LETTERS

Rec8 phosphorylation and recombination promote the step-wise loss of cohesins in meiosis

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During meiosis, cohesins—protein complexes that hold sister chromatids together—are lost from chromosomes in a step-wise manner[1]. Loss of cohesins from chromosome arms is necessary for homologous chromosomes to segregate during meiosis I. Retention of cohesins around centromeres until meiosis II is required for the accurate segregation of sister chromatids. Here we show that phosphorylation of the cohesin subunit Rec8 contributes to step-wise cohesin removal. Our data further implicate two other key regulators of meiotic chromosome segregation, the cohesin protector Sgo1 and meiotic recombination in bringing about the step-wise loss of cohesins and thus the establishment of the meiotic chromosome segregation pattern. Understanding the interplay between these processes should provide insight into the events underlying meiotic chromosome mis-segregation, the leading cause of miscarriages and mental retardation in humans.

During the meiotic cell cycle, DNA replication is followed by two rounds of chromosome segregation, in which homologues segregate during the first division and sister chromatids are partitioned in the second. Loss of cohesins from chromosome arms allows the segregation of homologous chromosomes during meiosis I by causing the resolution of meiotic recombination events, which hold homologous chromosomes together before anaphase I (ref. 2). Maintenance of cohesins around centromeres beyond anaphase I and cohesin removal during meiosis II are essential for accurate segregation of sister chromatids. Members of the MEI-S332/Sgo1 (Shugoshin) protein family protect cohesins around centromeres from removal during meiosis I by recruiting protein phosphatase 2A (PP2A) to centromeric regions[3–10].

Cohesins are removed from chromosomes by a protease known as separase (Esp1 in yeast). After the ubiquitin-dependent destruction of its inhibitor securin (Pds1 in yeast) mediated by the anaphase promoting complex/cyclosome (APC/C), separase cleaves a subunit of the cohesin complex, thereby triggering anaphase[11]. The Polo kinase Cdc5 contributes to cohesin removal by promoting cleavage-independent cohesin dissociation during meiotic prophase[12] and cohesin cleavage by separase[13–16]. Phosphorylation of the cohesin subunit and separase target Rec8 is furthermore decreased in cells lacking Cdc5 (ref. 14), raising the possibility that Rec8 phosphorylation is important for the protein's cleavage.

To determine the importance of Rec8 phosphorylation in cohesin cleavage we mapped the phosphorylation sites of Rec8 isolated from cells arrested in metaphase I either by depletion of the APC/C activator Cdc20 (ref. 14), or by expression of a non-degradable version of Pds1 from the meiosis-specific *DMC1* promoter (Supplementary Fig. 1). In both arrests, Rec8 is highly phosphorylated (data not shown). We also isolated Rec8 from cells depleted for Cdc5 to identify Cdc5-dependent phosphorylation events. The overall coverage of Rec8 was 85% (Supplementary Fig. 2) leading to the identification of 24 phosphorylation sites (Supplementary Table 1; Supplementary Information). Eleven of these sites were phosphorylated in Cdc20-depleted cells or Pds1dBΔ-expressing cells but not in Cdc5-depleted cells, suggesting that these sites are phosphorylated by Cdc5 *in vivo*. This analysis defined the motif $S/E/D–X_{0–2}–N(Q)–X_{0–2}–Sp(Tp)–X_3-\pi$ (where π represents a polar amino acid, and p indicates a phosphorylated amino acid residue) as the consensus sequence for Cdc5-dependent phosphorylation sites (Supplementary Table 2). Additional features of the region surrounding these sites are discussed in the Supplementary Information.

We mutated the phosphorylated sites within Rec8 to amino acids that can no longer be phosphorylated. Mutation of individual phosphorylation sites to alanine did not affect sporulation efficiency (data not shown). Mutation of multiple phosphorylation sites to alanine led to a delay in prophase I (Supplementary Fig. 3a), which is indicative of a partial loss of Rec8 function[17]. This result suggests that phosphorylation of Rec8 may be important for the protein's prophase functions.

