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Separase is required for chromosome segregation during meiosis I in *Caenorhabditis elegans*

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Background: Chromosome segregation during mitosis and meiosis is triggered by dissolution of sister chromatid cohesion, which is mediated by the cohesin complex. Mitotic sister chromatid disjunction requires that cohesion be lost along the entire length of chromosomes, whereas homolog segregation at meiosis I only requires loss of cohesion along chromosome arms. During animal cell mitosis, cohesin is lost in two steps. A nonproteolytic mechanism removes cohesin along chromosome arms during prophase, while the proteolytic cleavage of cohesin's Scc1 subunit by separase removes centromeric cohesin at anaphase. In *Saccharomyces cerevisiae* and *Caenorhabditis elegans*, meiotic sister chromatid cohesion is mediated by Rec8, a meiosis-specific variant of cohesin's Scc1 subunit. Homolog segregation in *S. cerevisiae* is triggered by separase-mediated cleavage of Rec8 along chromosome arms. In principle, chiasmata could be resolved proteolytically by separase or nonproteolytically using a mechanism similar to the mitotic "prophase pathway."

Results: Inactivation of separase in *C. elegans* has little or no effect on homolog alignment on the meiosis I spindle but prevents their timely disjunction. It also interferes with chromatid separation during subsequent embryonic mitotic divisions but does not directly affect cytokinesis. Surprisingly, separase inactivation also causes osmosensitive embryos, possibly due to a defect in the extraembryonic structures, referred to as the "eggshell."

Conclusions: Separase is essential for homologous chromosome disjunction during meiosis I. Proteolytic cleavage, presumably of Rec8, might be a common trigger for the first meiotic division in eukaryotic cells. Cleavage of proteins other than REC-8 might be necessary to render the eggshell impermeable to solutes.

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Background

The faithful transmission of chromosomes during both meiosis and mitosis is fundamental to the survival and reproduction of all living organisms. Errors during this process result in aneuploidy. Whereas sister chromatids segregate from each other at the metaphase to anaphase transition in mitotic cells, homologous chromosomes do so at the equivalent stage of the first meiotic division. Missegregation of chromosome 21 during meiosis is the cause of Down's Syndrome, whereas that of other chromosomes is the cause of many spontaneous fetal abortions [1]. Aneuploidy, which can be caused by mistakes in chromosome segregation during mitosis, is also associated with many forms of human cancer [2].

Recent work has identified proteins that link sister chromatids until metaphase and then break these linkages at the metaphase to anaphase transition. A multisubunit protein complex called "cohesin" [3–8] is believed to be the "glue" that holds sister chromatids together from their production during DNA replication until their disjunction at the onset of anaphase [9]. Sister chromatid separation is mediated by a CD clan cysteine protease called "separase," [10] whose activity depends on prior association with an inhibitory chaperone called "securin" [11]. Activation of separase shortly before the metaphase to anaphase transition due to destruction of securin by a ubiquitin protein ligase called the "anaphase-promoting complex" (APC) [12–14] causes cleavage of cohesin's Scc1 subunit and hence the dissolution of sister chromatid cohesion. In mouse, *Drosophila*, and humans, but not in yeast, the final separation of sister chromatids during anaphase is preceded by the resolution of chromosome arm cohesion during prophase and prometaphase, when the bulk of cohesin dissociates from chromosomes [7, 8, 15, 16]. In human cells, this is due to a mechanism that involves neither proteolytic cleavage by separase nor the APC [8, 17]. It appears that only cohesin, mainly in the vicinity of centromeres, which resists dissociation by the "prophase pathway" is later cleaved by separase [16]. The "prophase

pathway" is largely, if not completely, absent in the yeast *S. cerevisiae*, and, as a consequence, the bulk of Scc1 remains on chromosomes until the metaphase to anaphase transition, whereupon it is cleaved by separase [10].

At first sight, the segregation of homologous chromosomes during the first meiotic division appears quite different from the disjunction of sister chromatids during mitosis or during the second meiotic division. Reciprocal recombination, also known as "crossovers," between homologous chromatids creates chiasmata, which hold maternal and paternal chromosomes together. Meanwhile, the attachment of sister kinetochores to spindles with the same polarity, also known as "monopolar attachment," ensures that homologs and not sister chromatids are pulled in opposite directions once maternal and paternal kinetochore pairs attach to spindles from opposite poles. Thus, during metaphase I, it is chiasmata and not sister chromatid cohesion per se which resist the tendency of kinetochore-attached microtubules to pull homologous chromosomes toward opposite poles of the cell [18]. Sister chromatid cohesion, nevertheless, has a crucial role during this process. The ability of chiasmata to connect homologs depends on sister chromatid cohesion distal, with respect to the centromere, to crossovers. The disjunction of homologs to opposite poles at the onset of anaphase I has long been thought to be triggered by resolution of chiasmata, which could be triggered by the destruction of cohesion between sister chromatid arms [19].

Sister chromatid cohesion during meiosis is also mediated by cohesin. However, in both *S. cerevisiae* and in *C. elegans*, cohesin's scissile Scc1 subunit is replaced by a meiosis-specific variant called "Rec8" [20, 21]. In *S. cerevisiae*, the resolution of chiasmata depends on cleavage by separase of Rec8 located along chromosome arms [22]. Meanwhile, Rec8 in the vicinity of centromeres is resistant to separase [20, 22, 23]. Indeed, Rec8's persistence until anaphase I is essential for the disjunction of sister chromatids to opposite poles at anaphase II. Thus, the mechanism by which homologs disjoin at meiosis I in yeast is fundamentally similar to that by which sister chromatids disjoin during mitosis, namely, cleavage of Scc1 or Rec8 by separase [10, 22].

It is still unclear whether such a parsimonious and elegant solution applies to all eukaryotic organisms. The finding in grasshoppers that bivalents, homologous chromosomes connected by chiasmata, transferred to a meiosis II spindle apparatus disjoin at the same time as native sister chromatid pairs [24] is consistent with the notion that the same fundamental process, namely, separase-mediated cleavage, triggers meiosis I and II. Furthermore, mutational inactivation of the APC's Cdc16 or Apc4 subunits causes *C. elegans* embryos to arrest in metaphase I [25, 26]. In contrast, recent experiments in *Xenopus* have raised the possibility that chromosome segregation at meiosis I in vertebrate cells takes place in the absence of APC activity [27, 28] and in the presence of high levels of separase's inhibitor securin. Indeed, it is not at all obvious why cells that are capable of removing cohesin from chromosomes without cleavage of its scissile subunit would need Rec8 cleavage for the destruction of chromatid arm cohesion during meiosis I. Homolog disjunction at anaphase I could in principle be mediated by a mechanism that resembles the mitotic "prophase pathway."

