The conserved kinetochore protein shugoshin protects centromeric cohesion during meiosis

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Meiosis comprises a pair of specialized nuclear divisions that produce haploid germ cells. To accomplish this, sister chromatids must segregate together during the first meiotic division (meiosis I), which requires that sister chromatid cohesion persists at centromeres. The factors that protect centromeric cohesion during meiosis I have remained elusive. Here we identify Sgo1 (shugoshin), a protector of the centromeric cohesin Rec8 in fission yeast. We also identify a homologue of Sgo1 in budding yeast. We provide evidence that shugoshin is widely conserved among eukaryotes. Moreover, we identify Sgo2, a paralogue of shugoshin in fission yeast, which is required for faithful mitotic chromosome segregation. Localization of Sgo1 and Sgo2 at centromeres requires the kinase Bub1, identifying shugoshin as a crucial target for the kinetochore function of Bub1. These findings provide insights into the evolution of meiosis and kinetochore regulation during mitosis and meiosis.

In eukaryotes, sister chromatid cohesion is established during S phase and is maintained throughout G2 until the M phase. During mitosis, this cohesion is destroyed along the entire length of the chromosome, allowing sister chromatids to segregate to opposite sides of the cell (equational division), and ensuring that each daughter cell receives one copy of each chromosome. In contrast, meiosis consists of two rounds of chromosome segregation following a single round of DNA replication, leading to the formation of four haploid gametes from a diploid germ cell. During meiosis I, homologous chromosomes (homologues) pair up in order to recombine, forming chiasmata in which one sister chromatid from one homologue is covalently attached to a sister chromatid from the other homologue. Hence, for homologues to segregate at meiosis I, sister chromatid cohesion must be released along the chromosome arms to resolve chiasmata. However, sister chromatid cohesion is retained at the centromeres until meiosis II, when sister chromatids segregate as they do in mitosis, using the residual centromeric cohesion. Thus, meiotic divisions require sister chromatid cohesion to be released in two steps, yet the molecular basis for protection of centromeric cohesion only during meiosis I and only at the centromeres has remained unknown[1].

There are clues to the molecular nature of sister chromatid cohesion and the mechanism by which it is released at the onset of anaphase[1–5]. In various eukaryotes, sister chromatid cohesion depends on a multisubunit cohesin complex including Scc1 (Rad21 in the fission yeast *Schizosaccharomyces pombe*). Anaphase-promoting complex (APC)-dependent degradation of the securin Pds1 (Cut2 in *S. pombe*) allows release of the Esp1 (Cut1 in *S. pombe*) endopeptidase (separase), which in turn cleaves Scc1, releasing sister chromatid cohesion. During meiosis, the cohesin subunit Scc1 is replaced by a meiotic counterpart, Rec8 (refs 6–10). As Rec8 complexes reside only at centromeres after meiosis I and depletion of Rec8 disrupts centromeric cohesion, its presence at centromeres has been thought to confer the persistence of cohesion throughout meiosis I (ref. 11). Several lines of evidence[12,13] suggest that Rec8 along chromosome arms is cleaved by separase at anaphase I, whereas centromeric Rec8 is specifically protected until metaphase II. Budding yeast Spo13 has been implicated in the protection of centromeric Rec8 (refs 14, 15), but Spo13 is not centromeric and may function indirectly. *Drosophila* MEI-S332, a protein that resides at pericentromeric regions[16] and is required for the persistence of centromeric cohesion during meiosis I (ref. 17), has features of a candidate protector of meiotic centromeric cohesion, although the details of such protection have so far not been revealed[4]. Despite the completion of genome sequencing projects in several organisms, no homologues of Spo13 or MEI-S332 have emerged, preventing the formulation of a generalized view of protection. Concurrently, studies in fission yeast[18] have illuminated the importance of pericentromeric heterochromatin for recruiting centromeric Rec8 complexes and ensuring centromeric cohesion during meiosis I. However, pericentromeric heterochromatin cannot alone confer the specific protection of Rec8 at meiosis I compared with meiosis II. We now identify a meiosis-specific protein, Sgo1 (shugoshin, Japanese for 'guardian spirit'), that protects centromeric Rec8 from degradation during meiosis I.

Identification of Sgo1 in fission yeast

The replacement of the mitotic cohesin Rad21 with the meiotic version Rec8 is a prerequisite for protecting centromeric sister chromatid cohesion through anaphase of meiosis I (refs 19, 20). However, when we expressed Rec8 ectopically during mitosis, Rec8 localized largely at centromeres but disappeared at anaphase, with sister chromatids segregating to opposite sides of the cell (Fig. 1c, d). Moreover, the ectopic expression of non-cleavable Rec8 during mitosis (note that Rec8 is cleaved by separase Cut1 during meiosis[13]) resulted in an inability of sister chromatids to separate (Supplementary Fig. 1). Thus, in contrast to the situation during meiosis I, centromeric Rec8 is cleaved by separase during mitosis, resulting in sister chromatid separation. These observations prompted us to postulate a meiosis-I-specific centromeric protector of Rec8. To identify this factor, we searched for a gene that yields toxicity during mitotic growth only when co-expressed with Rec8. This screen identified a novel gene called *sgo1*$^+$ (open reading frame (ORF) SPBP35G2.03C). The hindrance of growth by Sgo1 was indeed dependent on Rec8, as Sgo1 had little effect on growth when co-expressed with Rad21 (Fig. 1a). Co-expression of *rec8*$^+$ and *sgo1*$^+$ frequently led to blocked nuclear division, as centromere-associated green fluorescent protein markers (*cen2–GFP*)[21] frequently segregated to the same side of a septated cell (Fig. 1b, c). To test the possibility that Sgo1 protects Rec8 from degradation at anaphase,

