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► **To cite this version:**

Elvira Nikalayevich, Safia El Jailani, Aude Dupré, Damien Cladière, Yulia Gryaznova, et al.. Aurora B/C-dependent phosphorylation promotes Rec8 cleavage in mammalian oocytes. *Current Biology - CB*, 2022, 10.1016/j.cub.2022.03.041 . hal-03637686

HAL Id: hal-03637686

<https://hal.science/hal-03637686>

Submitted on 11 Apr 2022

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Aurora B/C-dependent phosphorylation promotes Rec8 cleavage in mammalian oocytes

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Summary

To generate haploid gametes, cohesin is removed in a step-wise manner from chromosome arms in meiosis I and the centromere region in meiosis II, to segregate chromosomes and sister chromatids, respectively. Meiotic cohesin removal requires cleavage of the meiosis-specific kleisin subunit Rec8 by the protease Separase^{1,2}. In yeast and *C. elegans*, Rec8 on chromosome arms has to be phosphorylated to be cleaved in meiosis I³⁻⁷, whereas Rec8 at the centromere is protected from cleavage by the action of PP2A-B56⁸⁻¹⁰. However, in mammalian meiosis it is unknown whether Rec8 has to be equally phosphorylated for cleavage, and if so, the identity of the relevant kinase(s). This is due to technical challenges, as Rec8 is poorly conserved preventing a direct translation of the knowledge gained from model systems such as yeast and *C. elegans* to mammals. Additionally, there is no turn-over of Rec8 after cohesion establishment, preventing phosphomutant analysis of functional Rec8. To address the very basic question of whether Rec8 cleavage requires its phosphorylation in mammals, we adapted a biosensor detecting Separase activity to study Rec8 cleavage in single mouse oocytes by live imaging. Crucially, through phosphomutant analysis we identified phosphorylation sites in Rec8 promoting cleavage. We found that Rec8 cleavage depends on Aurora B/C kinase activity, and identified an aminoacid residue that is phosphorylated *in vivo*. Accordingly, inhibition of Aurora B/C kinase during meiotic maturation impairs endogenous Rec8 phosphorylation and chromosome segregation.

Keywords

Rec8, Cohesin, Aurora kinases, Oocytes, Meiosis, Separase, Bivalent segregation, Chromosome segregation, metaphase I, phosphorylation

Results and discussion

Yeast Rec8 is phosphorylated by CK1 δ/ϵ (Hrr25 in *S. cerevisiae*, Hhp2 in *S. pombe*) and DDK (Cdc7-Dbf4 in *S. cerevisiae*, possibly Hsk1/Dfp1 in *S. pombe*) for cleavage by Separase³⁻⁶. In *C. elegans* oocytes, Rec8 is phosphorylated by AIR-2 (Aurora B), which is recruited to the short chromosome arms in a Haspin kinase-dependent manner to bring about Rec8 cleavage⁷. We hypothesized that also in vertebrate meiosis one of these kinases may be required for Rec8 cleavage.

Aurora kinases are primarily regulated through their localization and interaction with their molecular partners, and not their individual substrate specificities. In mammalian oocytes three Aurora kinases are expressed, namely Aurora A, B and the oocyte-specific Aurora C^{11,12}. They are required for proper bipolar spindle formation, chromosome alignment and correction of faulty attachments. To address whether chromosome segregation defects -indicative of failure to cleave Rec8- are observed upon loss of Aurora B and C, oocytes entering meiosis I were treated with inhibitors targeting both Aurora B and C, namely AZD1152 and ZM447439¹³⁻¹⁷. Efficient Aurora B/C inhibition was verified by staining metaphase I chromosome spreads with a phospho-specific antibody against Histone H3pS (Serine) 10, a prominent substrate of both kinases also in oocytes^{15,16,18} (Figure 1A). Because of Aurora B/C's role in cytokinesis preventing polar body (PB) extrusion^{16,17,19}, we verified whether these oocytes nevertheless underwent metaphase-to-anaphase transition, a condition to analyse chromosome segregation. Exogenously expressed Securin-YFP degradation was assessed by live imaging. As previously published, degradation occurred in an accelerated manner in oocytes without Aurora B/C activity due to the absence of error correction signaling to the Spindle Assembly Checkpoint (SAC) (Figure 1B)^{17,19}. Securin-YFP degradation shows that inhibitor-treated oocytes are not cell-cycle arrested and proceed through meiosis I.

To determine whether bivalent chromosomes segregate in absence of Aurora B/C kinase activity, chromosome spreads were prepared from metaphase II oocytes treated with Aurora B/C inhibitors throughout meiotic maturation. Whereas control oocytes harbored only paired sister chromatids (dyads), the majority of oocytes treated with Aurora B/C inhibitors contained several bivalents (paired chromosomes), indicating failures in meiosis I chromosome segregation (Figure 1C-G). This phenotype is not due to absence of error correction resulting in chromosomes being attached to only one pole, because -as we have shown previously- bivalents attached to a monopolar spindle still separate homologous chromosomes²⁰. Hence, Aurora B/C kinase may be involved in efficient arm cohesin removal

in meiosis I. Indeed, bivalents in Aurora B/C inhibitor-treated oocytes maintain some Rec8 on chromosome arms, revealing failure of complete arm cohesin removal (Figure 1H).

