Δ oocytes were characterized in comparison to the Sep f/f oocytes as control.

(A and B) Confocal microscopic images of oocytes fixed at the indicated time after GVBD. Microtubules were visualized by immunofluorescence staining with an anti-tubulin antibody (green), and DNA was counterstained with propidium iodide (red). Magnified DNA images of the oocyte (O) and the polar body (P) are shown in the right panels in (B).

(C) DAPI-stained chromosome spreads prepared at the indicated time after GVBD.

(D) Frequencies of different classes of chromosome features contained in the oocytes. Oocytes were harvested at 14 hr after GVBD, except for Sep Δ oocytes with PBs (Sep Δ PBE) because the presence of their PBs is transient. PBE: oocytes that had extruded and maintained PBs, No PBE: oocytes that had not extruded PBs, PBR: oocytes that had extruded PBs and retracted them. Chromosome features were classified into the following different categories: approximately 20 univalents (~20× Uni), approximately 20 bivalents (~20× Bi), apparently less than 20 bivalents (<20× Bi), or other (Others). Numbers of oocytes examined are indicated (n).

associated with chromosome arms at metaphase I. To do this rigorously, we visualized the distribution of a functional version of the Rec8 protein containing nine tandem myc epitopes at its C terminus, expressed at levels comparable to Rec8 protein from endogenous *Rec8* genes from a bacterial artificial chromosome (BAC) transgene (N.R.K. and K.N., unpublished data). This *Rec8-myc* transgene (*TG Rec8-myc*) fully complemented the female sterility caused by a homozygous deletion of the *Rec8* locus (Xu et al., 2005) (see details in Experimental Procedures). The use of an epitope-tagged protein is the only way of ensuring that signals are in fact due to the protein being investigated. This is particularly important in a field where it has been difficult and frequently impossible to detect cohesin on chromosomes at metaphase during mitosis or meiosis using conventional antibodies (Waizenegger et al., 2000).

We first analyzed the distribution of Rec8-myc during meiotic prophase. To do this, we prepared chromosome spreads from fetal ovaries of E13.5 embryos heterozygous for *TG Rec8-myc* and stained them with anti-Myc, anti-Smc3, and anti-Sycp3 antibodies. During pachytene, both Rec8-myc (Figure 5A) and Smc3 (Figure S2A) colocalized with Sycp3, a major component of the lateral element of the synaptonemal complex. The pattern of Rec8-myc staining was similar to that obtained using an anti-Rec8 antibody (Prieto et al., 2004). We next analyzed Rec8-myc in chromosome spreads prepared from oocytes at metaphase I and metaphase II. On metaphase I bivalents prepared 6 hr after GVBD, Rec8-myc localized to interchromatid axes both proximal and distal (with regard to centromeres) to chiasmata but not at the chiasmata themselves (Figure 5B), similar to the localization of another cohesin subunit, Stag3, at this stage (Hodges et al., 2005). On metaphase II univalents from oocytes fixed 18 hr after GVBD, where little is known about cohesin's localization, Rec8-myc was absent from chromosome arms but present at small foci between sister centromeres (Figure 5B). This distribution is similar to those observed on metaphase I and II chromosomes from