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we examined the localization of Rec8 in the context of Sgo1 expression. We found that the Rec8 localization at centromeres persisted through anaphase only when Sgo1 was co-expressed (Fig. 1d). As Sgo1 is expressed exclusively in meiosis (DNA microarray data[22]; see below), the foregoing results allowed us to postulate that Sgo1 is a protector of Rec8 during meiosis.

Sgo1 protects centromeric cohesion at meiosis I

To examine whether Sgo1 is indeed required for the protection of Rec8 during meiosis, we deleted the entire ORF encoding *sgo1*$^+$ and examined the phenotype. *sgo1*-depleted cells (*sgo1*Δ) were viable and showed normal vegetative growth. To examine meiotic chromosome segregation, we marked centromere-linked sequences with GFP (*cen2–GFP*) on only one of the two homologues in a zygote and monitored the segregation of the GFP dots during meiosis. In normal meiosis, monopolar attachment of sister kinetochores to the spindle is established in metaphase I; therefore, sisters move together to the same side of the zygote (reductional division) in the following anaphase I (Fig. 2a). Thanks to the centromeric cohesion preserved throughout anaphase I, bipolar attachment is secured at meiosis II, thus leading to faithful disjunction (Fig. 2a). We found that meiosis I appeared normal in *sgo1*Δ cells, as sister chromatid pairs generally moved together to the same side of each zygote (Fig. 2b). Hence, monopolar attachment was intact in this mutant. Moreover, by marking *cen2–GFP* on both chromosomes, we determined that homologues underwent faithful disjunction at meiosis I (data not shown). At meiosis II in *sgo1*Δ cells, however, sister chromatids failed to segregate properly, undergoing non-disjunction in approximately 50% of cells (Fig. 2b). This value is consistent with random chromosome segregation at meiosis II.

To examine centromeric cohesion, we monitored *cen2–GFP* marked on both homologues in zygotes arrested before meiosis II, the stage at which centromeric cohesion is normally preserved in *sgo1*$^+$ cells. We found that *sgo1*Δ cells frequently displayed precocious centromeric dissociation as split *cen2–GFP* signals prevailed in the dyad nuclei (Fig. 2c). This result may explain why the second meiotic division is random in *sgo1*Δ cells, because cohesion is required for sister kinetochores to be properly recognized by spindle microtubules extending from opposite poles. Next we examined whether protection of Rec8 at centromeres is dependent on Sgo1 by monitoring Rec8–GFP at late anaphase I and prometaphase II. Notably, although Rec8 signals were centromeric in wild-type cells, the Rec8 signals had largely disappeared from the centromeres at these stages in *sgo1*Δ cells (Fig. 2d). All phenotypes of *sgo1*Δ cells are reminiscent of heterochromatin-deficient *S. pombe*, in which Rec8 localization to the pericentromeric regions is decreased and centromeric cohesion is lost during meiosis I, leading to random division at meiosis II (ref. 18). We then examined chromatin binding by Rec8 in cells arrested before meiosis I using a chromatin immunoprecipitation (ChIP) assay. In marked contrast to heterochromatin-deficient cells, Rec8 localization was intact in *sgo1*Δ cells at the pericentromeric regions as well as all other regions tested (Fig. 2e). As monopolar attachment requires centromeric Rec8 (refs 20, 23), the proper location of Rec8 before meiosis I is consistent with the fact that monopolar attachment is intact in *sgo1*Δ cells. More importantly, these results indicate that the loss of centromeric Rec8 after meiosis I is brought about not by an initial defect in Rec8 localization to centromeres but rather by a defect in the preservation of centromeric Rec8 during anaphase I. Previous results suggested that the Cut1 separase becomes active at the onset of anaphase I and cleaves most chromosomal Rec8, leaving only centromeric Rec8 intact[13]. Our current results advocate that Sgo1 has an essential role in protecting centromeric cohesion at anaphase I by safeguarding cohesin Rec8 from separase cleavage.