Loss of Aurora B/C can be complemented in part by Aurora A²¹, and complete loss of Aurora A interferes with proper spindle formation^{22,23}, leading to a cell cycle arrest in metaphase I²³. To address whether Aurora A is also required for chromosome segregation in meiosis I, we treated oocytes with the Aurora A inhibitor MLN8237²⁴ from entry into meiosis I onwards. Such as Aurora A knock-out oocytes, MLN8237-treated oocytes arrested in metaphase I, indicating that inhibition of Aurora A was efficient^{22,23} (Figure S1A). We added the Mps1 inhibitor Reversine to override the SAC arrest²⁵ which is at least partially responsible for the metaphase I arrest induced by Aurora A inhibition²³, and performed chromosome spreads 14 hours later. Figure S1A shows that inhibition of Aurora A and simultaneously overriding the SAC arrest rescued PB extrusion in 40 % of MLN8237 treated oocytes, similar to previously published data²³. Importantly, MLN8237 treated oocytes extruding PBs also segregated chromosomes (Figure S1B). Hence, we conclude that in presence of Aurora B/C, Aurora A is not required for cohesin removal in oocyte meiosis I.

It was attractive to speculate that Aurora B/C directly phosphorylate Rec8 to convert it into a Separase substrate. But it was just as likely that Aurora B/C activity were required for Separase activation itself. Separase activity can be analysed in live cells using a biosensor containing the mitotic kleisin subunit Rad21 sandwiched between two fluorescent tags and fused to H2B, to localize the sensor to chromosomes for quantification^{26,27}. Of note, in oocytes expression of this and similar constructs containing kleisin subunits does not produce cohesive cohesin complexes, as those are put in place long before entry into the first meiotic division, before birth of the female²⁸. Loss of co-localization of the two fluorophores on chromosomes indicates Separase cleavage of the sensor (Figure 2A). Notably, sensor cleavage was not perturbed by addition of any of two Aurora B/C kinase inhibitors from resumption of meiosis I onwards (Figure 2B). Thus, Separase is activated with comparable efficiency in the presence or absence of Aurora B/C activity. Aurora B/C kinases are therefore not required for Separase activation in meiosis I.

To ask whether Aurora B/C targets Rec8 for cleavage by Separase, we decided to adapt the Rad21 sensor assay to Rec8. For this, a sensor construct containing full length Rec8 was cloned and *in vivo* cleavage by Separase was analysed by quantifying loss of co-localization of the two fluorophores in oocytes. A recent

study had shown that a Rec8 sensor spanning 297-506aa is not cleaved *in vivo*, with the caveat that cleavage assays were performed in mitotic cells²⁹. In our hands, full-length Rec8 sensor was cleaved *in vivo* in meiosis I oocytes (Figure 3A). Importantly, cleavage was abolished when 39 out of 59 Serines and Threonines were mutated to Alanine (Figure 3A, Table S1), indicating that phosphorylation of at least some of those sites is required for Rec8 cleavage.

The low expression levels of the full-length sensor prompted us to test truncated versions of Rec8. Also, potential interference of other cohesin binding proteins when examining phosphorylation site mutations should be limited in truncated sensor constructs. Not surprisingly, a Rec8 fragment spanning the first 143aa and not containing any potential Separase cleavage sites was not cleaved at all in oocytes. A Rec8 fragment spanning 1-290aa and 143-290aa was not efficiently or not at all cleaved, respectively, even though they contain five potential minimal Separase cleavage sites, one of which allowed Rec8 cleavage *in vitro* in a previous study³⁰. The two most likely Separase cleavage sites (R434 and R454)³⁰ are found in Rec8 fragment 380-486aa, and indeed, this fragment was efficiently cleaved (Figure 3B, S2A and B). Cleavage was strongly reduced upon mutation of the two Separase cleavage sites (Rec8 380-486aa ExxD*2) (Figure 3B, Table S1). Accordingly, cleavage was Separase dependent, because it did not take place in conditional Separase knock-out oocytes³¹ (Figure 3C, Figure S2C). Therefore, a biosensor containing a Rec8 fragment can be used to dissect the requirements for Separase cleavage in oocytes, unlike expression of a truncated sensor in mitotic cells, which needed to be fused with the protein Meikin to be cleaved in a recent study²⁹.

Cleavage of the C-terminal Rec8 380-486aa fragment was abolished when all 17 Serines and Threonines were mutated to Alanine, indicating that phosphorylation of at least some of these residues is necessary for efficient Separase cleavage (Table S1, Figure 3B). Furthermore, sensor cleavage did not occur in the presence of Aurora B/C inhibitor (Figure 3D). Mutating only 5 Serines to Alanine around and including a conserved Aurora B/C consensus site -Serine S482- was enough to completely abrogate cleavage of the sensor, and mutating three (S466, 467 and 470) or two Serines (S479 and 482) to Alanine significantly decreased cleavage (Figure 3E). Substitution of Serine 479 or 482 to Aspartate, mimicking a phosphorylated residue, allowed efficient cleavage, in agreement with these sites being phosphorylated. However, we think that phosphorylation of other sites besides Serine 479 and 482 is required or takes place when these sites are mutated, because a fragment where 14 Serine/Threonine residues except in the Aurora B consensus at S482 (SRTS) were substituted to Alanine, is hardly cleaved (Figure 3F, compare