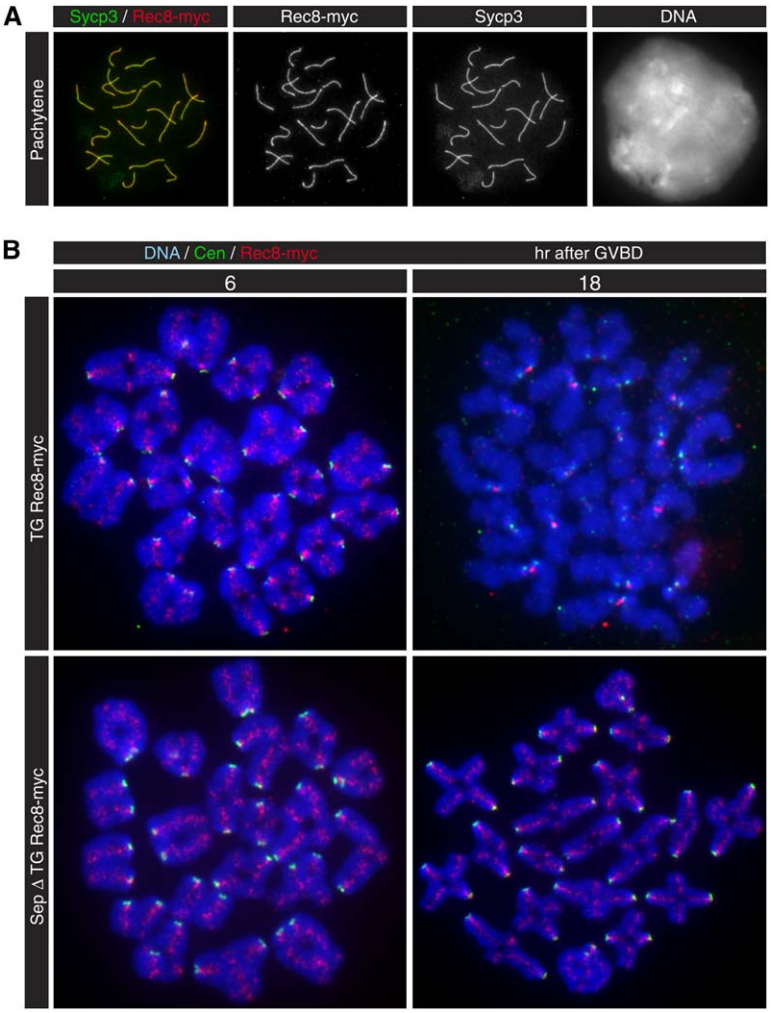

Figure 5. Separase Is Necessary for Removing Cohesin from Chromosome Arms at Anaphase I

(A) Localization of Rec8-myc on pachytene nuclei of oocytes expressing Rec8-myc. Chromosome spreads from fetal ovaries of E13.5 embryos heterozygous for the *Rec8-myc* transgene were prepared and stained with anti-Myc (green) and anti-Sycp3 (red) antibodies. DNA was counterstained with DAPI.

(B) Localization of Rec8-myc on chromosomes from *Separase*$^{flox/flox}$ oocytes also carrying a heterozygous *Rec8-myc* transgene (TG Rec8-myc) and *Separase*$^{flox/flox}$ *Zp3-cre* with *Rec8-myc* (Sep Δ TG Rec8-myc). Chromosome spreads were prepared from oocytes matured in culture for the indicated time after GVBD and stained with anti-Myc antibody (red) and CREST antiserum for marking centromeres (green). DNA was counterstained with DAPI (blue). Note that this spreading method produces large variations in chromosome morphology; we therefore refrain from drawing any more conclusions than are described in the Results.

mammalian spermatocytes (Eijpe et al., 2003; Lee et al., 2003). Crucially, no staining was observed on chromosomes obtained from mice lacking *TG Rec8-myc*. Signals attributable to Smc3 colocalized with Rec8-myc at least along the interchromatid axes in metaphase I (Figure S2B). These data are consistent with the notion that Rec8-containing cohesin complexes confer not only the sister chromatid cohesion distal to chiasmata that holds bivalents together during metaphase I but also the cohesion between sister centromeres that holds homologous chromatids together during metaphase II. The resolution of chiasmata that converts bivalents into univalents at anaphase I is accompanied by the selective loss of cohesin from chromosome arms in oocytes as well as in spermatocytes.

To address whether the lack of chiasma resolution in oocytes lacking separase is accompanied by a failure to remove cohesin from chromosome arms, we bred *Separase*$^{flox/flox}$ *Zp3-cre* females that were heterozygous for *TG Rec8-myc*. Localization of Rec8-myc on metaphase I bivalents was identical to that in oocytes of *Separase*$^{flox/flox}$ *TG Rec8-myc* control mice (Figure 5B). Importantly, we observed a similar if not identical localization on bivalents from *Separase*$^{flox/flox}$ *Zp3-cre TG Rec8-myc* mice when chromosomes were spread from oocytes incubated for 18 hr after GVBD (Figure 5B). At this stage, control oocytes contain only univalents. This implies that cohesin's removal from chromosome arms at anaphase I depends on separase. A corollary is that the nondisjunction of homologous chromosomes observed in oocytes lacking separase might be caused at least partly by their failure to cleave Rec8 along chromosome arms.

Oocytes Lacking Separase Are Not Arrested in Metaphase I

Since PBE is greatly impaired and neither chiasma resolution nor Rec8's removal from chromosome arms occurs in Sep Δ oocytes, it is theoretically possible that their lack of separase merely triggers a metaphase I arrest, possibly by activating a Mad2-dependent spindle assembly checkpoint (SAC). To investigate this, we examined the localization of Mad2 at kinetochores. Mad2 was recruited to the kinetochores of both Sep Δ and control Sep f/f oocytes 4 hr after GVBD (Figure 6A), as occurs in wild-type oocytes