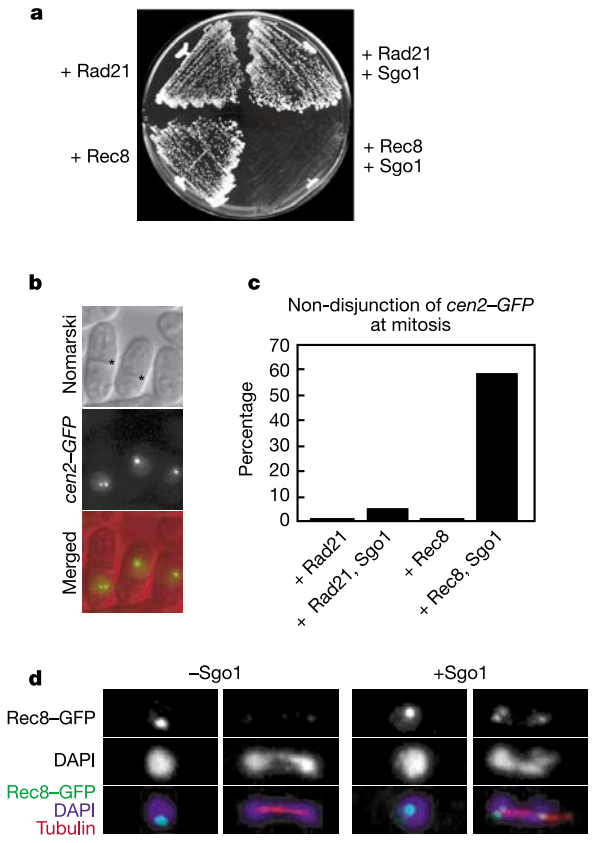

Figure 1 Co-expression of Sgo1 and Rec8 causes failure of sister chromatid separation during mitosis. **a**, The haploid *cen2–GFP* strains expressing the indicated genes by exogenous promoters (a constitutive promoter P*adh1* for *rad21*$^+$ or *rec8*$^+$, and a thiamine-repressible promoter P*nmt1* for *sgo1*$^+$) were streaked on a thiamine-depleted plate. **b**, Examples of P*adh1–rec8*$^+$ P*nmt1–sgo1*$^+$ cells cultured at 30 °C for 15 h after thiamine depletion. Note the non-disjunction of *cen2–GFP* in the septated cells (asterisk). **c**, The frequency of non-disjunction was counted among septated cells ($n > 100$). **d**, P*adh1–rec8*$^+$*–GFP* strains with or without P*nmt1–sgo1*$^+$ were cultured as in **b**, fixed with formaldehyde and stained with DAPI and antibodies against GFP and tubulin. Examples of each strain at interphase (left cell) and anaphase (right cell) are shown. Note that the Rec8 signal persists during anaphase mostly in *sgo1*$^+$-expressing cells (84%, $n = 129$) and only slightly in non-expressing cells (9%, $n = 129$).

Sgo1 localizes at centromeres during meiosis I

To detect the Sgo1 protein, we raised antibodies specific for Sgo1. Western blotting indicated that Sgo1 is expressed only around meiosis I (Fig. 3a). Immunofluorescence microscopy on cells at various stages of meiosis revealed that Sgo1 appears at late prophase of meiosis I and is fully localized as several punctate dots until metaphase I (Fig. 3b). These dots localize closely with the Mis6 kinetochore protein[24], indicating that Sgo1 is associated with centromeric regions (Fig. 3c). At the onset of anaphase I, Sgo1 signals decrease markedly. We found that Sgo1 remains undegraded at centromeres in APC-depleted cells arrested at metaphase I, but undergoes normal degradation in separase-defective cells (Supplementary Fig. 2), suggesting that Sgo1 degradation at anaphase I is regulated more directly by the APC rather than through separase. Although we detected residual Sgo1 signals at the centromeres in early anaphase I, they disappeared completely by the end of anaphase I (Fig. 3b). This suggests that a substantial amount of Sgo1 is required at the onset of anaphase I when separase is fully activated. As anaphase I progresses, however, smaller and smaller amounts of Sgo1 would be required. This idea is tenable if the separase activity is quickly downregulated or prevented from accessing chromosomes during anaphase I. Sgo1 does not reappear

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during meiosis II (Fig. 3b), consistent with the idea that Sgo1 is only required for the protection of Rec8 during meiosis I.

We have suggested previously that Rec8 localization at pericentromeric regions is especially important for the persistence of centromeric cohesion throughout meiosis I (ref. 18). If Sgo1 is a centromeric protector of Rec8, then it might be expected to localize there as well. To test this possibility, we delineated Sgo1 localization more precisely using a ChIP assay. Sgo1 does indeed associate with pericentromeric heterochromatin regions rather than central core regions along the centromere sequences (Fig. 3d). As immunoprecipitation experiments indicated that Sgo1 interacts with Rec8 complexes *in vivo* (Fig. 3f), the protection is probably carried out through close interaction. Together, our results indicate that Sgo1 resides at pericentromeric regions and operates to protect Rec8 from cleavage by separase at anaphase I (Fig. 3g).