Next, we examined the consequences of mutating the 11 residues whose phosphorylation was Cdc5-dependent (*rec8-psa*) to alanine. A defect in cohesin removal is expected to interfere with anaphase I entry[18] but cells expressing this allele did not exhibit a defect at this transition (Supplementary Fig. 3b), suggesting either that Cdc5-dependent phosphorylation of Rec8 was not important for Rec8 removal or that our mass-spectrometry analysis did not identify all Cdc5-dependent phosphorylation sites. The latter scenario was more likely, given that the coverage in the Cdc5-depletion arrest was only 65%. We therefore—in addition to the known Cdc5-dependent sites—mutated putative Cdc5-dependent phosphorylation sites that were found to be phosphorylated in the *pCLB2-CDC20* and/or *pDMC1-PDS1dBΔ* arrests but that were not covered in the Cdc5-depletion arrest, as well as three Cdc5-independent sites, to alanine (*rec8-17A*; Supplementary Table 1). Cells expressing this *rec8* mutant exhibited a 1-h prophase delay and a metaphase I delay that was not as great as that observed in cells expressing a non-cleavable version of Rec8 (Fig. 1a, Supplementary Fig. 4a). Entry into anaphase II was only slightly—if at all—delayed (Supplementary Fig. 4b), suggesting that Rec8 phosphorylation is less important for this cell cycle transition. *rec8* mutants in which all phosphorylated serines and threonines, except two recently identified sites (T291, S292), were mutated to alanine (*rec8-21A*) and mutants that had additional putative Cdc5 phosphorylation sites mutated to alanine (*rec8-24A*, *rec8-29A*; Supplementary Table 1) also appeared to be delayed in metaphase I, although the extent of the delay was difficult to assess owing to the severe prophase I delay exhibited by the mutants (Supplementary Fig. 3c). We conclude that mutating Rec8's phosphorylation sites leads to impairment in Rec8's prophase function and interferes with anaphase I entry.

The *rec8-17A* mutant was analysed in more detail because the

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metaphase I delay was the least obscured by the prophase delay in this mutant and because the mutant was likely to have most of its Cdc5-dependent phosphorylation sites mutated to alanine. Analysis of Pds1 by indirect *in situ* immunofluorescence revealed that *rec8-17A* cultures contained a significant fraction of metaphase I cells lacking Pds1 (Fig. 1c, Supplementary Fig. 5). We conclude that the metaphase I delay observed in the *rec8-17A* mutant is at least in part due to events occurring after the degradation of Pds1.

Next we determined whether the metaphase I delay observed in *rec8-17A* mutants was due to a Rec8 cleavage defect. In wild-type cells, the carboxy-terminal Rec8 cleavage product accumulated 4 h after induction of sporulation (Fig. 1b). The Rec8-17A protein assembled onto chromosomes normally (Fig. 1d, e) but cleavage did not occur until 7 h (Fig. 1a, b). This delay was only due in part to the prophase I delay, which was 90 min (Fig. 1a). Our results indicate that cleavage of the Rec8-17A mutant protein is delayed not only owing to prophase I defects but also owing to defects occurring after the degradation of Pds1. We conclude that phosphorylation of Rec8 is important for its timely cleavage during meiosis I.

We also examined the effects of eliminating meiotic recombination on *rec8-17A* mutants. Surprisingly, deletion of *SPO11*, a gene required to form the recombination-initiating double-strand breaks[19,20] or expression of a catalytic dead version of Spo11 *(spoII-Y135F)*[19] abolished the delay in Rec8 cleavage and cell cycle progression of *rec8-17A* or *rec8-29A* mutants (Fig. 2a, Supplementary Fig. 6, 7). Our results suggest that in the absence of recombination Rec8 phosphorylation is not as important for cohesin removal as it is when recombination occurs.