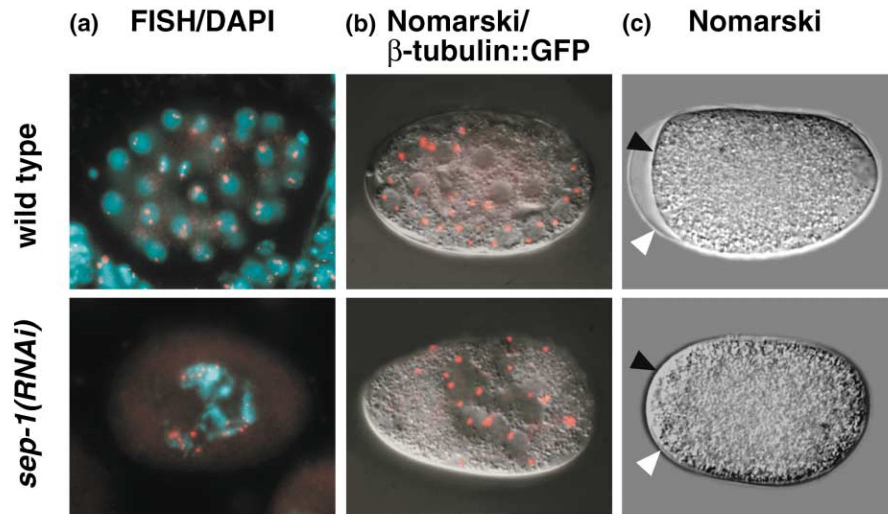
To address the actual mechanism used to resolve chiasmata in animal cells, we investigated the role of separase during meiosis in *C. elegans*. We chose this organism for two reasons: first and foremost because of the ease and rigor with which it is possible to deplete enzymes by RNA interference (RNAi) [29] and second because *C. elegans* is the only organism except for yeast in which it is known that REC-8 is required for sister chromatid cohesion during meiosis and is found along chromosome arms of bivalents at metaphase I [21]. We show here that separase is needed for the timely disjunction of homologs at the onset of anaphase I in *C. elegans*, which suggests that Rec8 cleavage might be a common trigger for chromosome segregation during meiosis I.

Results

Separase is required for sister chromatid separation during embryogenesis

To study the role of separase during the first meiotic division in *C. elegans*, we analyzed the consequences of separase depletion by RNAi or separase inactivation due to mutation of its gene. A database search revealed the presence of two open reading frames encoding predicted separase homologs in the *C. elegans* genome, namely, Y47G6A.12 (called *sep-1*) and ZK430.5. Both genes encode proteins containing the conserved histidine and cysteine residues that are believed to form the catalytic dyad of this class of proteases [10]. RNAi was used to inactivate, separately, both of these homologs. Interference with open reading frame ZK430.5 caused no detectable phenotype, while interference with *sep-1* caused 100% embryonic lethality 26 hr after double-stranded RNA (dsRNA) injection. The dead embryos contained a large mass of DNA in the center of a single cell, which had failed to undergo cleavage divisions (Figure 1a). Fluorescence in situ hybridization (FISH), using a probe directed to the 5S rDNA locus on the right end of chromosome V, revealed the presence of multiple FISH signals within the single-cell *sep-1(RNAi)* embryo. These observations suggest that inactivation of *sep-1* prevented both chromosome segregation and cytokinesis but did not interfere with the DNA replication cycle. *sep-1(RNAi)* also did not interfere with centrosome duplication as the single-cell separase-depleted embryos accumulated multiple centrosomes (Figure 1b). The continuation of centrosome duplication,

Figure 1



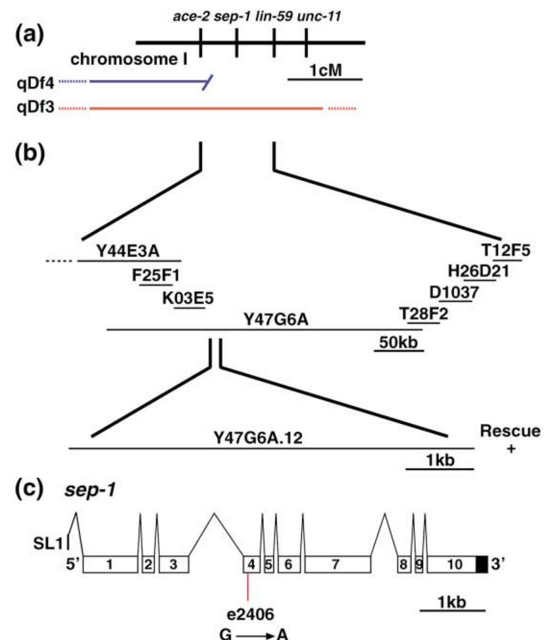
Phenotypes of *sep-1(RNAi)* embryos. *sep-1(RNAi)* was performed by dsRNA injection. **(a)** FISH directed to the 5S rDNA locus on chromosome V (red) was performed on wild-type and *sep-1(RNAi)* embryos. DNA is stained by DAPI (turquoise). **(b)** Overlay of Nomarski (gray) and β-tubulin::GFP images (red) of wild-type and *sep-1(RNAi)* embryos. **(c)** Nomarski images of wild-type and *sep-1(RNAi)* early embryos mounted in embryonic growth medium. The white and black arrowheads point to the eggshell and cell membrane, respectively.

in the absence of chromosome segregation in *sep-1(RNAi)* embryos, is similar to the situation in *S. cerevisiae* separase mutants (known as *esp1*), which were first identified on the basis of their accumulation of extra spindle pole bodies [30]. Two additional *sep-1(RNAi)* phenotypes were noticeable. The embryos lacked polar bodies and, furthermore, they had a swollen appearance without the usual gap between the eggshell and cell membrane, even before they had attempted any cleavage divisions, suggesting that they might be sensitive to their osmotic environment (Figure 1c).

The *sep-1(e2406)* temperature-sensitive maternal effect mutant worm contains a mutation that maps to the same genomic region as Y47G6A.12 (Figure 2a,b). Sequencing of the genomic locus identified a G to A transition mutation at position 1349 resulting in an amino acid substitution of cysteine to tyrosine in a nonconserved region of the protein (Figure 2c). The terminal phenotype of *sep-1(e2406)* mutant embryos fertilized after *sep-1(e2406)* homozygous mutant worms were shifted to the restrictive temperature was similar though not identical to that produced by depleting separase by RNAi (Figure 3).

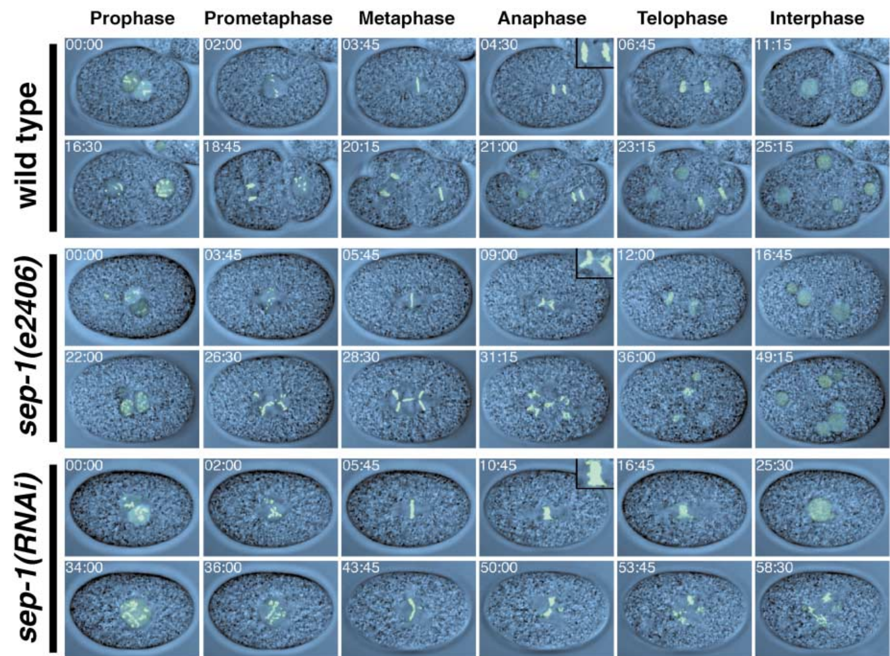
The *sep-1(e2406)* mutant and *sep-1(RNAi)* embryonic phenotypes were investigated using time-lapse microscopy of strains expressing a histone H2B transgene tagged with green fluorescent protein (GFP) (Figure 3). Despite earlier defects during oocyte meiosis (see below), the separase-defective embryos formed mitotic spindles on schedule, as visualized by Nomarski images, and both maternal and paternal chromosomes congressed to an apparently normal metaphase plate. Matters went awry only at the metaphase to anaphase transition. Chromatin either failed completely to segregate to opposite poles in *sep-1(RNAi)* embryos or did so only very slowly in *sep-1(e2406)* mutant embryos. Despite these massive defects in chromatid segregation and the complete lack of cytokinesis, chromosomes decondensed, mitotic spindles broke down, and embryos embarked on a second round of mitosis, again without success. The chromatid segregation defect of *sep-1(RNAi)* embryos was consistently more severe than that

Figure 2



Mapping *sep-1(e2406)*. **(a)** Genetic map in the region of *sep-1*. **(b)** Cosmids in the region of *sep-1*. Injection of cosmids did not provide rescue of the mutant phenotype, while a 6 kb PCR product from the YAC, Y47G6A, did. **(c)** Gene structure of *sep-1*, indicating the point mutation of *sep-1(e2406)*.