Sgo2 is a mitotic Sgo1 paralogue in fission yeast

By means of a conventional BLAST search of genome databases, we identified Sgo1-like proteins from *Saccharomyces cerevisiae* and *Neurospora crassa*, suggesting that Sgo1 is a conserved protein (see below). In the same search, we identified an *S. pombe* Sgo1 paralogue, which we call Sgo2 (ORF SPAC15A10.15). We disrupted the *sgo2*$^+$ gene and found that *sgo2*Δ cells are viable but show sensitivity to the spindle-destabilizing drug thiabendazole (TBZ) (Fig. 4a). Consistently, *sgo2*Δ cells display an increased incidence of chromosome mis-segregation at mitosis (Fig. 4c). These mitotic phenotypes are remarkable, as *sgo1*Δ cells never show such a defect (Fig. 4a). To investigate its cellular distribution, the endogenous *sgo2*$^+$ gene was tagged with GFP. In proliferating cells, Sgo2–GFP was observed as two or three dots within the nucleus (Fig. 4e); however, it localized closely with the centromere protein Mis6 at

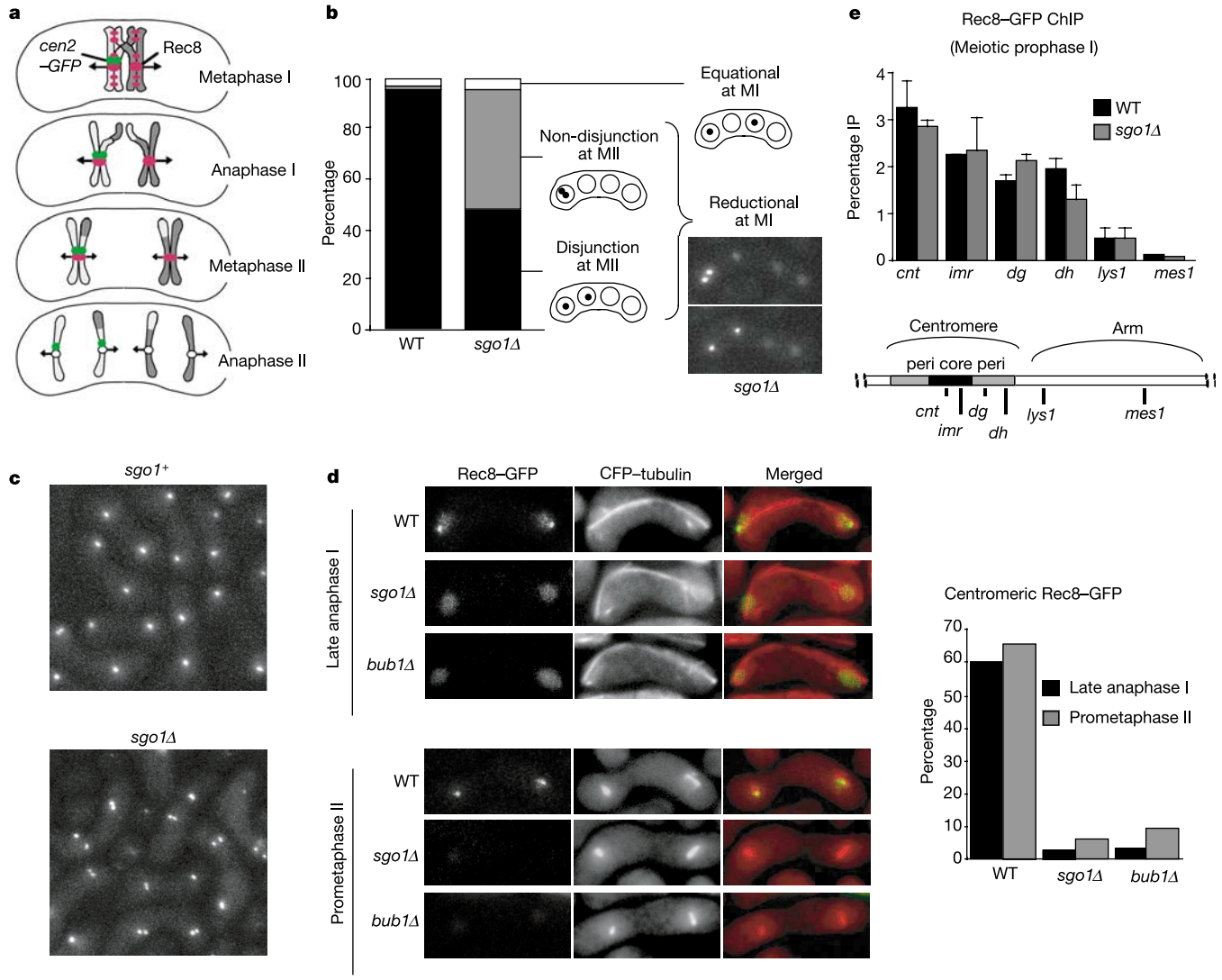

Figure 2 Sgo1 is required to protect Rec8 and thereby cohesion at centromeres during anaphase of meiosis I. **a**, Schematic drawing of the behaviour of homologous chromosomes (white and grey) in normal meiosis I and II. Expression of *cen2–GFP* (green oval) is marked on one of the homologous chromosomes and the location of the cohesin Rec8 (red oval) is indicated. **b**, One of the homologues marked with *cen2–GFP* was monitored for segregation during meiosis in wild-type (WT) and *sgo1*Δ cells ($n > 170$). Examples of *sgo1*Δ cells are shown (bottom right panel). **c**, Cells of both homologues marked with *cen2–GFP* were arrested before meiosis II by introducing the *mes1*Δ mutation, and were examined for *cen2–GFP* dots. **d**, The Rec8–GFP signal was monitored at late anaphase I ($n > 30$) and at prometaphase II ($n > 100$) in the indicated cells, and the frequency of the cells displaying centromeric Rec8–GFP was counted. The spindles were visualized by expressing cyan fluorescent protein (CFP)–Atb2 (α2-tubulin)[43]. **e**, A ChIP assay with anti-GFP antibodies was used to measure Rec8–GFP levels throughout the indicated chromosome sites in the arrested cells before meiosis I (*mei4*Δ arrest). The bottom panel shows a schematic representation of *S. pombe* chromosome I as well as the primers used (*cnt*, *imr*, *dg*, *dh*, *lys1*, *mes1*).

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metaphase and disappeared during anaphase (Fig. 4d, e). ChIP assays showed that Sgo2 chromatin association was detectable only in synchronous populations of mitotic cells, in which Sgo2 localized to the pericentromeric regions (Fig. 4f). Reinforcing this localization, *sgo2* deletion confers a marked defect in chromosome segregation if combined with the heterochromatin-deficient *swi6*Δ mutation, which by itself slightly impairs kinetochore function[25] (Fig. 4b, c). These results