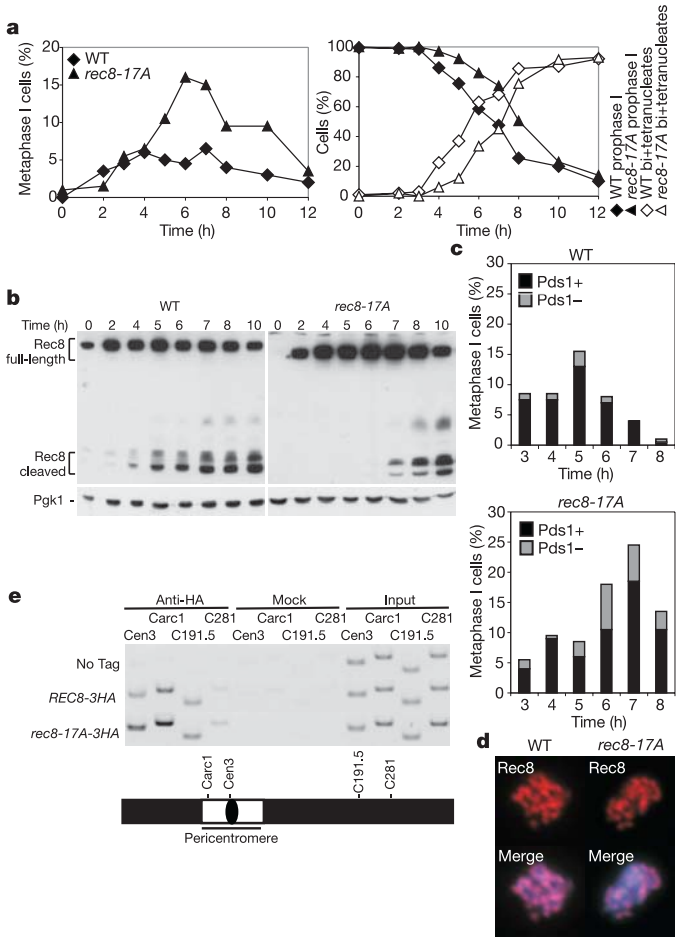

Figure 1 | Rec8 cleavage is delayed in *rec8-17A* cells. a, **b**, Wild-type (WT) (A14655, diamonds) and *rec8-17A* mutant (A14746, triangles) cells lacking the ubiquitin ligase Ubr1 to facilitate detection of the Rec8 cleavage product were induced to sporulate to determine the percentage of metaphase I cells (**a**, left panel), of prophase (**a**, right panel, solid symbols) and of the sum of bi- and tetra-nucleate cells (**a**, right panel, open symbols). Rec8-3HA and Pgk1 (loading control) were analysed by western blotting (**b**). **c**, WT (A14923) and *rec8-17A* mutant (A14861) cells both carrying a *PDS1-18MYC* fusion were induced to sporulate and Pds1 staining was determined in all metaphase I cells. **d**, The localization of Rec8 is shown on chromosome spreads of WT cells and *rec8-17A* mutants. Rec8 is shown in red, DNA in blue in the merge. **e**, WT *REC8-3HA* (A1972) and *rec8-17A-3HA* (A13559) and a control strain (A4962) were induced to sporulate and processed for chromatin immunoprecipitation after 4 h. Polymerase chain reaction (PCR) analysis of immunoprecipitated samples (anti-haemagglutinin; anti-HA), mock-treated samples, and input DNA (1:250) are shown along with a diagram indicating locations of chromosome III primer sets. C281 is a cohesin-poor location.