Figure 3



Time-lapse analysis of the early mitotic divisions in *sep-1(e2406)* and *sep-1(RNAi)* embryos. Nomarski (blue) and H2B::GFP (green) images overlaid from the early mitotic divisions of wild-type, *sep-1(e2406)*, and *sep-1(RNAi)* embryos mounted on 2% agarose pads in M9 buffer. The *sep-1(e2406)* mutant embryos filmed were fertilized at the restrictive temperature and were derived from homozygous *sep-1(e2406)* mutant worms shifted to the restrictive temperature at the early adult stage. The time at which each image was acquired is indicated in the top left-hand corner. Inlays show anaphase figures at higher magnification. *sep-1(RNAi)* was performed by soaking in dsRNA.

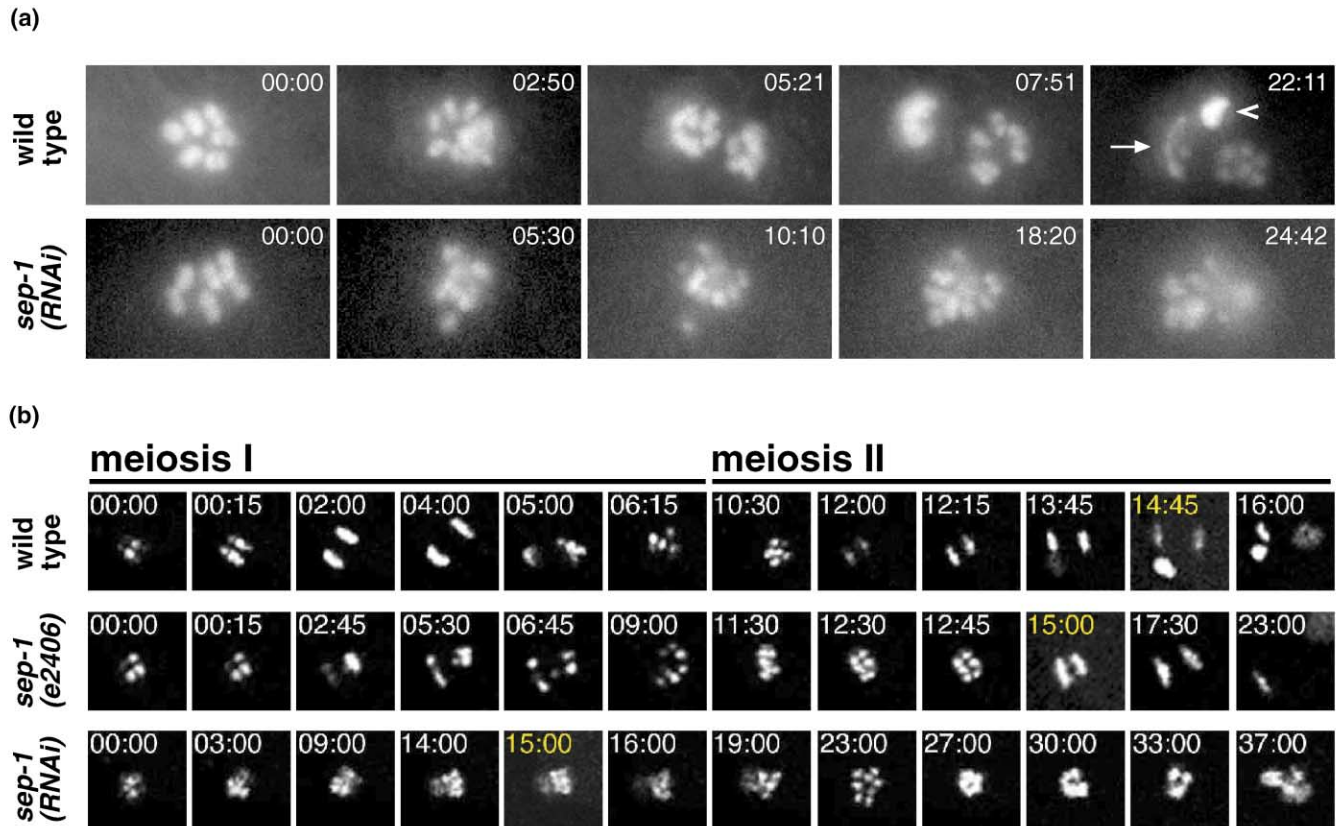
of *sep-1(e2406)* mutant embryos, suggesting that the *sep-1(e2406)* mutation does not completely inactivate separase at the restrictive temperature. The mitotic phenotype of inactivating separase in worms is consistent with the protease being specifically required for sister chromatid separation, as found in *S. cerevisiae* mutants [14].

The SEP-1 separase is required for proper homolog disjunction during meiosis I

In utero fluorescence time-lapse microscopy of worms containing a histone H2B::GFP fusion gene integrated into their genome allowed real-time visualization of chromosome segregation during both meiotic divisions. The early stages of meiosis I, up to and including the alignment of homologs on the first meiotic spindle, were unaffected by separase RNAi (see below). The first defect was only visible at the metaphase to anaphase transition of meiosis I, when embryos whose separase had been depleted by RNAi failed to segregate homologs in a timely manner (Figure 4a). In wild-type cells, the six bivalents align in a characteristic fashion on the first meiotic spindle during metaphase I, which occurs soon after fertilization. Soon thereafter, that is, within a few minutes, homologous chromosomes disjoin. One set forms the first polar body (Figure 4a; arrowhead), whereas the other undergoes the second meiotic division, producing a second polar body (Figure 4a; arrow) and what will become the female pronucleus. The characteristic alignment of bivalents on the first meiotic spindle occurred normally in *sep-1(RNAi)* embryos (Figure 4a; zero time point), but the chromosome segregation that should have ensued was delayed by at least 25 min (n = 3) or never occurred at all (n = 4) (Figure 4a,b). The *sep-1(e2406)* mutation also disrupted the first meiotic division, but its effect was less severe, with the result that a second meiotic division was clearly visible (n = 7) (Figure 4b).