suggest that Sgo2 cooperates with pericentromeric heterochromatin factors to ensure chromosome segregation at mitosis. Moreover, we found that Sgo2 persists throughout meiosis (Supplementary Fig. 4) and that *sgo2*Δ cells have a modest increase in non-disjunction of homologues at meiosis I (about 15%), suggesting that Sgo2 is also important for promoting proper meiosis I. Notably, the role of Sgo2 in meiosis does not overlap with that of Sgo1, as *sgo1*Δ neither yields an apparent defect at meiosis I (Fig. 2b) nor enhances the defect of *sgo2*Δ (data not shown).

Shugoshin location controlled by Bub1

A conserved centromere-associated kinase, Bub1, is thought to have a function in protecting Rec8 during meiosis, as centromeric Rec8 cannot be detected after meiosis I in fission yeast *bub1* mutants[26] (Fig. 2d). Although *bub1* mutation has pleiotropic effects in meiotic chromosome segregation[26], we thought that Sgo1 function might be targeted by Bub1 activity. To address this issue, we examined Sgo1–GFP signals in *bub1*Δ cells undergoing meiosis. Notably, *bub1*Δ cells were almost completely devoid of punctate centromeric Sgo1–GFP signals, showing instead a diffuse fluorescence within the nucleus (Fig. 3e). Identical results were obtained using the Bub1(K762R) point mutation (not shown), which abolishes the kinase activity[27]. As substantial levels of Sgo1 protein were detected in meiotic *bub1*Δ cells by western blot analysis (Fig. 3e), Bub1 primarily regulates localization rather than stability of the Sgo1 protein. Therefore, the observed defects in centromeric protection in *bub1*Δ cells might be explained by an impaired location of Sgo1. Moreover, these results suggest that Bub1 might regulate the timing of Sgo1 loading to and the disappearance from centromeres, as Bub1 localizes at centromeres during a similar period in meiosis I (ref. 26).

In parallel experiments, we found that mitotic Sgo2 localization at centromeres was similarly disrupted in *bub1* mutants (Fig. 4d), whereas protein levels of Sgo2 were unchanged (Supplementary Fig. 3c). It has been suggested that loss of Bub1 function leads to a weakness in kinetochore function[28]. In this regard, the Bub1(K762R) mutation shows a synthetic defect in growth with *swi6*Δ, a mutation that also impairs slightly kinetochore function through its role in pericentric heterochromatin formation. We found that *sgo2*Δ similarly showed a synthetic defect in growth with *swi6*Δ (Fig. 4b), exhibiting severe mis-segregation of chromosomes at mitosis (Fig. 4c). As the *sgo2*Δ *bub1*Δ double mutant showed no cumulative defects in growth or in TBZ sensitivity (Fig. 4a), these genetic analyses confirm that Sgo2 and Bub1 function in tandem to ensure chromosome segregation in mitosis. Taken together, these results reveal that Sgo1 and Sgo2 localization at centromeres is a crucial function of Bub1 kinase in meiosis and mitosis, respectively.