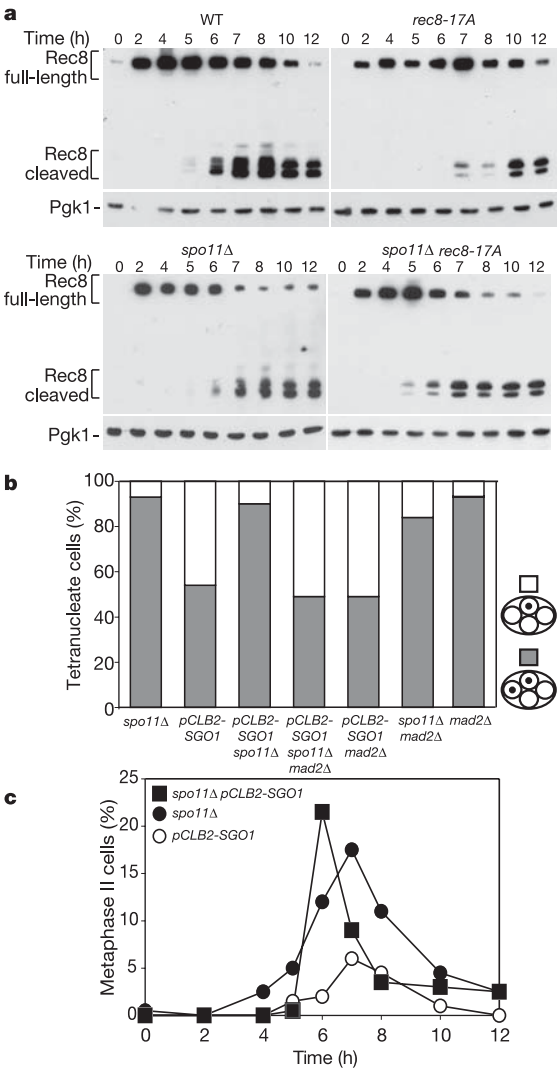

Figure 2 | Elimination of recombination abolishes the Rec8 cleavage delay in *rec8-17A* owing to retention of arm cohesion past meiosis I. a, WT (A14655), *spo11Δ* (A14755), *rec8-17A* (A14746) and *spo11Δ rec8-17A* (A14847) cells were induced to sporulate and Rec8-3HA was analysed by western blotting. **b**, *spo11Δ* (A9498), *spo11Δ pCLB2-SGO1* (A14938), *pCLB2-SGO1* (A11251), *spo11Δ pCLB2-SGO1 mad2Δ* (A15345), *spo11Δ mad2Δ* (A15490), *mad2Δ* (A15494) and *pCLB2-SGO1 mad2Δ* (A15344) cells carrying *CEN5-GFP* dots were induced to sporulate to determine GFP dot segregation in tetrads ($n = 100$). **c**, *spo11Δ* (A9498, closed circles), *spo11Δ pCLB2-SGO1* (A14938, squares) and *pCLB2-SGO1* (A11251, open circles) cells were induced to sporulate to determine the percentage of metaphase II cells.

Why does elimination of recombination suppress the Rec8 cleavage defect of *rec8-17A* mutants? In *spo11Δ*, *spo11Δ rec8-17A* and *spo11-Δ rec8-29A* mutants, loss of cohesins from chromosome arms and from centromeric regions occurs almost simultaneously, as is shown by the absence of binucleate cells with cohesins concentrated around centromeres in chromosome spreads (Supplementary Fig. 8a–d). This raises the possibility that in this mutant the bulk of cohesin removal occurs during meiosis II, when phosphorylation appears less important for Rec8 cleavage (Supplementary Fig. 4b). To test this hypothesis we examined the effects of deleting *SPO11* in Sgo1-depleted cells.

In Sgo1-depleted cells, the second meiotic division is random, owing to the absence of cohesion between sister chromatids. This phenotype can be observed when cells carry a tandem array of Tet operator sequences near the centromere on one of the two homologues and also express a Tet repressor–green fluorescent protein (TetR–GFP) fusion that binds to these repeats (*CEN5* GFP dots[21]). When meiosis II segregation is random, 50% of tetrads contain GFP dots in only one of the four spores and 50% of tetrads contain a GFP signal in two of the four spores (Fig. 2b, Supplementary Fig. 8d). Remarkably, 90% of *spo11Δ pCLB2-SGO1* cells segregate sister chromatids correctly (a GFP signal in two of the four spores; Fig. 2b). Furthermore,