to the vertical dotted lines representing standard deviations of the negative control). Crucially, phosphomimetic substitutions on Serine 479 and 482 allowed some cleavage of Rec8 380-486 14S/T-A, indicating that 1) phosphorylation of those sites (S479 and S482) does not take place in the 14S/T-A mutant and 2) phosphorylation of S479 and S482 promotes cleavage. Rec8 380-486aa peptide but not Rec8 380-486 17S/T-A produced in bacteria was thiophosphorylated by Aurora B-INCENP *in vitro*, indicating that Aurora B/C may indeed directly phosphorylate Rec8 (Figure 3G). Rec8 380-486aa sensor carrying 4 Aspartate substitutions around the conserved Aurora B/C consensus sites was cleaved in presence of Aurora B/C inhibitor, albeit with low efficiency, again indicating that other kinases and phosphorylation sites additionally contribute to Rec8 cleavage (Figure 3H). Altogether, our data strongly indicate that Aurora B/C-dependent phosphorylation of Rec8 contributes to Separase-dependent cleavage of Rec8, with Serine 479 and 482 being prominent, but not the only sites.

Next, we asked whether endogenous Rec8 is indeed phosphorylated in oocyte meiosis I in an Aurora B/C-dependent manner. In meiosis I, cohesin on chromosome arms is removed during the first division to allow separation of chromosomes, whereas a tiny fraction of cohesin in the centromere region is protected to maintain sister chromatids together until meiosis II^{1,2}. First, we wanted to determine the amount of endogenous centromeric Rec8 present in meiosis I and maintained until meiosis II. To address how much Rec8 is effectively protected in the centromere region, we stained endogenous Rec8 with a polyclonal antibody on chromosome spreads, obtained from meiosis I and II metaphase oocytes. Kinetochores and underlying centromeres were stained with CREST serum. The Rec8 signal overlapping with CREST and in-between the two CREST signals was quantified and normalized against CREST. We found that less than 20% of Rec8 present between and overlapping with the two centromere signals is preserved from meiosis I to meiosis II (Figure 4A). In other words, 80 % of Rec8 is removed from this region already in meiosis I and may thus be phosphorylated, similar to Rec8 localized to arms. Hence, phospho-specific antibodies directed against the sites we identified should allow detection of phosphorylated Rec8 on chromosome arms, but not to distinguish phosphorylated from unphosphorylated Rec8 in the centromere region.

We produced phospho-specific antibodies in rabbits against the main sites identified with the Rec8 Separase sensor, namely S467, S470, S479, and S482 (see Figure S3A for scheme and peptide sequences). Aliquots of the sera were immunopurified against the phospho-peptides (alone or in pairwise combination). The sera were tested on Rec8 380-486aa sensor constructs expressed in oocyte

meiosis I, either in wild type form or containing Alanine substitutions. S479 and S482 were recognized by the serum purified against those sites, but not when mutated to Alanine (Figure S3B and C). Sera directed against pS467 and pS470 did not recognize the sensor construct in its wild type form, and were thus not further used (Figure S3D).

Crucially, sera directed against pS482 and pS479-pS482 allowed detection of endogenous arm-localized cohesin in metaphase I, recognizable by the typical cross-shaped pattern that is usually observed when staining for Rec8 (Figure 4B). To assess presence of phosphorylated Rec8 on arms, we divided our spreads into three categories, as the intensity of the signal was highly variable, depending on the quality of the individual spreads, the degree of chromosome condensation and could therefore not be quantified (Figure 4C). As negative controls we used either pre-serum, or the serum pre-absorbed with phospho-peptide pS479-pS482 (Figure 4D). A prominent signal was also detected in the centromere region (Figure 4B). However, centromeres have the potential to generate unspecific background signals, with multiple Aurora kinase substrates being present there⁸ that may cross-react with the serum we generated. To address this issue, we compared chromosome spreads from embryos at the 8-cell stage undergoing mitosis, with meiosis II chromosome spreads. Because Rec8 is meiosis-specific and substituted by Scc1 from the first mitosis onwards³², this allowed us to distinguish the signal specific for Rec8 on paired sister chromatids in meiosis II from a background signal present at centromeres of paired sister chromatids in mitosis, on samples prepared exactly the same manner. Indeed, a strong and unspecific signal colocalizing with CREST was detected on mitotic spreads. In addition, a weak signal in-between sister centromere dots exactly where centromeric cohesin is localized (Figure 4A), was detected in meiosis II (Figure S3E). However, this signal is expected to be buried beneath the unspecific signal on fused kinetochores in meiosis I²⁰, preventing assessment of pRec8 staining in the centromere region in meiosis I. Together with the fact that the centromere region can be expected to contain both phosphorylated and unphosphorylated Rec8 (Figure 4A), we thus decided to limit our analysis to arm cohesin, as the pattern of cross-shaped staining is only observed for proteins of the cohesin complex. The signal with Rec8 p482 antibody on arms is gone upon arm Rec8 cleavage in anaphase I, further indicating that the serum specifically recognizes Rec8 there (Figure S3F). When normalized to CREST, the background signal at centromeres did not decrease, indicating that the signal there is unspecific (Figure S3G). Thus, endogenous Rec8 on arms is phosphorylated on S482 (the Aurora B consensus site). However, no conclusions concerning protected Rec8 in the centromere region can be drawn in meiosis I. It is nonetheless interesting to note that pRec8 is detected between CREST dots in meiosis II (Figure S3E),

suggesting that phosphorylation of S482 is involved in centromeric cohesin removal at anaphase II as well.