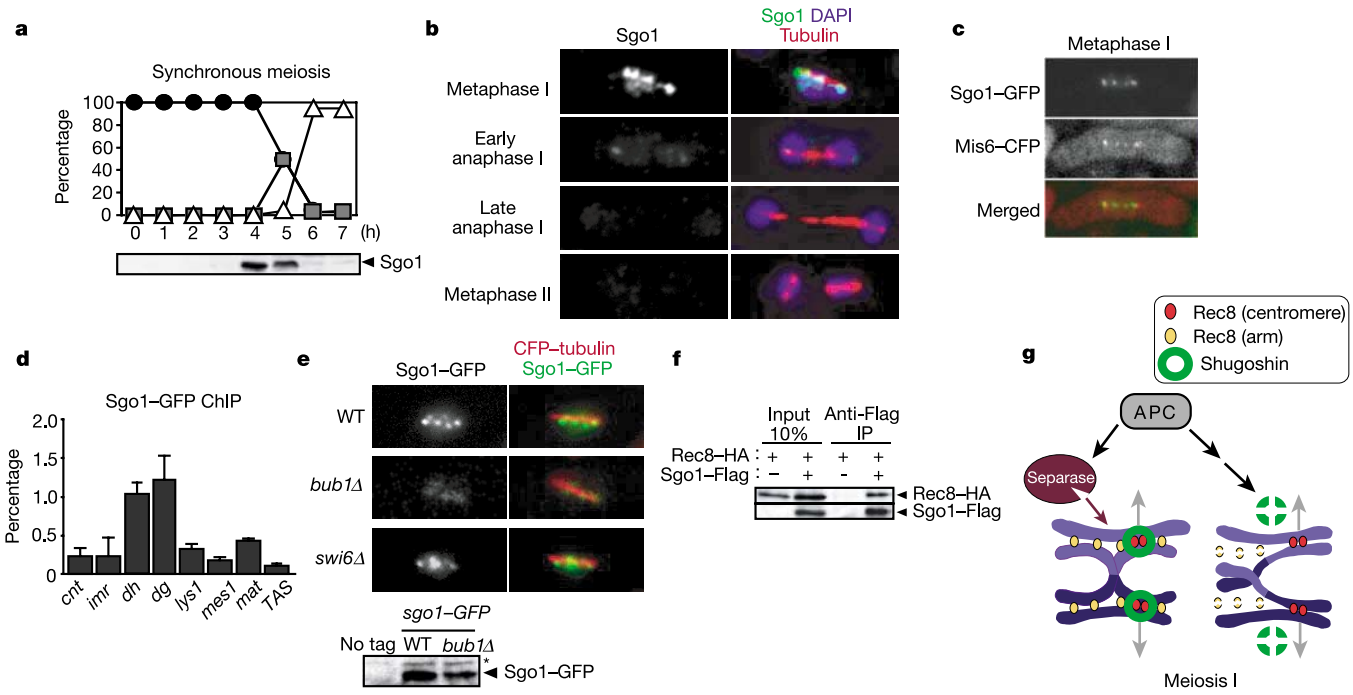

Figure 3 Sgo1 localizes at pericentromeric regions during meiosis I. **a**, Synchronous meiosis of diploid *pat1-114/pat1-114* cells[13] was sampled. Meiotic nuclear division was monitored by DAPI staining, and the protein level of Sgo1 was detected by western blotting using anti-Sgo1 antibodies. Circle, one nucleus; square, two nuclei; triangle, 3–4 nuclei. **b**, Sgo1 (green) was counterstained with tubulin (red) and DAPI (blue) in the meiotic cell at the indicated stages. **c**, An *sgo1*$^{+}$*–GFP* cell co-expressing *mis6*$^{+}$*–CFP* was examined under fluorescence microscopy. Sgo1–GFP (green) and Mis6–CFP (red) are merged. **d**, A ChIP assay with anti-GFP antibodies was used to measure Sgo1–GFP levels throughout the indicated chromosome sites in cells arrested at metaphase I. We used the same primers as for Fig. 2e together with additional primers at *mat* (heterochromatin region at the mating-type locus) and *TAS* (telomere-associated sequence). **e**, Sgo1–GFP (green) was detected at metaphase I in the indicated cells expressing CFP–Atb2 to visualize spindles (red). Western blot analysis of Sgo1–GFP was carried out on the indicated strains arrested at metaphase I. The asterisk indicates a nonspecific band. **f**, Rec8–HA was expressed with or without Sgo1–Flag in proliferating cells and the extracts were immunoprecipitated with anti-Flag antibody. **g**, A model for the action of shugoshin in meiosis. Shugoshin localizes at centromeres in meiosis I depending on Bub1 kinase, and it protects centromeric Rec8 complexes from cleavage by separase at the onset of anaphase I, thereby preserving centromeric cohesion until meiosis II. Shugoshin is degraded depending on APC during anaphase I.

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Characterization of a Sgo1 homologue in budding yeast