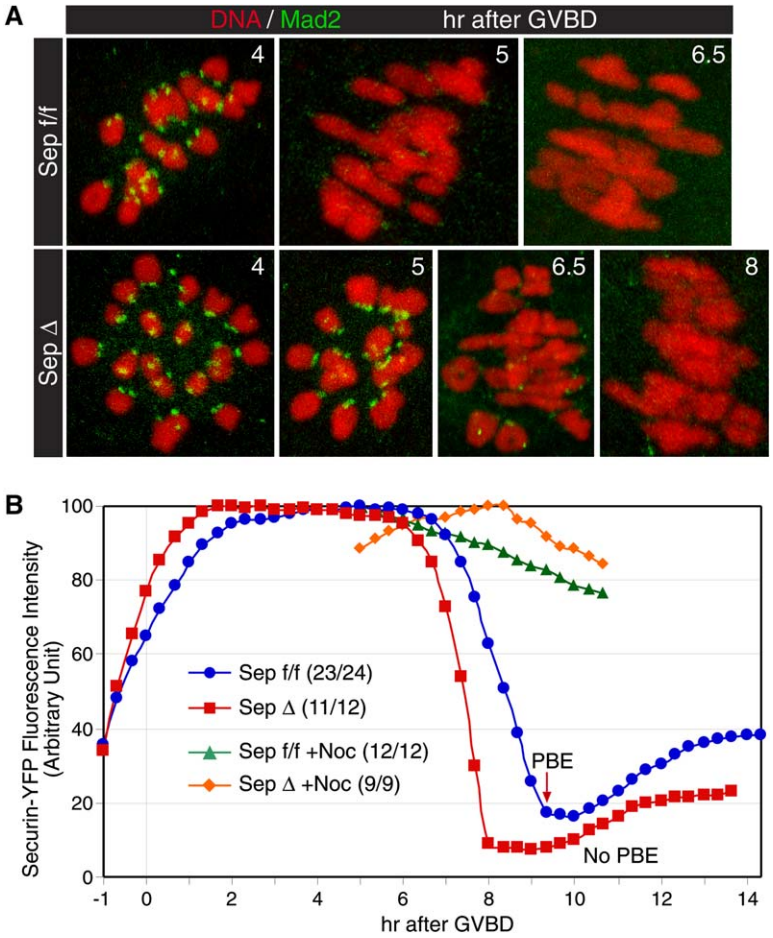

Figure 6. Oocytes Lacking Separase Are Not Arrested in Metaphase I

Sep Δ oocytes were characterized in comparison with control Sep f/f oocytes.

(A) Confocal microscopic images of immunofluorescence staining visualizing Mad2 at kinetochores before metaphase I. Oocytes were fixed at the indicated time in culture after GVBD. Mad2 was visualized by anti-Mad2 antibody (green), and DNA was counterstained with propidium iodide (red).

(B) Time-lapse fluorescence measurement of securin-YFP expressed from mRNA injected at GV stage. The maximum values of the YFP fluorescence signal in each oocyte during the time course were set to 100, and transitions of relative intensity in a representative oocyte were plotted. The ratio of the number of oocytes that showed a transition pattern similar to the indicated curve (n) to the number of oocytes successfully analyzed (N) is given as n/N in parentheses. Measurements in oocytes cultured in the presence of nocodazole (+Noc) were started from 5 hr after GVBD at the same time that the oocytes were transferred to 4 μM nocodazole-containing medium.

(Wassmann et al., 2003). In Sep f/f oocytes, the amount of Mad2 at kinetochores declined by 5 hr and was undetectable 6.5 hr after GVBD. This decline was modestly delayed in Sep Δ oocytes, in which some Mad2 persisted at kinetochores until 5 hr and small amounts even until 6.5 hr; nevertheless, none could be detected by 8 hr after GVBD. The modest delay in Mad2's departure from kinetochores was not accompanied by any major delay in activation of the APC/C because fluorescence due to injection of mRNA encoding a securin-yellow fluorescent protein (YFP) fusion protein declined dramatically 6–9 hr after GVBD in Sep Δ as well as Sep f/f oocytes (Figure 6B), as described in wild-type oocytes (Herbert et al., 2003). PBE accompanied the decline in Sep f/f but not Sep Δ oocytes. In both cases, activation of the SAC by the addition of nocodazole halted the decline (Figure 6B). We also measured histone H1 kinase activity, which is presumably associated with Cdk1-cyclin B1 complexes (Kubiak et al., 1992). We observed a decline in histone H1 kinase activity in Sep Δ oocytes irrespective of whether or not they extruded PBs (Figure S3). We conclude that many of the events normally associated with anaphase I (namely, disappearance of Mad2 from kinetochores, securin degradation, Cdk1 inactivation, and even early steps of PB formation) can occur in oocytes lacking separase. Despite this, chiasmata are never resolved.