To see whether individual chromosomes were disjoined when chromatin showed signs of having been pulled apart in *sep-1(RNAi)* worms, we used FISH to detect segregation of the 5S rDNA locus on chromosome V. During metaphase I, both wild-type and *sep-1(RNAi)* embryos contained either one (Figure 5) or two FISH signals. When interpreting FISH signals in metaphase I and in diakinesis, it must be considered that the pattern of FISH signals observed depends on the position of the crossover relative to the FISH probe. There is usually one crossover per chromosome per meiosis in *C. elegans*, and this crossover occurs in the terminal third region of chromosomes in 90% of autosomal crossovers [31]. Microtubules in meiosis I attach telocentrically to the chromosome end that is furthest from the point of crossing over [32]. If the crossover occurs near the FISH target, two closely associated FISH signals are seen near the center of the bivalent (Figure 5; arrows), while a crossover at the opposite end of the chromosome to the FISH target results in two FISH signals at either end of the bivalent (Figure 5; arrowheads). Each set of chromosomes produced by the first meiotic division in wild-type cells contained either one 5S FISH signal, corresponding to two adjacent chromatids, or two signals close together (Figure 5). This symmetry was rarely, if ever, observed in *sep-1(RNAi)*

Figure 4



Fluorescence time-lapse analysis of meiotic chromosomes in *sep-1(RNAi)* and *sep-1(e2406)* embryos. The meiotic divisions in wild-type, *sep-1(RNAi)*, and *sep-1(e2406)* embryos were followed by in utero fluorescence time-lapse microscopy using a strain with an integrated H2B::GFP fusion gene. The time at which the images were acquired is indicated at the top of each image. **(a)** The arrowhead and arrow show the first and second polar bodies, respectively. Images were acquired at a 100× magnification. *sep-1(RNAi)* was performed by dsRNA injection. **(b)** The *sep-1(e2406)* mutant embryos filmed were fertilized at the restrictive temperature and were derived from homozygous *sep-1(e2406)* mutant worms shifted to the restrictive temperature at the early adult stage. The 15 min time points are shown in yellow. *sep-1(RNAi)* was performed by soaking in dsRNA.

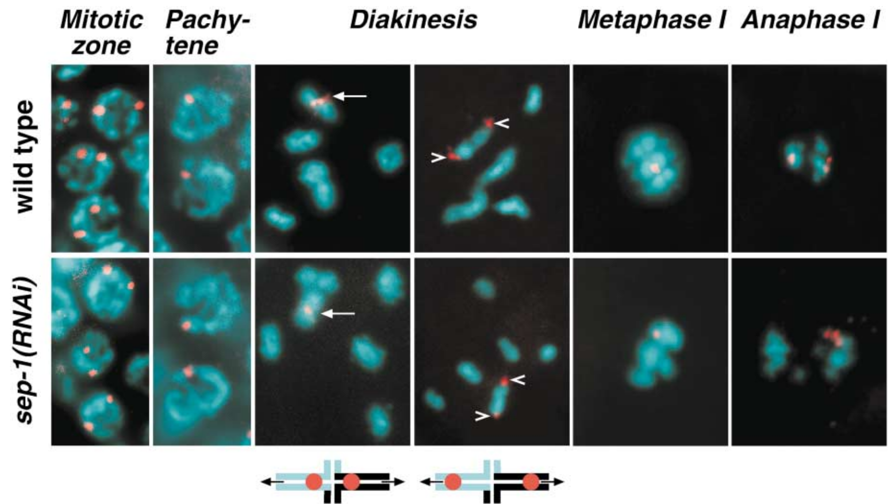
embryos. To be sure that we analyzed only those embryos that had clearly attempted to undergo anaphase I, we scored anaphase I figures in which there was some evidence of at least partial chromosome segregation. In 50% of such anaphase figures from *sep-1(RNAi)* embryos (n = 19), FISH signals were completely absent in one half of the chromatin mass (Figure 5). Inactivation of separase must, therefore, interfere with disjunction of homologs at the first meiotic division in *C. elegans*.

Premeiotic mitoses and homolog pairing in meiotic prophase I are unaffected in *sep-1(RNAi)* worms

To ensure that the failure in *sep-1(RNAi)* embryos to undergo anaphase I was not due to an earlier defect, we used FISH and DAPI staining to analyze earlier phases of meiosis as well as the preceding proliferative germline mitoses. As in wild-type, premeiotic mitotic germ cells in the gonad of *sep-1(RNAi)* worms contained two FISH signals per nucleus, whereas pachytene cells contained a single FISH signal (Figure 5). This suggests that RNAi treatment did not affect either premeiotic mitoses or chromosome synapsis. Diakinesis in wild-type is characterized by the presence of six end-to-end oriented bivalents corresponding to the six homologous chromosomes physically linked by chiasmata. Two FISH signals either near the center of the bivalent (Figure 5, arrows) or at either end of the bivalent (Figure 5, arrowheads) were observed in both wild-type and *sep-1(RNAi)* worms. We therefore conclude that diakinesis is normal in *sep-1(RNAi)* worms.

Our observation that chromosome segregation during premeiotic mitoses was unaffected in *sep-1(RNAi)* worms does not mean that separase activity is not required during gonadal development. Analysis of *sep-1(e2406)* worms showed precisely the opposite to be the case. Homozygous *sep-1(e2406)* mutant worms derived from heterozygous animals, shifted as larvae to the restrictive temperature, were sterile and failed to produce any normal meiotic

Figure 5



FISH analysis of the different stages of meiosis I in *sep-1(RNAi)* embryos. FISH directed to the 5S rDNA locus on chromosome V (red) was performed on wild-type and *sep-1(RNAi)* worms. DNA is stained by DAPI (turquoise). Arrows and arrowheads represent centrally and terminally positioned 5S FISH signals, respectively. Schematic illustrations of alternative FISH signal (red) patterns are shown below the diakinesis panels. Black arrows represent the direction of bivalent disjunction in meiosis I. *sep-1(RNAi)* was performed by dsRNA injection.

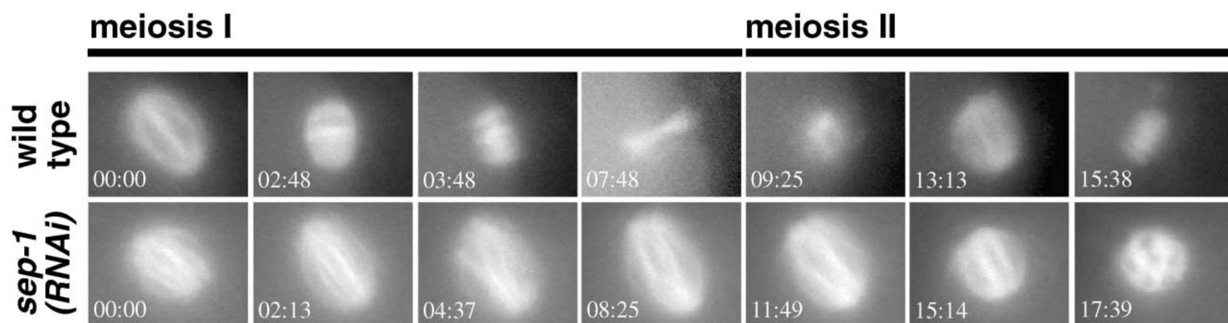
cells (data not shown). It is not surprising that gonadal development is unaffected by RNAi treatment, because worms are injected with dsRNA at the early adult stage when the gonads are already fully developed. The absence of a *sep-1(RNAi)* phenotype in the gonads was fortunate, as it allowed the study of the function of separase in *sep-1(RNAi)* embryos at the metaphase to anaphase transition of the first meiotic division in the absence of any prior mitotic or meiotic defect.

The first meiotic spindle appears normal in *sep-1(RNAi)* embryos

Two lines of evidence suggest that the failure to segregate chromosomes at the first meiotic division in *sep-1(RNAi)* embryos cannot be attributed to any obvious defect of the meiotic spindle. First, kinetic motion of chromosomes as they "struggle" to segregate in the first meiotic division indicates that pulling forces are exerted on the chromosomes and that these are, eventually, sufficient to segregate chromatin, albeit in a highly aberrant manner. Second, the meiotic spindle appeared normal when visualized by in utero fluorescence time-lapse microscopy using a line of worms with an extrachromosomal array containing an α-tubulin::GFP fusion gene (Figure 6). Although the first meiotic spindle appeared normal in *sep-1(RNAi)* embryos, it persisted for much longer than in wild-type embryos, which is consistent with the observation that chromosomes are pulled back and forth for a period exceeding the time it takes for both meiotic divisions in wild-type (Figure 4a). We conclude that the chromosomes of *sep-1(RNAi)* embryos come under tension on what appears to be a normal meiosis I spindle but, nevertheless, fail to disjoin.