S482 corresponds to the minimum consensus site for Aurora B/C. If endogenous Rec8 were phosphorylated in an Aurora B/C-dependent manner, detection of arm-localized Rec8-p482 should fail in oocytes treated with Aurora B/C inhibitor, and this was indeed the case (Figure 4E). Total Rec8 staining was not affected by addition of ZM447439, showing that it is the phosphorylation of Rec8 and not Rec8 itself that is lost (Figure 4F). Together with the failure to correctly segregate chromosomes in meiosis I in absence of Aurora B/C activity we conclude that mouse Rec8 is phosphorylated in an Aurora B/C kinase-dependent manner on S482 for Separase cleavage in meiosis I.

The Aurora B/C consensus motif we identified here in mouse oocytes can also be found in human Rec8, suggesting conservation of the mechanism for Rec8 cleavage in mammals. However, we think that also other sites are phosphorylated by Aurora B/C, and do not exclude potential roles for additional kinases, such as CK1 δ or DDK, which are required for Rec8 cleavage in yeast³⁻⁶. Aurora kinases have been shown to substitute for each other, and it is quite likely that in the absence of Aurora B/C, Aurora A can take over their role to a certain degree. This and the contribution of other kinases may explain why inhibition of Aurora B/C does not completely block bivalent segregation in meiosis I, and why meiosis II bivalents obtained upon Aurora B/C inhibition harbor only small amounts of Rec8 (Figure 1H). Aurora kinases function as catalytic subunits of the CPC (Chromosomal passenger complex) and have been shown to compete for CPC binding, with Aurora A having highest affinity, followed by Aurora B and C. Aurora C is localized to the interchromatin axis in a Haspin kinase-dependent manner, whereas Aurora B is restrained to the centromere region. It is attractive to speculate that because of its localization Aurora C is the major kinase required for Rec8 cleavage on arms in meiosis I. Indeed, an increase in active Aurora C-CPC complexes has been shown to cause precocious sister chromatid segregation in meiosis I, in agreement with Aurora C promoting cleavage²¹.

We have discovered recently that an event we termed kinetochore individualization takes place in anaphase I and may require cleavage of centromeric Rec8²⁰. However, we do not think that Aurora B/C dependent phosphorylation of Rec8 is required, because sister kinetochores still individualize in oocytes treated with Aurora B/C inhibitor (Figure 1D and E). Future work will aim at identifying how Rec8 accessibility is regulated, and the role phosphorylation plays in cleavage of distinct Rec8 fractions.

Chromosome segregation in human oocytes is highly error prone, one reason being weakening of cohesin complexes with age, causing high rates of pregnancy loss³³. Insights into the molecular mechanisms of cohesin removal will be crucial to better understand how these errors occur.

Author contributions

Sensor assays were mostly done by EN, antibody stainings by SEJ and SAT with participation of CF and EN. EB contributed to Figure 1, SAT did sensor assays in Figure S2 and AD *in vitro* kinase assays. DC provided expert technical help, and SAT participated in supervising. Statistical analysis was done by EN, EB, SAT, SEJ and CF. Figures were prepared by all authors. Overall supervision, funding acquisition and project administration were done by KW. The manuscript was written by KW with substantial input from all authors.

Acknowledgements

We thank members of the MOM team for discussion and helpful suggestions, Eileen Breunig for preliminary experiments, Olaf Stemmann for Rad21 sensor construct, mRec8-39A plasmid and hosting EN in his lab for several months, Kim Nasmyth for the Separase conditional knock-out mouse strain, Scott Keeney for Rec8 polyclonal antibody and Léa Lemaire (Covalab) for useful advice on the generation of phospho-specific antibodies. EN was supported by a postdoctoral fellowship by the FRM (Fondation de la Recherche Médicale SPF20150934093) and EMBO (short term fellowship ASTF 426 – 2015). The KW lab obtained funding for this project by ANR (ANR-16-CE92-0007-01, ANR-19-CE13-0015), FRM (Equipe FRM DEQ 20160334921, Equipe FRM DEQ 202103012574), and core funding by the CNRS and Sorbonne Université.

Declaration of interests

The authors declare that they have no conflict of interest.

Main Figure legends

Figure 1

Aurora B/C activity is required for homologous chromosome separation in meiosis I.

(A) Chromosome spreads from mouse oocytes in prometaphase I (GVBD+4h) stained for DNA (with Hoechst, blue), centromeres (with CREST antibody, red), and Histone H3 phosphorylated on Ser10 (H3pS10, green). Scale bar (white) is 10 μm . The graph on the right shows the corresponding quantification of H3pS10 fluorescence intensity detected on chromosome arms. Each dot is a measurement from one chromosome arm corrected for background. Bars and whiskers are means and SD.

(B) Accelerated Securin-YFP degradation in oocytes treated with AZD1152 or ZM447439. Securin-YFP fluorescence intensity in the cytoplasm was measured in time lapse images of mouse oocytes undergoing maturation. n is the number of oocytes. Hours after Germinal Vesicle Breakdown (GVBD) are indicated. Time points were taken every 20 minutes. Each dot and whiskers (shaded areas) are means and SD.