Separase Proteolytic Activity Is Required for Chiasma Resolution but Not for PBE

To investigate whether removal of cohesin from chromosome arms at anaphase I depends on separase's proteolytic activity, we tested whether the meiotic defects of Sep Δ oocytes can be suppressed by injection (at the GV stage) of mRNA encoding wild-type or mutant separase (Figure 7A). Under the conditions used for these experiments, few if any Sep Δ oocytes were observed to extrude PBs. Neither mock injection nor injection of mRNA carrying a frameshift mutation at amino acid 212 (separase 1-212) had any effect, but injection of wild-type separase mRNA restored PBE in 57% (n = 169) of oocytes (Figure 7B). Strikingly, injection of mRNA encoding separase with a mutation of its catalytic cysteine residue (C2028S) (Stemmann et al., 2001; Uhlmann et al., 2000) caused 44% of oocytes (n = 144) to undergo PBE—an efficiency only slightly less than that of wild-type mRNA. Unlike wild-type oocytes and Sep Δ oocytes injected with wild-type mRNA, chromosomes were unequally distributed between oocytes and their PBs after

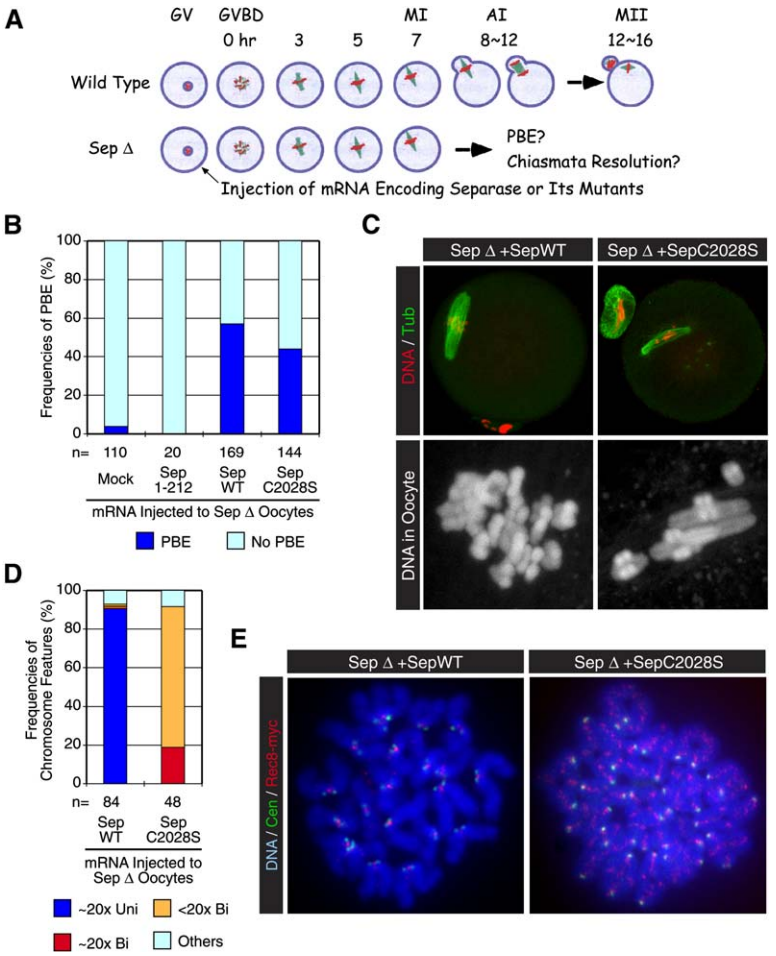

Figure 7. Separase Proteolytic Activity Is Required for Chiasma Resolution

(A) Schematic outline of the experimental procedure. Oocytes lacking separase (Sep Δ) at GV stage were injected with mRNA encoding wild-type or mutant separase. Injected mRNAs encoded wild-type separase (Sep WT), a mutant carrying a serine amino acid replacement at its catalytic cysteine residue (Sep C2028S), or a mutant carrying a frameshift mutation at amino acid residue 212 (Sep 1-212). Water (solvent of mRNA) was injected as a mock control (Mock). Injected oocytes were cultured for 16 hr after release into inhibitor-free medium, and PBE was scored. Chromosome spreads were prepared from oocytes that had extruded PBs and were stained with DAPI to classify chromosome features.

(B) PBE frequencies of either mRNA- or mock-injected oocytes. Numbers of oocytes examined are indicated (n).

(C) Confocal microscopic images of mRNA-injected Sep Δ oocytes fixed at 20 hr after release. Microtubules were visualized by immunofluorescence staining with an anti-tubulin antibody (green), and DNA was counterstained with propidium iodide (red). Magnified images of chromosomes in the oocytes are shown in the lower panels.