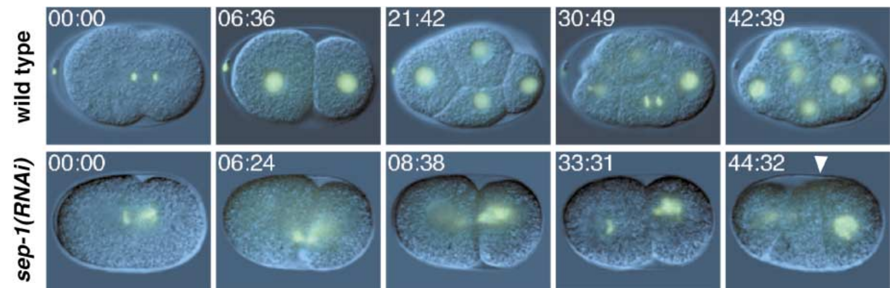
We cannot, of course, exclude the possibility that some

Figure 6



Fluorescence time-lapse analysis of the meiotic spindle in *sep-1(RNAi)* embryos. The meiotic spindles in wild-type and *sep-1(RNAi)* embryos were followed by in utero fluorescence time-lapse microscopy using an α-tubulin::GFP expressing line. The time at which each image was acquired is indicated in the bottom left-hand corner. *sep-1(RNAi)* was performed by dsRNA injection.

Figure 7



Rescue of the embryonic cytokinesis defect in *sep-1(RNAi)* embryos. Nomarski (blue) and H2B::GFP (green) images overlaid from the early mitotic divisions of wild-type and *sep-1(RNAi)* embryos. The white arrowhead points to the persistent cleavage furrow. Embryos were filmed in M9 buffer in the absence of a coverslip rather than on 2% agarose pads in M9 buffer, thus allowing cytokinesis to proceed in *sep-1(RNAi)* embryos. The time at which each image was acquired is indicated in the top left-hand corner. *sep-1(RNAi)* was performed by dsRNA injection.

more subtle aspect of the meiosis I spindle is defective in *sep-1(RNAi)* worms.

Separase is not required for cytokinesis in the early embryo

The lack of cytokinesis caused by separase inactivation in *C. elegans* (Figures 1a,b and 3) is striking because it differs from findings in *S. cerevisiae* [33] and *Schizosaccharomyces pombe* [34] where separase mutant cells form septa despite having failed to separate sister chromatids [14]. At first glance, this suggests that separase might be directly involved in promoting cell cleavage in *C. elegans* as well as sister chromatid separation. However, the finding that *sep-1(RNAi)* embryos are also swollen, even at very early stages after fertilization, raises an alternative and more mundane explanation. As it has been observed that excessive turgor can inhibit cell cleavage in early embryos (S. Kaitna and M.G., personal communication), the lack of cytokinesis in embryos might be an indirect consequence of an increase in turgor caused by separase inactivation. To test this, we sought medium conditions in which *sep-1(RNAi)* embryos were no longer swollen. We found that the gap between the eggshell and the cell membrane, which is normally seen in wild-type, is restored in *sep-1(RNAi)* embryos when they are incubated in M9 buffer in the absence of a coverslip (Figure 7). Embryonic growth medium (EGM) with different concentrations of egg salts was found to be an unsuitable medium for *sep-1(RNAi)* embryos, as the embryos died shortly after being dissected into the medium (data not shown). The fact that the swollen appearance of embryos can only be rescued in the absence of a coverslip suggests that the *sep-1(RNAi)* embryos are also sensitive to mechanical pressure. *sep-1(RNAi)* embryos incubated in M9 buffer formed apparently normal cleavage furrows at the same time as wild-type (Figure 7), even though they still completely failed to segregate their chromosomes. As a consequence, chromatin lying in the plane of cleavage was cut by the narrowing furrow, producing a "cut" phenotype reminiscent of fission yeast separase mutants [34]. We conclude that separase is not directly required for cytokinesis in the early embryo and that the lack of cell cleavage in *sep-1(RNAi)* embryos is a secondary consequence of their osmotic sensitivity. Our data do not exclude the possibility that separase activity might be required for cytokinesis during postembryonic divisions in cells with a much larger chromatin to mass ratio. They nevertheless suggest that separase does not have a direct role in promoting cell cleavage beyond ensuring that sister chromatids have been removed from the path of the cleavage furrow.

Discussion

Separase is required for mitosis and meiosis I

Separase is a conserved CD clan cysteine protease known to be required for separating sister chromatids during both mitosis [10] and meiosis [22] in ascomycetes. Crucial substrates include cohesin's Scc1 subunit and its meiotic counterpart Rec8. Recent work suggests that cleavage of Scc1 by separase [16] is also required for sister chromatid separation [17] in vertebrate cells. However, the direct consequences of inactivating separase in animal cells have not, thus far, been investigated. We show here that separase inactivation in *C. elegans*, either by mutation or by RNAi, prevents chromosome segregation during early embryonic divisions without affecting other aspects of the cell cycle. Inactivation of separase in *C. elegans* embryos completely blocks chromosome segregation without affecting centrosome duplication, DNA replication, chromosome condensation/decondensation, spindle formation, or cytokinesis. These results confirm that the function of separase during mitosis is highly conserved between yeast and animal cells and is primarily concerned with sister chromatid separation.

The primary goal of this study was, in fact, to address separase's role during the first meiotic division in *C. elegans*. In the yeast *S. cerevsiae*, cleavage of Rec8 along chromosome arms by separase is crucial for the resolution of chiasmata [22]. We wished to address whether animal cells use a similar mechanism or whether they instead utilize a separase-independent process, analogous to the one responsible for removing the bulk of cohesin from

chromosome arms during prophase and prometaphase in mitosis. Our finding that chromosome segregation during anaphase I but not any other preceding meiotic event is dependent on separase is consistent with the notion that proteolytic cleavage mediated by separase is a common feature of the first meiotic division in eukaryotic cells. A Rec8-like protein required for meiotic sister chromatid cohesion has been identified in *C. elegans* [21]. *C. elegans* REC-8 is associated with chromosomal axes from zygotene until metaphase I and persists on chromosomes until the onset of anaphase II. REC-8 is possibly only removed from those parts of the bivalents distal (with regard to the meiosis I kinetochore) to crossovers, and it is, therefore, difficult to detect a significant reduction in REC-8's abundance on chromosomes at the first metaphase to anaphase transition by immunofluorescence. Thus, we have not been able to address whether inactivation of separase affects the distribution or abundance of REC-8 as cells attempt to undergo anaphase I. REC-8 is, nevertheless, a good candidate for separase's main target during meiosis I. Our recordings of separase-depleted embryos suggest that homologs struggle to separate for at least 25 min longer than it takes for this process to be completed in wild-type embryos. FISH shows that even in embryos that manage some degree of chromosome segregation, homologous sequences on chromosome V fail to segregate from each other in at least 50% of what appear to be anaphase I figures. The RNAi phenotype is fully penetrant at the level of embryonic lethality (for embryos laid at least 26 hr after dsRNA injection), but this could disguise differences in the behavior of individual chromosomes at anaphase I.

If the failure of *sep-1(RNAi)* embryos to disjoin homologous chromosomes at meiosis I were due to their failure to resolve chiasmata, then the lack of chromosome segregation should be rescued by eliminating recombination. We examined the effect of separase inactivation on chromosome segregation during meiosis I in the *spo-11* mutant background which lacks meiotic recombination [35, 36]. Interpretation of our results was confounded due to the inherent variability in the timing and frequency of meiotic divisions in the *spo-11* mutant.