(C) Chromosome spreads from mouse oocytes in prometaphase I (GVBD+4h) and metaphase II (GVBD+18h), stained for DNA (with Hoechst, grey) and centromeres/kinetochores (CREST antibody, red). Lower panels are enlarged areas marked with yellow dash line squares and a schematic of a bivalent (left) and a sister chromatid pair (right). On the schematic the chromosome arms are in blue and centromeres/kinetochores are in red. Scale bar (white) is 10 μm .

(D) Chromosome spreads from mouse oocytes treated with 10 μM Aurora B/C inhibitor AZD1152 from GVBD onwards, in prometaphase I (GVBD+4h) and metaphase II (GVBD+18h), stained for DNA (with Hoechst, grey) and centromeres (with CREST antibody, red). Lower panels are enlarged areas marked with yellow dash line squares and a schematic of bivalents. On the schematic the chromosome arms are in blue and centromeres/kinetochores are in red. Of note, centromeres/kinetochores are separated on meiosis II bivalents due to kinetochore individualization. Scale bar (white) is 10 μm .

(E) Chromosome spreads from mouse oocytes treated with 20 μM Aurora B/C inhibitor ZM447439 from GVBD onwards, in metaphase II (GVBD+18h), stained for DNA (with Hoechst, white) and centromeres/kinetochores (with CREST antibody, red). Lower panels are an enlarged area as marked with a yellow dash line square and a schematic of a bivalent. On the schematic the chromosome arms are in blue and centromeres/kinetochores (individualized) are in red. Scale bar (white) is 10 μm .

(F) Frequency of bivalent separation in meiosis I, quantified from chromosome spreads of metaphase II oocytes (GVBD+18h). Chromosomes were observed in control oocytes and oocytes treated with Aurora B/C inhibitors (10 μ m AZD1152 or 20 μ M ZM447439) and classified as “bivalents” (homologous chromosomes together in form of a bivalent), or “dyads” (homologous chromosomes separated into sister chromatid pairs). The number for dyads is divided by 2 to compare to the number of “not separated bivalents”. n is the number of analyzed bivalents. *** $p < 0.0001$, calculated with Fisher’s exact test.

(G) Same data as (F), but numbers are per oocyte. Only groups of chromosomes for one oocyte in the same field were quantified. n is the number of oocytes. On each graph all frequencies are displayed as numbers on the corresponding bars. *** $p < 0.0001$, calculated with Fisher’s exact test.

(H) Chromosome spreads such as in (E) and stained with polyclonal Rec8 antibody. ZM447439 was added from GVBD onwards.

Related to Figure S1.

Figure 2

Aurora B/C activity does not inhibit Separase-dependent cleavage of Rad21 sensor.

A) Schematic of sensor construct (top), example of time lapse microscopy acquisition of a mouse oocyte expressing the Rad21 sensor, +/- 40 minutes (min) relative to anaphase I onset (middle) and an example of the quantification of GFP/Cherry ratio (on the right). Anaphase I is labeled as 0 minutes timepoint. Scale bar (white) is 20 μ m. Cleavage of the sensor is visible by the disappearance of the GFP signal from the chromosomes, while the Cherry signal remains. Quantification of the GFP/Cherry ratio measures mean signal from the area occupied by the chromosomes corrected for the background signal from the cytoplasm. Sensor cleavage efficiency is quantified as per cent decrease of the GFP/Cherry ratio when comparing the last timepoint before anaphase I onset and the lowest point during anaphase I.

B) Cleavage of Rad21 sensor in control oocytes and oocytes treated with the indicated inhibitors of Aurora B/C. Shown are examples of time lapse microscopy images of mouse oocytes expressing the Rad21 sensor, +/- 40 minutes relative to onset of anaphase I. Scale bar (white) is 20 μ m.

C) Quantification of cleavage efficiency of Rad21 sensor from experiment in (B). n is the number of oocytes analysed. Bars and whiskers are mean and SD. n.s. – not significant, according to Mann-Whitney test.

Figure 3

Cleavage efficiencies of Rec8 sensor constructs in oocyte meiosis I.

The sensor constructs that were analysed are indicated on the left with corresponding schemes, and the corresponding *in vivo* cleavage efficiencies when expressed in oocytes, are on the right. Negative control (ctrl) indicates a sensor construct without Rec8 sequence and no cleavage site. In the schemes, potential Separase cleavage sites are indicated in green, single amino acid substitutions in red, multiple amino acid substitutions indicated in grey, and the relative position of the Rec8 fragment being tested is shown by its position on the black line (not in scale). On each graph n is the number of oocytes, bar and whiskers are means and SD. Vertical dotted lines mark standard deviation of the negative control sensor. n.s. – not significant, * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$ according to t test performed for each condition compared to negative control.

A) Cleavage of sensor construct containing full-length Rec8, and Rec8 harboring 39 Serine or Threonine to Alanine substitutions.

B) Cleavage efficiencies of four Rec8 fragments (1-143aa, 143-290aa, 1-290aa, 380-486aa), and Rec8 380-486aa harboring mutations in two Separase cleavage sites, or 17 Serine or Threonine mutated to Alanine.

C) Cleavage efficiencies of Rec8 380-486aa sensor construct in Separase control (Sep^{+/+}) and Separase knock-out (Sep^{-/-}) oocytes. *** $p < 0.0001$ according to t test comparing Sep^{-/-} to Sep^{+/+}.