(D) Frequencies of different classes of chromosome features contained in the oocytes that had been injected with the indicated mRNAs and extruded PBs. Chromosome features were classified into the following different categories: approximately 20 univalents (~20× Uni), approximately 20 bivalents (~20× Bi), apparently less than 20 bivalents (<20× Bi), or other (Others). Numbers of oocytes examined are indicated (n).

(E) Localization of Rec8-myc on chromosomes from mRNA-injected *Separase*$^{flox/flox}$ *Zp3-cre TG Rec8-myc* oocytes that extruded PBs. Chromosome spreads were prepared at 16 hr after the release and stained with anti-Myc antibody (red) and CREST antiserum for marking centromeres (green). DNA was counterstained with DAPI (blue).

injection with C2028S mRNA and apparently present as bivalents rather than univalents (Figure 7C). To ascertain the state of chromosomes with greater certainty, we prepared chromosome spreads from Sep Δ oocytes injected with wild-type and C2028S mRNA. Ninety percent contained (approximately) 20 univalents after PBE when injected with wild-type mRNA, but none contained appreciable numbers of univalents when injected with C2028S mRNA. Instead, all post-PBE Sep Δ oocytes injected with C2028S mRNA contained only bivalent chromosomes, though usually fewer than 20 (Figure 7D). Importantly, this failure of chiasma resolution in oocytes stimulated to undergo PBE with C2028S mRNA was accompanied by the persistence of Rec8-myc protein along chromosome arms when the experiment was repeated with *Separase*$^{flox/flox}$ *Zp3-cre TG Rec8-myc* oocytes (Figure 7E) (n = 15). We conclude that a catalytically inactive separase can promote PBE but not the removal of cohesin from chromosome arms or the resolution of chiasmata.

DISCUSSION

We describe here a novel approach for studying the role of mitotic proteins during meiosis I in mouse oocytes. We show that expression of Cre recombinase from a zona pellucida promoter (*Zp3-cre*) causes efficient deletion of both maternal and paternal floxed alleles of the separase gene (*Separase*$^{flox/flox}$) specifically in oocytes. This prevents their first meiotic division and thereby causes complete female sterility without interrupting in any way the mitotic divisions required to produce the germ cells that give rise to oocytes. *Zp3-cre* is expressed only after completion of premeiotic DNA replication and meiotic recombination, which has both advantages and disadvantages. An important advantage is that it permits analysis of protein function during chromosome segregation without the potential complication of having interfered with a function that takes place earlier in the meiotic process, for example during recombination. A corollary is that the *Zp3-cre* system cannot be used to study early meiotic functions. As

a consequence, our finding that *Separase*$^{flox/flox}$ *Zp3-cre* mice produce normal numbers of oocytes with normal numbers of chiasmata does not imply that separase is not required for recombination. *Zp3-cre* causes inactivation of the separase gene only after recombination has already been completed. Another great advantage of the system is that the defects caused by gene deletion can be rescued by injection of mRNA made in vitro into oocytes, which enables analysis of the function of mutant forms of the protein in question without further strain construction. The *Zp3-cre* system should be applicable to many genes that have a function during later stages of oocyte maturation.

Our study of *Separase*$^{flox/flox}$ *Zp3-cre* oocytes has enabled us to answer in a definitive manner a long-standing and controversial question, namely whether proteolytic cleavage mediated by separase is required for resolving chiasmata at the onset of anaphase I in mammals. The resolution of chiasmata is one of the most important events during meiotic chromosome segregation. Moreover, it is a process that in human oocytes is accompanied by errors that give rise to aneuploidy, which causes Down's syndrome, miscarriages, and infertility (Hassold and Hunt, 2001). In yeast, cleavage by separase of cohesin's meiosis-specific α-kleisin subunit Rec8 is essential for destroying sister chromatid cohesion along chromosome arms that holds bivalent chromosomes together from the point at which crossovers are formed during pachytene until the onset of anaphase I (Buonomo et al., 2000; Kitajima et al., 2003). It is thought that Rec8 cleavage actually triggers meiosis I chromosome segregation. Therefore, it was somewhat surprising that meiosis I in *Xenopus* oocytes was unaffected either by depletion of the APC/C's activator Cdc20 using antisense RNA (Taieb et al., 2001) or by microinjection of antibodies against Cdc20 or the APC/C's Cdc27 subunit (Peter et al., 2001). These observations led to the proposal that chiasma resolution in vertebrates does not involve the APC/C-separase pathway and might instead be mediated by a process analogous to the prophase pathway that removes cohesin from chromosome arms in a separase-independent manner during mitosis (Hauf et al., 2005). The subsequent finding that meiosis I in mouse oocytes is blocked by microinjection of mRNA encoding nondegradable securin was inconsistent with the conclusion drawn from the *Xenopus* studies (Herbert et al., 2003). Nevertheless, the notion that meiosis I in vertebrates is triggered by a separase-independent process has still been given serious credence (Yu and Koshland, 2005).