Recent data suggest that the APC is also required for the onset of anaphase I in *C. elegans* [25, 26]. It is likely that at least one of the APC's functions during meiosis I may be to activate separase by mediating the proteolysis of yet to be identified securin molecules and/or cyclins. The dependence of meiosis I in *C. elegans* on both the APC and separase is similar to the situation in yeast but contrasts with the recent finding that meiosis I in *Xenopus* takes place in the absence of APC activity [27, 28]. Clearly, further experiments will be required to address whether or not chiasmata are resolved by a universal mechanism in all eukaryotes, namely, cleavage of Rec8 by separase.

A novel role for separase in the *C. elegans* early embryo

Our study has revealed a potentially novel function for separase in the osmotic regulation of the *C. elegans* early embryo. Both *sep-1(RNAi)* and *sep-1(e2406)* mutant embryos are osmosensitive and lack a normal gap between the cell membrane and its eggshell, which suggests that they are under abnormal osmotic pressure. This defect as well as their subsequent failure to undergo cell cleavage is at least partly suppressed when the embryos are dissected into M9 buffer in the absence of a coverslip, even though their chromosome segregation defect is not ameliorated. We, therefore, propose that the failure of separase-depleted embryos to undergo cytokinesis is an indirect effect of their increased turgor, which hinders the establishment and/or constriction of cleavage furrows. Mutational inactivation of APC subunits, which should hinder separase activation, also results in an osmosensitive phenotype [25]. Other proteins required for meiotic chromosome segregation, such as INCENP, ICP-1, do not form osmosensitive embryos when depleted by RNAi [37], suggesting that the osmosensitivity does not result from inhibiting chromosome segregation in meiosis. Our observations raise the possibility that separase may be required for some aspect of eggshell formation after fertilization in *C. elegans*, as well as for triggering the first meiotic division.

Fertilization of oocytes by sperm is the trigger for the oocyte's meiotic maturation and for the formation of an eggshell [38, 39]. Oocyte meiotic maturation involves the alignment of the six diakinesis bivalents on the metaphase plate. The first and second meiotic divisions then follow in quick succession. Formation of the eggshell initiates in the spermatheca and is completed around 30 min later [39, 40]. In a related nematode, the eggshell is composed of three layers, namely, a vitelline membrane, a chitinous layer, and a lipid-rich layer [41]. The latter renders the early embryo impermeable to most solutes. Both *sep-1(RNAi)* and *sep-1(e2406)* mutant embryos have a visible eggshell which, nevertheless, appears to be defective, causing embryos to be excessively permeable to solutes. It is striking that both the first meiotic division and the eggshell are defective in *sep-1(RNAi)* and *sep-1(e2406)* mutant embryos and that both of these processes are triggered by the fertilization of oocytes by sperm. This raises the possibility that the sperm carries with it a signal that, directly or indirectly, activates separase, which in turn causes disjunction of homologous chromosomes at anaphase I, as well as ensuring the formation of an impermeable eggshell. The sperm-supplied SPE-11 protein that is required for both completion of oocyte meiosis and for proper eggshell formation [40, 42] could, in theory, be such a candidate signal. The fact that APC *emb-27(g48)* mutant embryos are also osmotically sensitive [25] suggests that the APC and separase act in the same pathway, that is, the osmosensitivity of APC mutants could be due

to their failure to activate separase. It is possible that cleavage of REC-8 or of some other protein could be required for correct eggshell formation. Separase causes cleavage of the *S. cerevisiae* kinetochore protein Slk19 [43] as well as Scc1 at the onset of anaphase during mitosis [44], which suggests that the worm protease might also have multiple substrates.

Changes in the extracellular structures of the egg upon fertilization are not specific to *C. elegans*. In fact, such changes occur in many animals and are often associated with the prevention of polyspermy. In *Xenopus*, fertilization causes the conversion of the vitelline envelope to the fertilization envelope which can no longer be penetrated by sperm [45–47]. Mammalian oocytes are also surrounded by an extracellular structure called the "zona pellucida" [48]. Following fertilization in mouse, modification of the zona pellucida glycoproteins ZP2 and ZP3 results in a block to polyspermy [49–52]. The novel role of separase in the formation or maintenance of the extracellular structures of the eggshell might thus be of wider significance.

Materials and methods

Strains and alleles

All *C. elegans* strains were derived from wild-type Bristol N2 and were cultured as previously described [53]. Strains and alleles used: Bristol N2, CB5119 [*sep-1(e2406*ts*)*], MG155(*xsIs3*[*H2B::GFP*]), MG240 (*spo-11(ok79)/dpy-20 unc-24* IV; *xsIs3*[*H2B::GFP*] I), WH204 (*unc-119(ed3)*; *ojIs1*[β*-tubulin::GFP unc-119(+)*]), RE249 (qDf4/szT1 I; +/szT1[*lon-2(e678)*] X), JK323 (qDf3/szT1[*lon-2(e678)*] I; +/szT1 X), *fog-1(e2121)*, *ace-2(g72)*, *unc-11(e47)*, *dpy-5(e61)*, *ace-1(p1000),* AZ212 (*unc-119*; *ruIs32*[*H2B::GFP*]), and WH0213 (*sep-1(e2406*ts*)*/hT2[*qIs48*] I; *unc-119*; *ruIs32*[*H2B::GFP*]/hT2[*qIs48*] III). Bristol N2 containing an extrachromosomal array of α-tubulin::GFP [54] was also used. The α-tubulin::GFP worms as well as strains MG155 and MG240 were maintained at 25°C to avoid germline silencing of the GFP fusion genes. The permissive and restrictive temperatures for *sep-1(e2406*ts*)* are 16°C and 25°C, respectively.

Mapping and cloning and molecular analysis of sep-1

Two separate three-factor crosses were performed to map *sep-1* between *fog-1* and *unc-11* and to the right of *ace-2* on chromosome I. From *fog-1 unc-11/sep-1* heterozygotes, we obtained *fog-1* (23/38) *sep-1* (15/38) *unc-11*, and from *ace-2/sep-1 dpy-5; ace-1* heterozygotes we obtained *ace-2* (0/42) *sep-1* (42/42) *dpy-5.* The deficiency qDf4 complements *sep-1(e2406)*, whereas qDf3 does not. The interpolated map position of *sep-1* places it between *ace-2* and *lin-59*. In order to identify *sep-1*, we disrupted gene function by RNAi [29] of predicted genes in the region beginning with Y47G6A and found that disruption of Y47G6A.12 phenocopied the *sep-1(e2406*ts*)* one-cell-arrest mutant phenotype. Three independent 6 kb genomic fragments containing only Y47G6A.12 were amplified from single N2 worms using the Expand™ Long Template PCR System (Roche Diagnostics GmbH, Germany), pooled together, and used to transform balanced *sep-1* heterozygotes as before. We obtained rescue of the postembryonic defects in all lines tested (15 lines) at both an intermediate temperature of 20°C and the restrictive temperature of 25°C. Automated DNA sequencing of the same 6 kb region from three independent templates of the mutant *sep-1(e2406*ts*)* was performed using the ABI 377XL sequencer at the UW Biotechnology Center. We found a single transition mutation from G to A at position 1349 of the predicted gene that results in the amino acid conversion of a cysteine to a tyrosine. We concluded that Y47G6A.12 was *sep-1.*

To determine the gene structure of *sep-1*, we sequenced one of the longest cDNAs available, yk429h5, and also RT-PCR products from RNA (using 5′/3′ RACE Kit, Roche Diagnostics GmbH, Germany). The obtained sequence confirmed the GeneFinder predicted gene structure in Wormbase and also revealed the presence of the *trans*-spliced leader SL1 at the 5′ end of *sep-1*.