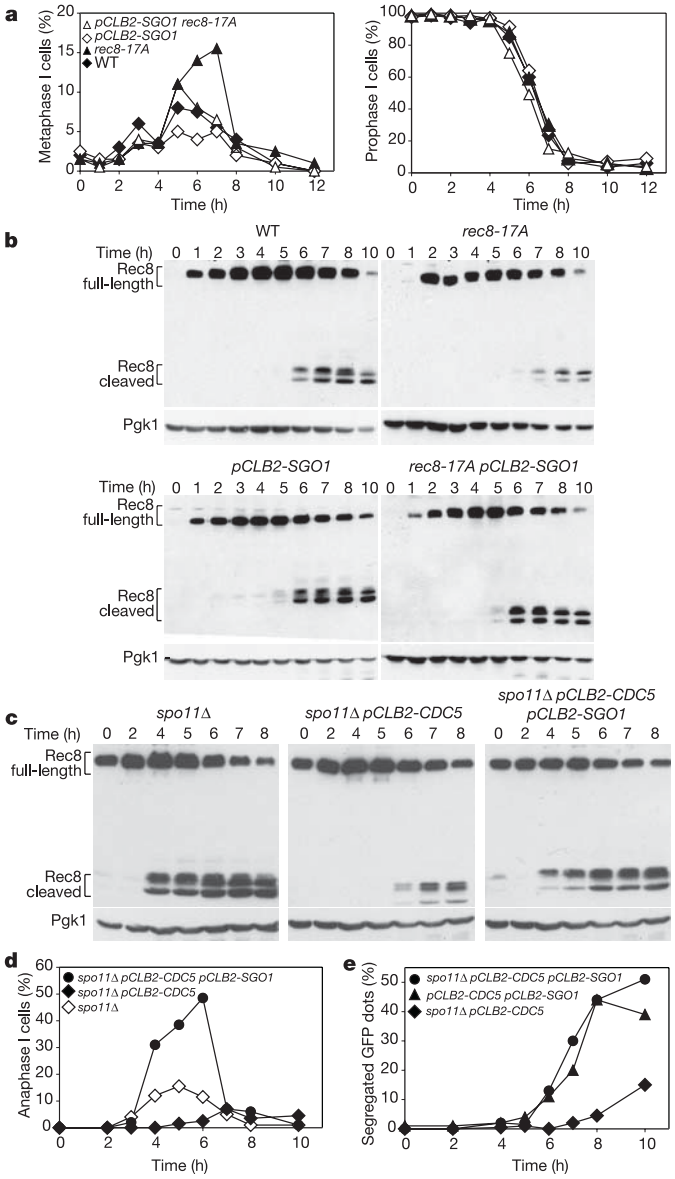

Figure 3 | Depletion of Sgo1 partially alleviates the need for Rec8 phosphorylation and Cdc5 in Rec8 cleavage and anaphase I entry. **a**, **b**, WT (A15086, closed diamonds), *rec8-17A* (A14750, closed triangles), *pCLB2-SGO1* (A15085, open diamonds) and *pCLB2-SGO1 rec8-17A* (A15084, open triangles) were induced to sporulate to determine the percentage of metaphase I cells (**a**, left panel), prophase I cells (**a**, right panel) and Rec8-HA protein by western blot analysis (**b**). **c**, **d**, *spo11Δ* (A15022, open diamonds), *spo11Δ pCLB2-CDC5* (A15025, closed diamonds), and *spo11Δ pCLB2-CDC5 pCLB2-SGO1* (A15000, closed circles) cells were induced to sporulate to determine the percentage of anaphase I cells (**d**) and Rec8 protein levels (**c**). **e**, *spo11Δ pCLB2-CDC5* (A14657, closed diamonds), *pCLB2-CDC5 pCLB2-SGO1* (A14870, closed triangles), and *spo11Δ pCLB2-CDC5 pCLB2-SGO1* (A14776, closed circles) cells all carrying *CEN5-GFP* dots were induced to sporulate to determine the percentage of cells with GFP dots separated by at least 2 μm ($n = 200$ per time point). Note that in metaphase I-arrested *spo11Δ pCLB2-CDC5* mutants, two juxtaposed GFP dots are visible because sister kinetochores attach to opposite poles rather than the same pole in meiosis I and the tension exerted by the spindle leads to separation of *CEN5* GFP dots[14,15].