Analysis of *Separase*$^{flox/flox}$ *Zp3-cre* oocytes (Sep Δ) has now finally settled this crucial issue. Depletion of separase using the *Zp3-cre* system clearly prevents resolution of chiasmata and the formation of permanent PBs. These effects cannot be caused by activation of the SAC because the kinetics of securin proteolysis appears unaffected by the lack of separase. Importantly, the resolution of chiasmata is restored in most oocytes by microinjection of mRNA encoding wild-type separase but not separase containing a frameshift mutation. PBE is also restored (though possibly not completely) by microinjection of mRNA encoding separase whose catalytic cysteine residue has been replaced by serine (C2028S). Crucially, all chromosomes persist as bivalents containing unresolved chiasmata and Rec8 along chromosome arms even when PBE has been promoted by C2028S mRNA. The lack of chiasma resolution in oocytes that express C2028S separase cannot therefore be due to a general block to meiotic progression and is very likely a direct consequence of separase failing to cleave proteins associated with meiosis I bivalents. Our work on oocytes leaves open the identity of these proteins. However, the finding that Rec8 persists on chromosome arms in these oocytes suggests that cohesin's α-kleisin subunits are likely targets of the protease. It is therefore possible (but still not fully proven) that the disjunction of chromosomes during anaphase involves the same fundamental chemistry during mitosis and meiosis in most eukaryotic organisms, namely cohesin cleavage.

One of our more surprising findings is the observation that Sep Δ oocytes often fail to extrude PBs. As expected, this defect is suppressed by injection of wild-type separase mRNA. More unexpectedly, it is also suppressed, albeit slightly less well, by mRNA encoding a catalytically dead protease (C2028S). This suggests that separase using a mechanism that does not rely on proteolytic cleavage has a role in triggering the formation of PBs following chiasma resolution. Since injection of mRNA encoding securin that cannot be degraded by the APC/C also hinders PBE (Herbert et al., 2003), it is likely that separase can only promote PBE once it has been liberated from its inhibitory chaperone. This implies that activation of separase by the APC/C, once bivalents have aligned on the meiotic spindle and the SAC has been turned off, triggers not only disjunction of bivalents but also the ensuing cell division that sequesters one half of the genome and extinguishes any further prospect of inheritance. This dual function of separase is reminiscent of the situation in budding yeast, where destruction of securin not only triggers sister chromatid separation but also releases the Cdc14 phosphatase from the nucleolus in a protease-independent manner (Buonomo et al., 2003; Stegmeier et al., 2002; Sullivan and Uhlmann, 2003). This event plays a role in downregulation of Cdk1, resolution of sister rDNAs, association of aurora B kinase with the midzone of anaphase spindles, and anaphase spindle stabilization (Higuchi and Uhlmann, 2005). Unlike mouse oocytes, separase is not essential for cytokinesis in yeast because Cdc14's release from the nucleolus is also supported by a separase-independent process called the mitotic exit network (Stegmeier et al., 2002). We have little idea at present how separase promotes PBE in oocytes. The finding that separase binds cyclin B1 and thereby inhibits Cdk1 (Gorr et al., 2005) suggests that its liberation from securin might help to lower Cdk1 activity, though whether separase is sufficiently abundant for such a function to be of physiological relevance is unclear.

Though it makes biological sense, our finding that PBE depends on separase is in fact surprising because elimination of separase function in mouse embryonic fibroblasts by inducing deletion of the very same floxed allele does not prevent cytokinesis despite eliminating sister chromatid separation (Wirth et al., 2006). Cytokinesis is likewise unaffected in fruit-fly embryos by mutations in *three rows* and *pimples* that compromise separase function (Pandey et al., 2005). This implies that, as in yeast, animal cells possess a separase-independent pathway capable of promoting cytokinesis following activation of the APC/C. For some reason, this pathway is insufficient for the equivalent task in oocytes. We suggest therefore that studying the process of cytokinesis in oocytes may be particularly revealing as to its physiology. We suspect that PBE is dependent on two or more processes, each of which is individually sufficient in somatic cells. If so, it may be easier to implicate particular proteins in the process of cytokinesis by studying the dependence of PBE on such proteins.