RNA-mediated interference

RNAi was performed either by injection (Figures 1, 4a, 5, 6, and 7) or by soaking (Figures 3 and 4b). For injection, dsRNA was prepared as follows: PCR products (~500 bp) corresponding to *sep-1* cDNA and ZK430.5 predicted cDNA were amplified from *C. elegans* cDNA using primer pairs V24 (5′-CGTACCTTGTCATCTGTCC-3′) and V25 (5′-CGATTTCACCATCAGTGAC-3′); and V19 (5′-CAAGACCAAACGATTCCCG-3′) and V20 (5′-CATCCCATAAGCATCCAAC-3′), respectively. The PCR products were cloned into the pGEM-T vector (Promega, Madison, Wisconsin). Single-stranded RNAs were generated by in vitro transcription using the T3 and SP6 MeGAscript™ kits (Ambion, Austin, Texas), annealed to form dsRNAs, and were injected into both gonad arms of young adult hermaphrodites as previously described [29]. The animals were allowed to recover on NGM plates for at least 26 hr at 25°C prior to imaging.

sep-1 dsRNA used in soaking experiments was made as above, but the PCR product was amplified from the Kohara clone yk429h5, using primers MK11 (5′-TTGTAAAACGACGGCCAG-3′) and MK12 (5′-CATGATTACGCCAAGCTC-3′). DsRNA was dissolved in M9 buffer at a concentration of ~1 mg/ml. About 30 L4 larvae were soaked in 30 μl of dsRNA in an Eppendorf tube for 24 hr at 20°C. The animals were allowed to recover on NGM plates for 14–24 hr prior to imaging.

Microscopy

Two live cell-imaging setups were used for fluorescence time-lapse microscopy.

For Figures 4a, 6, and 7, a Zeiss Axiovert microscope equipped with an AttoArc2 HBO 100 W light source dimmed to 20%, a CoolSnap FX (Roper Scientific) camera, and GFP filter (480/40 bandpass excitation filter, a 505LP beamsplitter and a 505LP-emission filter from Chroma) were used for time-lapse imaging. All images were taken with a 100×/1.3 neofluor objective. MetaMorph (Universal Imaging) was used to control the camera and to process acquired images. Time points were taken every 10–20 s and at each time point, fluorescent images from three to five different focal planes (typically 1.5–2.5 μm apart), and one Nomarski image were acquired. The images from the various focal planes were projected into a single image using the Metamorph maximum intensity algorithm. For Figures 3 and 4b, the Optical Workstation (constructed by D. Wokosin and J. White) using two-photon excitation with the Ti:sapphire laser tuned to 900 nm was used. Single focal plane images were obtained using the Bio-Rad MRC1024 software with manual adjustment of the *z* axis.

For in utero filming of the meiotic divisions, whole worms were mounted either on 2% agarose pads in 1 mg/ml levamisole diluted in M9 buffer [53] (Figures 4a and 6) or in 0.1% tricaine, 0.01% tetramisole [38] (Figure 4b). In order to film embryonic mitotic divisions, embryos were dissected into M9 buffer and filmed either on 2% agarose pads in M9 buffer (Figure 3) or in M9 buffer in the absence of a coverslip (Figure 7). EGM (Figure 1c) was prepared as described [55], without L-Tyrosine, amino acid stock, penicillin-streptomycin, base mix, L-Glutamine, BME vitamins, Na_2HPO_4, $MgSO_4$, or chicken egg yolk.

Fluorescence in situ hybridization

FISH was performed using a 133 bp PCR-labeled digoxygenin-11-dUTP probe directed to the 5S rDNA locus on chromosome V. The FISH procedure was performed as previously described [21], with the following modification: instead of cutting worms open, pressure was applied to whole worms with a coverslip so that embryos were extruded from the uterus.

Supplementary material
Supplementary material including time-lapse movies of Figures 3, 4, 6, and 7 is available at http://images.cellpress.com/supmat/supmatin.htm.