We identified a single Sgo1 homologue in *S. cerevisiae*, ScSgo1 (ORF YOR073W), which has so far not been analysed. We examined the cellular localization of ScSgo1 by tagging endogenous *ScSGO1* with GFP, and found that the pattern of ScSgo1 localization closely resembles that of *S. pombe* Sgo2 in mitosis and Sgo1 in meiosis (Supplementary Fig. 5). To examine the function of ScSgo1, we disrupted the *ScSGO1* gene. *Scsgo1*Δ cells were viable but grew slowly and showed sensitivity to the spindle-destabilizing drug benomyl (Fig. 5a), suggesting that kinetochore function might be impaired. We used a colony-sectoring assay to compare the rates of chromosome loss in *Scsgo1*Δ cells with those in wild-type cells. Whereas less than 2% of wild-type colonies contained red sectors (which indicate chromosome loss), approximately 40% of the *Scsgo1*Δ colonies contained such sectors (Fig. 5b). We conclude that ScSgo1 has a crucial role at kinetochores for ensuring mitotic chromosome segregation. *Scsgo1*Δ cells showed significant defects in the initiation of meiosis, as many cells arrested with a single nucleus in the meiotic condition. Among the leaked tetranucleate products of meiosis, however, the distribution pattern of *cenV–GFP* was consistent with proper segregation at meiosis I but random segregation at meiosis II (Fig. 5c). We also found that tagging chromosomal *ScSGO1* with a 13-Myc tag at its carboxy terminus, which by itself yields no detectable defects in mitotic growth or meiosis I, resulted in impaired segregation at meiosis II (34% non-disjunction indicating 68% random segregation) (Fig. 5d). Moreover, the Myc-tagged ScSgo1 cells showed frequent separation of sister centromeres at late meiotic anaphase I (Fig. 5e), indicating that centromeric cohesion was not properly protected. Together, these results support the idea that ScSgo1 has a crucial role in protecting centromeric cohesion throughout meiosis I, thereby ensuring normal progression to meiosis II, as does fission yeast Sgo1.

Conservation of shugoshin among eukaryotes

Our BLAST searches identified only three Sgo1-like proteins, all in fungi: *S. pombe* Sgo2, *S. cerevisiae* ScSgo1 and *N. crassa* B23G1.060. As we found two conserved regions among these proteins, we used the Block Maker and MAST programs[29,30] to search for related proteins under conditions of two-block sequences. This approach yielded several candidate proteins from various eukaryotes including fly, nematode, plant, mouse and human (Fig. 6). Notably, the list included *Drosophila* MEI-S332, a previously characterized protein essential for preserving centromeric cohesion in meiosis[17], although the similarity score is marginal (*E*-value = 10). All other proteins in the list show a short stretch of similarity in the C-terminal basic regions, whereas the primary sequences in the first block are not conserved except that they all contain a putative coiled-coil. The space and sequences between these two blocks diverge among the proteins. As these blocks were previously identified to be important for MEI-S332 function[31], we investigated the significance of the conserved regions in Sgo1. We changed several amino acids individually within these similarity blocks to alanines and investigated the function of the mutant proteins *in vivo* (Supplementary Fig. 6). We found that three conserved amino acids known to be critical for MEI-S332 function[31] were also required for Sgo1 function (N13, V34 and S384 in MEI-S332; N29, I50 and S294 in Sgo1) (Fig. 6, marked as arrowheads). Other conserved amino acids within the second block (P293, R296, K298, L299, R300 in Sgo1) were again all required for Sgo1 function (Fig. 6, asterisks), whereas non-conserved residue T297 could be changed to alanine without loss of function (Fig. 6, circle). These results underscore that the marginal