The recent finding that the sterility of SMC1β-deficient female mice is accompanied and very possibly caused by the precocious loss of chiasmata proves that sister chromatid cohesion has a key role in holding bivalents together during meiosis I, which is essential for co-orienting homologous centromeres (Hodges et al., 2005). The incidence of unpaired univalents and sometimes even single chromatids before the first meiotic division in SMC1β-deficient oocytes increases progressively with maternal age, which is reminiscent of human trisomy. Defects in maintaining sister chromatid cohesion might therefore contribute to the mistakes during meiosis I in human oocytes that give rise to trisomy. Such defects might be caused by a failure to maintain sufficient sister chromatid cohesion during the long arrest of oocytes in prophase. Our finding that separase mediates chiasma resolution in mammalian oocytes raises the possibility that misregulation of this key enzyme, possibly in conjunction with the inherent difficulty of maintaining sister chromatid cohesion for long periods, could also contribute to the mistakes during meiosis I that give rise to trisomy.

EXPERIMENTAL PROCEDURES

Mouse Strains and In Vitro Culture of Oocytes

Generation of the *Separase*^{flox} mice was recently described (Wirth et al., 2006). A transgenic mouse line that expresses Cre recombinase from the *Zona pellucida 3* promoter (*Zp3-cre*) (Lewandoski et al., 1997) was purchased from the Jackson Laboratory. A transgenic line that expresses Rec8 from a bacterial artificial chromosome (BAC), with nine tandem copies of the human c-*myc* epitope at its C terminus, will be described in a separate report (N.R.K. and K.N., unpublished data). Mouse strains used had mixed backgrounds of C57BL/6J, 129/Sv, and CBA/J. Fully grown mouse GV-stage oocytes surrounded by cumulus cells were isolated by disaggregating ovaries of females older than 11 weeks in M2 medium (Specialty Media or Sigma) supplemented with 100 μg/ml dibutyryl cyclic AMP (dbcAMP) (Sigma) at 37.5°C. Oocytes released from most of the surrounding cumulus cells were cultured in drops of medium (~50 μl) covered with mineral oil (Sigma). Oocytes were matured in M16 medium (Specialty Media or Sigma) at 37.5°C in the presence of 5% CO_2 or in M2 medium at 37.5°C in air. Only oocytes that had undergone GVBD within 90 min following release into dbcAMP-free medium were selected (time 0 after GVBD) and cultured further for experiments. Oocytes were cultured in medium containing nocodazole at different concentrations and for different lengths of time as indicated in the figure legends.

Examination of Functionality of the *Rec8-myc* Transgene

To test whether the *Rec8-myc* transgene is functional, we performed complementation tests using a *Rec8* null allele (*Rec8*$^{-}$) that causes complete sterility in homozygous mice of both sexes (Xu et al., 2005). We bred *Rec8*$^{-/-}$ mice that were heterozygous for *TG Rec8-myc*. The average litter size of crosses between *TG Rec8-myc Rec8*$^{-/-}$ females and C57BL/6J males was 9.7 (10 deliveries of 3 females). The average litter size of crosses between *TG Rec8-myc Rec8*$^{-/-}$ males and C57BL/6J females was 7.8 (7 deliveries of 2 males). These are comparable to the average litter size of 7.7 obtained from breeding C57BL/6J in our laboratory (28 deliveries of 10 breeding pairs). In addition, the heterozygous BAC-mediated *Rec8-myc* transgenic allele also suppresses late embryonic sublethality and severe postnatal growth defects seen in *Rec8*$^{-/-}$ mice (Xu et al., 2005). These results demonstrate that Rec8-myc is potentially as functional as wild-type Rec8 protein.

In Situ Immunofluorescence and Time-Lapse Live Microscopy

For in situ immunofluorescence studies, oocytes were harvested at the indicated time after GVBD and fixed following zona pellucida removal by Tyrode's acidic solution (Kubiak et al., 1992; Wassmann et al., 2003). For visualization of the spindle and Mad2, rat anti-α-tubulin antibody (clone YL1/2, Serotec) and rabbit anti-Mad2 antibody (Wassmann et al., 2003) were used as primary antibodies, respectively, and appropriate secondary antibodies conjugated with FITC (KPL or Biosystems) were used. Chromosomal DNA was counterstained with 2 μg/ml propidium iodide (Molecular Probes). Confocal microscopic images were acquired with a Leica TCS4D confocal microscope. Time-lapse live microscopy was performed with a Leica DM IRBE microscope equipped with a Micromax 1300 YHS CCD camera (Princeton Instruments). Fluorescence and DIC images were acquired using MetaMorph software (Universal Imaging) at 20 min intervals. For visualizing DNA, oocytes were cultured in M2 medium containing 2 ng/ml bisbenzimide *H* 33342 (Hoechst 33342) (Sigma) at 37°C following a 2 hr preculture in Hoechst-containing M2 medium. For quantification of the YFP signal, the excitation light of a 100 W mercury lamp was decreased to 10% by a neutral density filter and an exposure was taken for 300 ms. The signal intensity was calculated as the sum of pixels in a defined region against mean background levels using ImageJ 1.32j software (NIH).