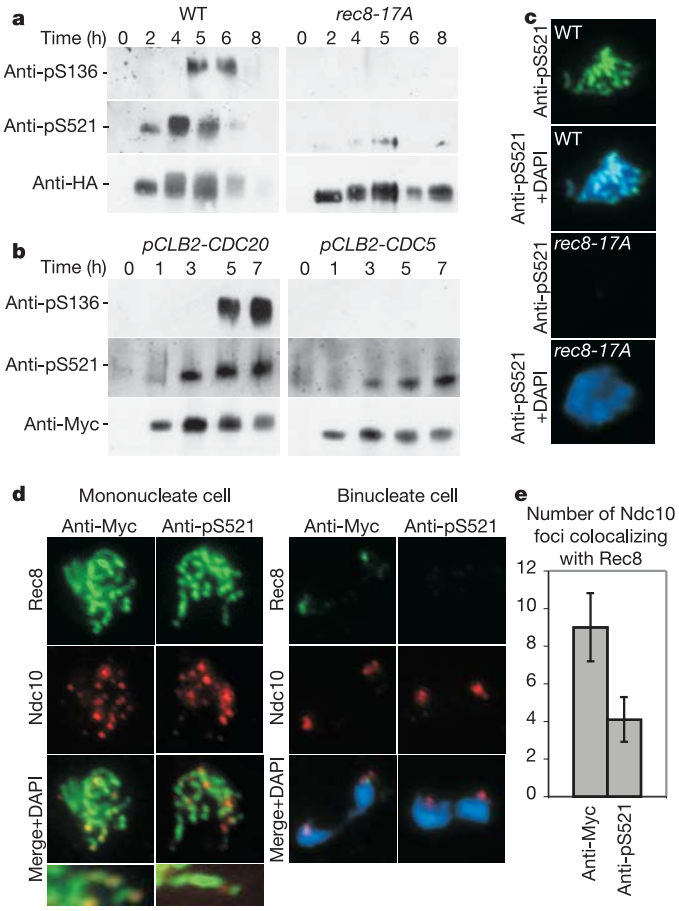

Figure 4 | Serine 521 phosphorylation is reduced around centromeres during meiosis I. **a**, WT (A1972) and *rec8-17A* mutant (A13559) cells were induced to sporulate and Rec8-HA immunoprecipitates were probed with either anti-HA or anti-phospho S136 (anti-pS136) or anti-phospho S521 (anti-pS521) antibodies. **b**, Rec8-Myc was immunoprecipitated from *pCLB2-CDC20* (A5441) and *pCLB2-CDC5* (A9858) cells and probed with either anti-Myc or anti-pS136 or anti-pS521 antibodies. **c**, Anti-pS521 staining was analysed on chromosome spreads in WT (A14655) and *rec8-17A* mutant (A14746) cells 4 h after sporulation induction. Anti-pS521 staining is shown in green and DNA in blue. **d**, **e**, WT cells carrying a *REC8-MYC* fusion and a *NDC10-HA* fusion (A3640) were spread and the distribution of Rec8 was determined either using anti-Myc or anti-pS521 antibodies. Examples of prophase and binucleate cells are shown. Rec8 is shown in green, Ndc10 in red and DNA in blue. **e**, The number of Ndc10 foci overlapping with the anti-Myc and anti-pS521 staining ($n = 12$). Error bars represent standard deviation.

deletion of *SPO11* restored metaphase II to Sgo1-depleted cells (Fig. 2c, Supplementary Fig. 8e). Similar results were obtained with other recombination mutants, such as *spo11-YF*, *rad50S* (double-strand break resection defective[22]) or *dmc1Δ* (strand invasion defective[23]) mutants in which recombination-induced linkages between homologues are abolished (Supplementary Fig. 9). This observation, together with the finding that chromosome segregation was again random in *spo11Δ pCLB2-SGO1 mad2Δ* triple mutants (Fig. 2b), provided insight into why cohesin removal did not occur during meiosis I in the absence of recombination: in the absence of linkages between homologues, chromosomes fail to attach properly to the meiosis I spindle. This leads to the activation of the spindle assembly checkpoint, which in turn prevents the removal of cohesins from chromosomes. Cells nevertheless undergo anaphase I and progress into meiosis II because chromosomes lack the necessary linkages to prevent meiosis I spindle elongation[24,25]. This results in metaphase II chromosomes with cohesins on chromosome arms. These observations, together with the finding that Rec8 phosphorylation is not important for Rec8 cleavage during meiosis II, explain why elimination of recombination abolishes the Rec8 cleavage delay in the *rec8-17A* mutant and demonstrate a role for recombination in establishing the step-wise loss of cohesins from chromosomes.