D) Cleavage efficiencies of Rec8 380-486aa sensor construct with or without being exposed to Aurora B/C inhibitor AZD1152 from GVBD onwards. *** $p < 0.0001$ according to t test comparing inhibited oocytes to control.

E) Cleavage efficiencies of Rec8 380-486aa harboring Alanine substitutions in two, three and five potential phosphorylation sites (5S-A: S466, 467, 470, 479, 482A; 3S-A: S466, 467, 470A; and 2S-A: S479, 482A) or Aspartate substitutions (SRTD: S482D; DRTS: S479D). S482 corresponds to an Aurora B consensus site. * $p < 0.01$, *** $p < 0.0001$ according to t test performed for each condition compared to negative control.

F) Cleavage efficiencies of Rec8 380-486aa harboring 14 Alanine substitutions except Serine 479 and 482, which were either Serine (SRTS) or Aspartate (DRTD). ** $p < 0.001$ according to t test comparing SRTS to DRTD.

G) *In vitro* kinase assay using bacterially expressed GST-Rec8 380-486aa (WT) and GST-Rec8 380-486aa 17S/T-A (A) as substrates for GST-Aurora B-INCENP. Above: incorporation of γ P into Rec8 and Aurora B

(autophosphorylation) are revealed by detection with an antibody against thiophosphate ester. Below: Rec8 and Aurora B are revealed with an anti-GST antibody.

H) Cleavage efficiencies of Rec8 380-486aa wild type (SELS...SRTS) or carrying Aspartate substitutions for Serine 467, 470, 479, 482 (DELD...DRTD) in the presence of AZD1152 from GVBD onwards. * $p < 0.01$ according to t test comparing wild type to phosphomimetic.

Related to Figure S2 and Table S1.

Figure 4

Phospho-specific antibodies recognize Rec8 in an Aurora B/C kinase dependent manner.

A) Oocytes in metaphase I and metaphase II were fixed for chromosome spreads right next to each other, in the same wells, and stained with polyclonal Rec8 antibody (magenta), anti-CREST serum (centromeres/kinetochores, green), and Hoechst (DNA, in blue). A representative spread and a zoom on one bivalent are shown for each condition on the left. n indicates the number of oocytes analysed, scale bars are 10 μm . Below, quantifications of Rec8 signal overlapping with CREST, or inbetween the two CREST signals, as indicated in the scheme. *** $p < 0.0001$, by Mann-Whitney Test.

B) Chromosome spreads of oocytes in prometaphase I were stained with the indicated sera to detect endogenous Rec8 phosphorylated on S482, or both S479 and S482 (magenta), together with anti-CREST serum (centromeres/kinetochores, green), and Hoechst (DNA, in blue). A representative spread is shown for each condition. n indicates the number of oocytes analysed, scale bars are 10 μm .

C) Spreads stained with phospho-specific anti-Rec8 antibodies were classified according to the examples of stainings shown in (B).

D) Serum specific for pS479pS482 was pre-absorbed with peptide pS479 pS482, and used to stain chromosome spreads as described in (B). The results of quantifications according to the classification in c) are shown.

E) Chromosome spreads of control prometaphase I oocytes and oocytes treated with ZM447439 from GVBD onwards, were stained with serum directed against pS482 to detect endogenous Rec8 phosphorylated on S482 (magenta), together with anti-CREST serum (centromeres/kinetochores, green), and Hoechst (DNA, in blue). The graph shows quantifications of the indicated number of chromosomes, according to classification in (C). A representative spread with a zoom on one chromosome is shown for each condition. n indicates the number of oocytes analysed, scale bars are 10 μm .

F) Chromosome spreads prepared and stained as in e), except that a polyclonal Rec8 antibody was used. Chromosomes with Rec8 localized on arms were quantified as in (E), according to the classification in (C). A representative spread with a zoom on one chromosome is shown for each condition. n indicates the number of chromosomes analysed, scale bars are 10 μm .

Related to Figure S3.

STAR Methods

LEAD CONTACT

Further information and requests for resources and reagents should be directed and will be fulfilled by the lead contact, Katja Wassmann (katja.wassmann@upmc.fr)

MATERIALS AVAILABILITY

All unique reagents generated in this study are available from the lead contact without restriction. The study did not generate new mouse lines.

DATA AND CODE AVAILABILITY

All data reported in this paper will be shared by the lead contact upon request.

This paper does not report original code.

Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals

Adult Swiss mice were purchased from Janvier, France, at 7 weeks of age and kept in the conventional animal facility of UMR7622 (authorization B75-05-13) in an enriched environment and with food and water access *ad libitum*. Separase^{-/-} mice and litter mate control mice (C57BL/6-Tg) were house-bred in the animal facility of UMR7622. The local ethical committee (comité d'éthique en experimentation animale Charles Darwin, no 5, Ile-de-France) authorized the project (registered under 03263.02), in accordance with national guidelines and regulations. Female mice were sacrificed by cervical dislocation between 8-16 weeks of age, ovaries were dissected and GV stage oocytes harvested. Mice were not involved in any procedures before sacrifice except for genotyping, and hormonal stimulation to obtain embryos.