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References

1. Hassold T, Hunt P: **To err (meiotically) is human: the genesis of human aneuploidy.** *Nat Rev Genet* 2001, **2:**280-291.
2. Sen S: **Aneuploidy and cancer.** *Curr Opin Oncol* 2000, **12:**82-88.
3. Michaelis C, Ciosk R, Nasmyth K: **Cohesins: chromosomal proteins that prevent premature separation of sister chromatids.** *Cell* 1997, **91:**35-45.
4. Guacci V, Koshland D, Strunnikov A: **A direct link between sister chromatid cohesion and chromosome condensation revealed through the analysis of MCD1 in S. cerevisiae.** *Cell* 1997, **91:**47-57.
5. Losada A, Hirano M, Hirano T: **Identification of Xenopus SMC protein complexes required for sister chromatid cohesion.** *Genes Dev* 1998, **12:**1986-1997.
6. Toth A, Ciosk R, Uhlmann F, Galova M, Schleiffer A, Nasmyth K: **Yeast cohesin complex requires a conserved protein, Eco1p(Ctf7), to establish cohesion between sister chromatids during DNA replication.** *Genes Dev* 1999, **13:**320-333.
7. Darwiche N, Freeman LA, Strunnikov A: **Characterization of the components of the putative mammalian sister chromatid cohesion complex.** *Gene* 1999, **233:**39-47.
8. Sumara I, Vorlaufer E, Gieffers C, Peters BH, Peters JM: **Characterization of vertebrate cohesin complexes and their regulation in prophase.** *J Cell Biol* 2000, **151:**749-762.
9. Uhlmann F, Nasmyth K: **Cohesion between sister chromatids must be established during DNA replication.** *Curr Biol* 1998, **8:**1095-1101.
10. Uhlmann F, Wernic D, Poupart MA, Koonin EV, Nasmyth K: **Cleavage of cohesin by the CD clan protease separin triggers anaphase in yeast.** *Cell* 2000, **103:**375-386.
11. Jallepalli PV, Waizenegger IC, Bunz F, Langer S, Speicher MR, Peters JM, *et al.*: **Securin is required for chromosomal stability in human cells.** *Cell* 2001, **105:**445-457.
12. Cohen-Fix O, Peters JM, Kirschner MW, Koshland D: **Anaphase initiation in Saccharomyces cerevisiae is controlled by the APC-dependent degradation of the anaphase inhibitor Pds1p.** *Genes Dev* 1996, **10:**3081-3093.
13. Funabiki H, Yamano H, Kumada K, Nagao K, Hunt T, Yanagida M: **Cut2 proteolysis required for sister-chromatid seperation in fission yeast.** *Nature* 1996, **381:**438-441.
14. Ciosk R, Zachariae W, Michaelis C, Shevchenko A, Mann M, Nasmyth K: **An ESP1/PDS1 complex regulates loss of sister chromatid cohesion at the metaphase to anaphase transition in yeast.** *Cell* 1998, **93:**1067-1076.
15. Warren WD, Steffensen S, Lin E, Coelho P, Loupart M, Cobbe N, *et al.*: **The Drosophila RAD21 cohesin persists at the centromere region in mitosis.** *Curr Biol* 2000, **10:**1463-1466.
16. Waizenegger IC, Hauf S, Meinke A, Peters JM: **Two distinct pathways remove mammalian cohesin from chromosome arms in prophase and from centromeres in anaphase.** *Cell* 2000, **103:**399-410.
17. Hauf S, Waizenegger IC, Peters JM: **Cohesin cleavage by separase required for anaphase and cytokinesis in human cells.** *Science* 2001, **293:**1320-1323.
18. Orr-Weaver TL: **The ties that bind: localization of the sister-chromatid cohesin complex on yeast chromosomes.** *Cell* 1999, **99:**1-4.
19. Maguire MP: **Letter: The need for a chiasma binder.** *J Theor Biol* 1974, **48:**485-487.
20. Klein F, Mahr P, Galova M, Buonomo SB, Michaelis C, Nairz K, *et al.*: **A central role for cohesins in sister chromatid cohesion, formation of axial elements, and recombination during yeast meiosis.** *Cell* 1999, **98:**91-103.
21. Pasierbek P, Jantsch M, Melcher M, Schleiffer A, Schweizer D, Loidl J: **A Caenorhabditis elegans cohesion protein with functions in meiotic chromosome pairing and disjunction.** *Genes Dev* 2001, **15:**1349-1360.
22. Buonomo SB, Clyne RK, Fuchs J, Loidl J, Uhlmann F, Nasmyth K: **Disjunction of homologous chromosomes in meiosis I depends on proteolytic cleavage of the meiotic cohesin Rec8 by separin.** *Cell* 2000, **103:**387-398.
23. Watanabe Y, Nurse P: **Cohesin Rec8 is required for reductional chromosome segregation at meiosis.** *Nature* 1999, **400:**461-464.
24. Paliulis LV, Nicklas RB: **The reduction of chromosome number in meiosis is determined by properties built into the chromosomes.** *J Cell Biol* 2000, **150:**1223-1232.
25. Golden A, Sadler PL, Wallenfang MR, Schumacher JM, Hamill DR, Bates G, *et al.*: **Metaphase to anaphase (mat) transition-defective mutants in Caenorhabditis elegans.** *J Cell Biol* 2000, **151:**1469-1482.
26. Furuta T, Tuck S, Kirchner J, Koch B, Auty R, Kitagawa R, *et al.*: **EMB-30: an APC4 homologue required for metaphase-to-anaphase transitions during meiosis and mitosis in Caenorhabditis elegans.** *Mol Biol Cell* 2000, **11:**1401-1419.
27. Peter M, Castro A, Lorca T, Le Peuch C, Magnaghi-Jaulin L, Doree M, *et al.*: **The APC is dispensable for first meiotic anaphase in Xenopus oocytes.** *Nat Cell Biol* 2001, **3:**83-87.
28. Taieb FE, Gross SD, Lewellyn AL, Maller JM: **Activation of the anaphase-promoting complex and degradation of cyclin B is not required for progression from meiosis I to II in Xenopus oocytes.** *Curr Biol* 2001, **11:**508-513.
29. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: **Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans.** *Nature* 1998, **391:**806-811.
30. Baum P, Yip C, Goetsch L, Byers B: **A yeast gene essential for regulation of spindle pole duplication.** *Mol Cell Biol* 1988, **8:**5386-5397.
31. Barnes TM, Kohara Y, Coulson A, Hekimi S: **Meiotic recombination, noncoding DNA and genomic organization in Caenorhabditis elegans.** *Genetics* 1995, **141:**159-179.
32. Albertson DG, Thomson JN: **Segregation of holocentric chromosomes at meiosis in the nematode, Caenorhabditis elegans.** *Chromosome Res* 1993, **1:**15-26.
33. McGrew JT, Goetsch L, Byers B, Baum P: **Requirement for ESP1 in the nuclear division of Saccharomyces cerevisiae.** *Mol Biol Cell* 1992, **3:**1443-1454.
34. Uzawa S, Samejima I, Hirano T, Tanaka K, Yanagida M: **The fission yeast cut1+ gene regulates spindle pole body duplication and has homology to the budding yeast ESP1 gene.** *Cell* 1990, **62:**913-925.
35. Keeney S, Giroux CN, Kleckner N: **Meiosis-specific DNA double-strand breaks are catalyzed by Spo11, a member of a widely conserved protein family.** *Cell* 1997, **88:**375-384.
36. Dernburg AF, McDonald K, Moulder G, Barstead R, Dresser M, Villeneuve AM: **Meiotic recombination in C. elegans initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis.** *Cell* 1998, **94:**387-398.
37. Kaitna S, Mendoza M, Jantsch-Plunger V, Glotzer M: **Incenp and an aurora-like kinase form a complex essential for chromosome segregation and efficient completion of cytokinesis.** *Curr Biol* 2000, **10:**1172-1181.
38. McCarter J, Bartlett B, Dang T, Schedl T: **On the control of oocyte meiotic maturation and ovulation in Caenorhabditis elegans.** *Dev Biol* 1999, **205:**111-128.
39. Ward S, Carrel JS: **Fertilization and sperm competition in the nematode Caenorhabditis elegans.** *Dev Biol* 1979, **73:**304-321.

40. Hill DP, Shakes DC, Ward S, Strome S: **A sperm-supplied product essential for initiation of normal embryogenesis in Caenorhabditis elegans is encoded by the paternal-effect embryonic-lethal gene, spe-11.** *Dev Biol* 1989, **136:**154-166.
41. Chitwood BG, Chitwood MB: *Introduction to nematology*. Baltimore: University Park Press; 1974.
42. Browning H, Strome S: **A sperm-supplied factor required for embryogenesis in C. elegans.** *Development* 1996, **122:**391-404.
43. Sullivan M, Lehane C, Uhlmann F: **Orchestrating anaphase and mitotic exit: separase cleavage and localization of Slk19.** *Nat Cell Biol* 2001, **3:**771-777.
44. Uhlmann F, Lottspeich F, Nasmyth K: **Sister-chromatid separation at anaphase onset is promoted by cleavage of the cohesin subunit Scc1.** *Nature* 1999, **400:**37-42.
45. Grey RD, Wolf DP, Hedrick JL: **Formation and structure of the fertilization envelope in Xenopus laevis.** *Dev Biol* 1974, **36:**44-61.
46. Wolf DP: **The cortical granule reaction in living eggs of the toad, Xenopus laevis.** *Dev Biol* 1974, **36:**62-71.
47. Gerton GL, Hedrick JL: **The vitelline envelope to fertilization envelope conversion in eggs of Xenopus laevis.** *Dev Biol* 1986, **116:**1-7.
48. Bleil JD, Wassarman PM: **Mammalian sperm-egg interaction: identification of a glycoprotein in mouse egg zonae pellucidae possessing receptor activity for sperm.** *Cell* 1980, **20:**873-882.
49. Schultz RM, Kopf GS: **Molecular basis of mammalian egg activation.** In *Current Topics in Developmental Biology.* Edited by Pedersen RA, Schatten G. San Diego: Academic Press; 1995:21-62.
50. Wassarman PM: **Zona pellucida glycoproteins.** *Annu Rev Biochem* 1988, **57:**415-442.
51. Moller CC, Wassarman PM: **Characterization of a proteinase that cleaves zona pellucida glycoprotein ZP2 following activation of mouse eggs.** *Dev Biol* 1989, **132:**103-112.
52. Miller DJ, Gong X, Decker G, Shur BD: **Egg cortical granule N-acetylglucosaminidase is required for the mouse zona block to polyspermy.** *J Cell Biol* 1993, **123:**1431-1440.
53. Brenner S: **The genetics of Caenorhabditis elegans.** *Genetics* 1974, **77:**71-94.
54. Oegema K, Desai A, Rybina S, Kirkham M, Hyman AA: **Functional Analysis of Kinetochore Assembly in Caenorhabditis elegans.** *J Cell Biol* 2001, **153:**1209-1226.
55. Edgar GE: Blastomere Culture and Analysis. In *Caenorhabditis elegans Modern Biological Analysis of an Organism.* Edited by Epstein HF, Shakes DC. San Francisco: Academic Press; 1995:303-321.