Sgo1 was shown to protect cohesins by recruiting PP2A to chromosomes[9,10]. If Sgo1 solely functioned to prevent centromeric cohesin removal by preventing the phosphorylation of Rec8, inactivation of *SGO1* should not affect the phenotype exhibited by Rec8-17A-expressing cells. Surprisingly, depletion of Sgo1 in Rec8-17A-expressing cells led to Rec8 cleavage, almost to the extent seen in wild-type cells, and an elimination of the metaphase I delay (Fig. 3a, b, Supplementary Fig. 10a) implicating Sgo1 in functions other than preventing Rec8 phosphorylation. An alternative explanation for this observation was that our mass-spectrometry analysis missed key phosphorylation sites, phosphorylation of which would allow for efficient cleavage of the Rec8-17A mutant protein in the absence of Sgo1. To test this possibility we examined Rec8 cleavage in cells depleted for Cdc5. Depletion of Sgo1 allowed Rec8 cleavage, and loss of Rec8 from chromosomes occurred (Fig. 3c; Supplementary Fig. 11). Chromosome segregation, as judged by the separation of *CEN5* GFP dots, and spindle elongation also took place (Fig. 3d, e, Supplementary Fig. 10b–d). It is possible that in the absence of Sgo1, low levels of Cdc5 and other protein kinases are capable of bringing about cohesin removal. We consider this possibility unlikely because both sister chromatid separation and spindle elongation occur with remarkable efficiency. Instead we suggest that Sgo1 affects cohesin cleavage by means in addition to preventing Rec8 phosphorylation.

To determine whether Rec8 phosphorylation contributes to establishing the step-wise nature of cohesin removal we raised two antibodies, one that recognizes phospho-serine 136 and one that recognizes phospho-serine 521 (Fig. 4a, Supplementary Fig. 12a). As predicted by the mass-spectrometry analysis, phosphorylation of S136 is Cdc5-dependent and phosphorylation of S521 is Cdc5-independent (Fig. 4a, b; Supplementary Fig. 12b). The anti-phospho-S521 antibody recognized phospho-S521 on chromosome spreads (Fig. 4c) and revealed that Rec8 phosphorylation on S521 mirrored the differential loss of arm and centromeric cohesins during meiosis I. Rec8 visualized using an antibody against a C-terminal tag (Rec8-Myc) appeared continuous and was found in long stretches on chromosome spreads, presumably representing chromosome axes. In contrast, the anti-phospho-S521 signal appeared fragmented (Fig. 4d) and frequently did not overlap with the kinetochore marker Ndc10 (Fig. 4e). Furthermore, the anti-phospho-S521 signal was absent from chromosome spreads of binucleate cells when only centromeric cohesins are left on chromosomes (Fig. 4d). S521 phosphorylation was not affected by depletion of Sgo1 or deletion of the centromere-associated kinase *BUB1* (data not shown). Our results show that S521 phosphorylation is reduced or perhaps even excluded from centromeric regions, but present on chromosome arms. However, Sgo1and Bub1 do not appear to regulate the phosphorylation state of S521 either because they only regulate the phosphorylation state of a subset of Rec8 phosphorylation sites or because they affect cohesins at centromeric regions through means other than preventing Rec8 phosphorylation. We do not know whether Rec8 is phosphorylated before its removal in metaphase II. We have not detected an anti-phospho-S521 signal in any binucleate cells. This result suggests that Rec8 phosphorylation on S521 may not be a prerequisite for Rec8 removal during meiosis II, which would be consistent with the observation that the *rec8-17A* mutant does not exhibit a delay in metaphase II.

Our studies not only produced an *in vivo*-derived consensus sequence for targets of Polo kinases but also provided insights into how cohesin removal is regulated in meiosis. Our results suggest that it is overall phosphorylation rather than phosphorylation of a specific site that is important for Rec8 cleavage. We also observed that the delay in Rec8 cleavage in the *rec8-17A* mutant was significantly shorter than that for cells lacking Cdc5, probably because cells depleted for Cdc5 exhibit additional defects[14,15], or because additional Cdc5 phosphorylation sites may exist in Rec8. Our results also revealed a role for recombination in establishing the step-wise loss of cohesion. Recombination establishes linkages between homologues, which are essential for silencing of the spindle checkpoint and thus the timely removal of cohesins from chromosome arms. Thus recombination not only ensures the correct attachment of bivalents to the meiosis I spindle but also, together with Rec8 phosphorylation and Sgo1, establishes the step-wise loss of cohesion, another key aspect of meiotic chromosome segregation.

METHODS

Strains and plasmids used in this study are described in Supplementary Table 3 and Supplementary Methods, respectively. Immunoblots were performed as described in ref. 26. Chromatin immunoprecipitation (ChIP) was performed according to ref. 8. Chromosomes were spread according to ref. 27 and indirect *in situ* immunofluorescence was performed according to ref. 28.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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