METHOD DETAILS

Mouse oocyte and embryo culture

Oocytes were harvested in M2 medium (homemade or Merck Millipore, MR-015P) and kept arrested at GV stage with 100µg/mL dbcAMP (dibutyryl cyclic AMP, Sigma-Aldrich, D0260). After release in dbc-AMP

free M2 medium, oocytes undergoing GVBD no later than 90min after release were used for experiments. Oocytes were cultured in M2 medium drops covered by mineral oil, manipulated on heating plates and kept in a temperature-controlled incubator at 38°C. For longer incubation, oocytes were cultured in M16 medium (MERK Millipore MR-10P or homemade) in a CO₂- and temperature-controlled incubator. To obtain 8-cell stage embryos in mitosis, superovulation was induced in female mice by intraperitoneal injection of PMSG (pregnant mare serum gonadotropin, 5 IU/female) and hCG (human chorionic gonadotropin, 5 IU/female) 48 hours later. Then, females were coupled with a male, verified by the presence of a vaginal plug. Pregnant female mice were sacrificed 12h after fertilization, embryos collected and incubated in EmbryoMax CZB medium (Sigma-Aldrich) with 1mM supplementary L-glutamine (Sigma-Aldrich) until they reached 8-cell stage, then incubated with 4 μM Nocodazole (Sigma Aldrich) for 6 hours to keep the cells arrested in the 4th mitosis.

Drug treatments

All inhibitors used were dissolved in DMSO (Sigma-Aldrich, D2650) and diluted to M2 or M16 medium at the indicated final concentrations. For Aurora B/C inhibition, ZM447439 (Tocris, 2458) was used at 20 μM, ZM447439 (Santa Cruz, sc-200696) was used at 2-20 μM and AZD1152 (Sigma Aldrich, SML0268) at 10μM. Cytokinesis failure but no metaphase I arrest was used as a positive control for only Aurora B/C (and not Aurora A) inhibition in every experiment. Optimal concentrations had to be determined for every lot of ZM447439. For Aurora A inhibition, MLN8237 (Bertin Bio-reagent, 13602) was used at 1μM, and metaphase I arrest was used in every experiment as control. To bypass the SAC, Mps1 inhibitor Reversine (Cayman Chemical, 10004412) was used at 0,5 μM, and also added to the oil covering the culture droplets. Acceleration of meiosis I was verified as control for efficient Mps1 inhibition.

Plasmids

Plasmids used are described in Table S1. Rad21 sensor construct was a gift from O. Stemmann (University of Bayreuth). Cloning of the plasmids was carried out using restriction enzymes (Thermo Fisher), Pfu polymerase (Promega) for PCR, and T4 ligation kit (Thermo Fisher). DNA was purified with QIAprep Spin Miniprep Kit, QIAquick PCR purification kit or QIAquick Gel Extraction kit (Qiagen). Cloning strategies for each construct are described in Table S1. All primer sequences are listed in Table S2. All primers were synthesized commercially (Eurofins). After cloning, plasmid sequences were verified through a commercial sequencing service (GATC).

Production of truncated GST-Rec8 proteins

Rec8 380-486aa and Rec8 380-486aa 17S/T-A were PCR cloned into pGEX-6P-2 (Primer sequences available upon request). Expression of GST-Rec8 proteins was induced in BL21(DE3)pLys bacteria (Promega, L1195) with 0.5 mM isopropyl- β -D-thiogalactopyranoside (Euromedex, EU0008-B) for 3 h at 37°C. After centrifugation at 10,000 $\times g$ for 15 min at 4°C, bacteria were lysed in PBS buffer supplemented with 1 mM PhenylMethylSulfonyl fluoride (Roche 39212624) in the presence of detergents (0.1% Triton X100 and 0.1% NP40) and sonicated. After ultracentrifugation (100,000 $\times g$, 4°C, 1 h), the supernatant containing recombinant GST-Rec8 proteins was loaded for 1h30 at 4°C on 1ml glutathione-agarose beads (Sigma G4510), previously equilibrated in PBS buffer. After washing, proteins were eluted with PBS and 10 mM glutathione. The purified proteins were dialyzed overnight against PBS buffer and frozen at -80°C for further use.

***in vitro* Kinase assays**

In vitro thiophosphorylation³⁴ of Rec8 proteins, either WT-Rec8 or 17S-A-Rec8, was performed by incubating 165 ng of recombinant GST-Rec8 proteins with 120 ng of recombinant Aurora B-INCENP (MERCK, item#14-835) in 30 μ l of kinase buffer (40 mM MOPS, pH 7.3; 100mM MgCl₂, 1mM EDTA; 0,1% β -Mercaptoethanol) supplemented with 5mM ATP γ S for 90 min at 30°C. Each reaction was incubated for 30 mins at RT with 12.5 mM p-nitrobenzyl mesylate (Abcam, ab138910) for alkylation and the reaction was stopped by adding Laemmli buffer. Incorporated thiophosphates (γ P) were immunoblotted using an antibody directed against alkylated thiophosphates (Abcam, ab92570, 1:2000). Membranes were stripped twice for 15 mins at RT in 0.1M Glycine pH2.2 supplemented with 1% SDS and then probed using a HRP-GST antibody (Sigma, 16-209, 1:5000). Western blots are representative of three independent experiments.

Generation of phospho-specific antibodies

Using the services of Covalab (France), 2 rabbits each were primed with three peptides against each phospho-epitope, respectively: pSElpS (S467 S470): pSELSLEAAEDEKSR, SELpSLEAAEDEKSR, pSElpSLEAAEDEKSR; and pSRTpS (S479 S482): EDEKSRtpSLIPPEWWA, EDEKpSRTSLIPPEWWA, EDEKpSRTpSLIPPEWWA (Covalab). Sera of the rabbits with the highest titer were immunopurified against the individual peptides according to instructions by Covalab, and used as indicated. To detect sensor fragments, spreads were performed on metaphase I spreads and the sera were diluted 1:40, and to

detect endogenous Rec8, spreads were performed on prometaphase I spreads and sera diluted 1:20 and 1:50.