Preparation and Staining of Chromosome Spreads

Chromosome spreads of mouse oocytes during prophase I and meiotic maturation were prepared using methods previously described (Hodges and Hunt, 2002; Peters et al., 1997); see Supplemental Data for further details. The dried chromosome spreads were stained with 1 μg/ml DAPI for DNA counterstaining, following immunofluorescence staining when required. Mouse anti-human c-*myc* epitope antibody (clone 4A6, Upstate), rabbit anti-Smc3 antibody (727, gift of J.-M. Peters, Vienna; Sumara et al., 2000), rabbit anti-Sycp3 antibody (Knuf, gift of C. Heyting, Wageningen, The Netherlands; Lammers et al., 1994), mouse anti-Sycp3 antibody (clone 10G11, Abcam), and CREST serum (gift of A. Kromminga, Hamburg, Germany) for marking centromeres were used as primary antibodies, and appropriate secondary antibodies conjugated with Alexa Fluor 488 or 568 (Molecular Probes) or Cy5 (Jackson ImmnoResearch) were used. Images were captured with an Axioplan 2 microscope (Carl Zeiss) equipped with an α Plan-FLUAR 100×/1.45 oil objective and a CoolSnap HQ CCD camera (Photometrics) and analyzed using MetaMorph software (Universal Imaging).

Synthesis and Microinjection of mRNAs

Mouse separase cDNA (GenBank/EMBL/DDBJ accession number AK129072) was identified by BLAST search using the amino acid sequences of its orthologs from other organisms. A cDNA clone encoding full-length mouse separase cDNA (clone name mKIAA0165) was kindly provided by the Kazusa DNA Research Institute (Chiba, Japan). Construction of plasmids for in vitro transcription of mRNAs encoding wild-type separase, a separase mutant with a site-directed mutation at its catalytic cysteine at amino acid 2028 replaced by serine (separase C2028S), and a truncated mutant with a frameshift mutation at amino acid 212 (separase 1-212) is described in the Supplemental Data. Construction of a plasmid for the mRNA encoding mouse securin (gene product of *Pttg1*, MGI ID 1353578) fused with YFP will be described elsewhere (K.W., unpublished data). Capped mRNAs were synthesized by in vitro transcription using an mMessage mMachine T3 kit (Ambion) and purified by RNeasy columns (QIAGEN). mRNAs prepared at a final concentration of 0.1 μg/μl in RNase-free water (Ambion) were microinjected using a FemtoJet microinjector (Eppendorf) with constant flow settings into the cytoplasm of GV-stage oocytes in M2 medium containing 100 μg/ml dbcAMP. Oocyte maturation was induced within 1 hr after microinjection. RNase-free water was injected as a mock control.

Time-Lapse Confocal Microscopy of Live Oocytes Expressing Histone H2B-mRFP

GV-stage oocytes were injected with 10 pl of 0.3 μg/μl mRNA encoding H2B-mRFP (M.S. and J.E., unpublished data) in M2 medium containing 250 μM dbcAMP using methods described elsewhere (Jaffe and Terasaki, 2004). Control Sep f/f oocytes were coinjected with 25 kDa Alexa 488-labeled dextran (Molecular Probes) to distinguish from Sep Δ oocytes. Following mRNA injection, oocytes were cultured for 2–3 hr at 37°C to allow H2B-mRFP expression and incorporation into chromosomes. Oocytes were cultured in M16 medium placed in an EMBL environmental microscope incubator (EMBL, GP 106), allowing cells to be maintained in a 5% CO_2 atmosphere at 37°C with humidity control during imaging. Time-lapse image acquisitions were performed using a customized Zeiss LSM510 META confocal microscope equipped with a 532 nm excitation laser, a long-pass 545 nm emission filter, a 40× C-Apochromat 1.2 NA water immersion objective lens (Carl Zeiss), and an in-house-developed 3D tracking macro (Rabut and Ellenberg, 2004).

Supplemental Data

Supplemental Data include Supplemental Results, Supplemental Experimental Procedures, Supplemental References, three figures, and two movies and can be found with this article online at http://www.cell.com/cgi/content/full/126/1/135/DC1/.

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