Microinjection

mRNA coding for Securin-YFP³⁵, or sensor constructs was transcribed using the mMessage Machine T3 or T7 Kit (Invitrogen) and purified with RNeasy mini-kit (Qiagen). GV oocytes were injected on an inverted Nikon Eclipse Ti microscope with self-made micropipettes (Narishige magnetic puller, PN-31) and a FemtoJet microinjector pump (Eppendorf) with constant flow. Oocytes were manipulated using a holding pipette (vacuTip from Eppendorf).

Chromosome spreads

After removal of the zona pellucida by successive transfer in acidic tyrode solution, chromosomes were spread by transferring oocytes in hypotonic solution (3 mM DTT, 0,15% Triton-X100, 1% Paraformaldehyde), placed on 8 wells glass slides(Fisher Scientific, 10588681)³⁶. Upon drying at room temperature, chromosome spreads were stored at -20°C until immunostaining. Thawed spreads were washed several times with PBS 1X, incubated 10 min with PBS-BSA 3%, and incubated with primary antibodies diluted in PBS-BSA 3% for 2-3h. The following antibodies were used at the indicated concentrations: Human CREST serum (Immunovision, HCT-100, at 1:50 or 1:100), Rabbit poly clonal anti-Rec8 (gift from Scott Keeney, 1:50 and 1:100), Rabbit anti-pRec8 (directed against the indicated phospho-peptides, 1:20, 1:40, 1:50), anti-phospho-Histone H3 (pS10H3, 1/100 dilution; Merck Millipore, 05-806). To detect endogenous Rec8 efficiently, spreads had to be performed in prometaphase I (GVBD+ 4-5 hours), before complete chromosome condensation. Spreads were washed several times with PBS and incubated with the following secondary antibodies diluted in PBS-BSA 3%, for at least 1 hour: donkey anti-human Cy3 (709-166-149, Jackson Immuno Research, 1:200), donkey anti-Human Alexa Fluor 488 (709-546-149, Jackson Immuno Research, 1:200), donkey anti-Rabbit Cy3 (711-166-152, Jackson Immuno Research, 1:200), donkey anti-Rabbit Alexa Fluor 488 (711-546-152, Jackson Immuno Research, 1:200). DNA was stained with Hoechst 33342 (Invitrogen, H21492, 50µg/mL). After several PBS washing steps chromosome spreads were mounted in Vectashield (Eurobio H-1000) or AF1 mounting medium (CityFluor) and stored at 4°C until acquisitions with a spinning disk confocal microscope.

Image Acquisitions

Live imaging of oocytes expressing Securin-YFP was performed on a Nikon eclipseTE 2000-E inverted microscope coupled to a Prime sCMOS camera, equipped with PreciSExite High Power LED Fluorescence, a Märzhäuser Scanning Stage and using a Plan APO (20x/0,75NA) objective. Time lapse was set up for 6h with 20 min intervals. 1 z-section was acquired for every time point in the GFP channel and in DIC. Live imaging of oocytes expressing sensor constructs was performed on a Zeiss Axiovert 200M inverted spinning disk confocal microscope, coupled to an EMCCD camera (Evolve 512, Photometrics), using a Plan-APO (63x/1,4 NA) oil objective. Time lapse was set up for 10 hours with 20 min intervals. For each timepoint, 11 z-stacks (3 μm optical section spacing) were acquired in 491nm channel for GFP or YFP and in 561nm channel for mCherry. Only oocytes expressing comparable levels of sensor constructs and undergoing metaphase-to-anaphase transition without delay were further analysed. All live imaging was done under perfectly temperature-controlled conditions, and microscopes were piloted by Metamorph software. Fixed chromosome spreads were acquired on the same Zeiss Axiovert 200M inverted spinning disk confocal microscope, but using a 100x/1.4 NA oil objective. 6 z sections of 0,4 μm interval were taken in each channel. Images were processed using Fiji software. For publication, images were treated in Adobe Photoshop to adjust brightness and contrast, all adjustments were applied to whole images uniformly, and to all images being compared.

QUANTIFICATION AND STATISTICAL ANALYSIS

Quantifications were performed with Fiji software. For quantification on chromosome spreads, the mean fluorescence intensities were measured on sum-projected images. A square is drawn around centromeres as depicted on the scheme in Figure 4A or on chromosome arm. The measured signal is corrected to background. Data plots and statistical analysis were obtained with Graphpad PRISM 8 software. Quantifications of cleavage efficiencies of different sensors were done as described²⁶. In brief, mean fluorescence signal for YFP (or GFP) and mCherry was measured in the box surrounding chromosomes during several timepoints before, during and after anaphase I, corrected for background fluorescence. The ratio between YFP (or GFP) and mCherry signals was calculated and the difference between pre-anaphase value (normalized to 100%) and first post-anaphase value derived. All experiments were done at least three times independently, the total number of oocytes analysed in each condition is indicated.

Table S1

List of Rec8 sensor constructs used in this manuscript. Related to STAR Methods.

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