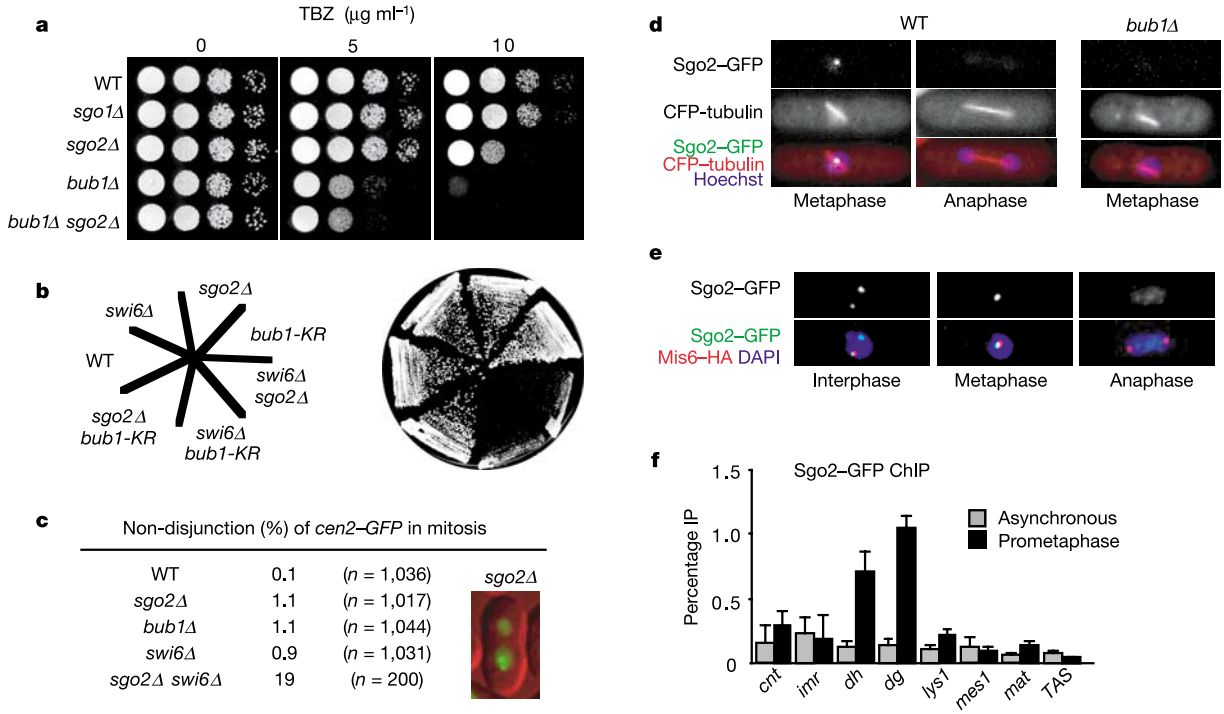

Figure 4 Sgo2 has a crucial role in mitotic division at the kinetochore. **a**, Serial dilutions of the indicated cultures were spotted onto YEA plates containing 0, 5 or 10 μg ml^{-1} TBZ and incubated at 30 °C for 3 days. **b**, The indicated strains were streaked on YEA plates and incubated at 30 °C for 3 days. **c**, The frequency of non-disjunction of *cen2–GFP* was monitored in the indicated proliferating cells. **d**, Sgo2–GFP (green) was detected at metaphase in wild-type and *bub1*Δ cells expressing CFP–Atb2 for the visualization of spindles (red). DNA was stained with Hoechst (blue). A wild-type cell at anaphase is also shown. **e**, The *sgo2*$^{+}$*–GFP mis6*$^{+}$*–HA* cells were fixed and stained with anti-GFP and anti-HA antibodies. Note that the Sgo2–GFP signal near Mis6–HA is faint or undetectable in interphase cells but is obvious in metaphase cells. **f**, A ChIP assay was used to measure Sgo2–GFP levels throughout the indicated chromosome sites in cells arrested at prometaphase or in asynchronous cells.

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structural similarity found between *S. pombe* Sgo1 and the other proteins in various eukaryotes is significant. Plants and mammals carry two shugoshin-like proteins, suggesting that the function of shugoshin may have diverged to accomplish mitosis and meiosis, as in fission yeast.

Discussion

Our results provide some mechanistic insight into how shugoshin protects centromeric cohesion in meiosis. Previously we have suggested that Rec8 complexes enriched at the pericentromeric regions are crucial for preserving centromeric cohesion through meiosis I (ref. 18). Here we show that the protector protein Sgo1 also localizes at pericentromeric regions during meiosis I (Fig. 3d), consistent with the notion that Sgo1 protects precisely those Rec8 complexes that preserve centromeric cohesion. We found that Rec8 localization is not dependent on Sgo1 and vice versa (Fig. 2e, not shown). This independency of localization ensures that the mechanism protects Rec8 only at centromeres and not along chromosome arm regions. We suggest that shugoshin shields Rec8 physically from separase action or counteracts it. In this regard, we found that strong overexpression of Sgo1 moderately interferes with mitotic growth even in the absence of Rec8 expression (not shown), and that mild expression of Sgo1 kills a *cut1* mutant[32] even at the permissive temperature for the *cut1* allele (Supplementary Fig. 7). These results suggest that Sgo1 itself might have some ability to counteract separase function *in vivo*, although further analysis is necessary to address the precise mechanism by which shugoshin counteracts separase at the centromeres. We have shown that the co-expression of Sgo1 and Rec8 leads to the inability of sister chromatids to separate in mitosis (Fig. 1), suggesting that Sgo1 itself has the ability to protect Rec8 from degradation. However, in budding yeast cells, which express only a single shugoshin homologue, expression of Rec8 does not lead to a block in mitosis unless Spo13 is also expressed[14,15]. This observation suggests that Spo13 is a potential meiotic activator of budding yeast shugoshin. As *S. pombe* cells expressing Rec8 instead of Rad21 exhibit normal mitotic growth (Fig. 1a), Sgo2 has no obvious activity in protecting Rec8 during mitosis. Thus, *S. pombe* Sgo1 appears to be well developed as a specialized Rec8 protector, obviating the need for meiosis-specific activators of shugoshin in fission yeast, and potentially explaining why Spo13 is not conserved.

Although fission yeast Sgo2 has some minor involvement in meiosis, it appears to function primarily in mitosis, whereas Sgo1 is dispensable for mitosis. Previous studies in *Drosophila* revealed that MEI-S332, presumably the only shugoshin in this organism, localizes to centromeres in mitosis and may have some role in strengthening cohesion[33]. Here we find that the single budding yeast shugoshin has an important role in mitotic chromosome segregation, as the shugoshin mutant shows obvious chromosome instability during proliferation (Fig. 5b). Fission yeast *sgo2*Δ cells also show a modest defect in mitotic chromosome segregation and it becomes marked if an HP1 homologue, Swi6, is simultaneously depleted (Fig. 4b, c). Therefore, Sgo2 has an important role at mitotic kinetochores in a redundant capacity with centromeric heterochromatin. As heterochromatin is required to recruit large amounts of cohesin to centromeres[34,35], our results suggest that mitotic shugoshin may also have a close functional relationship with cohesin. One proposal would be that Bub1 senses a lack of

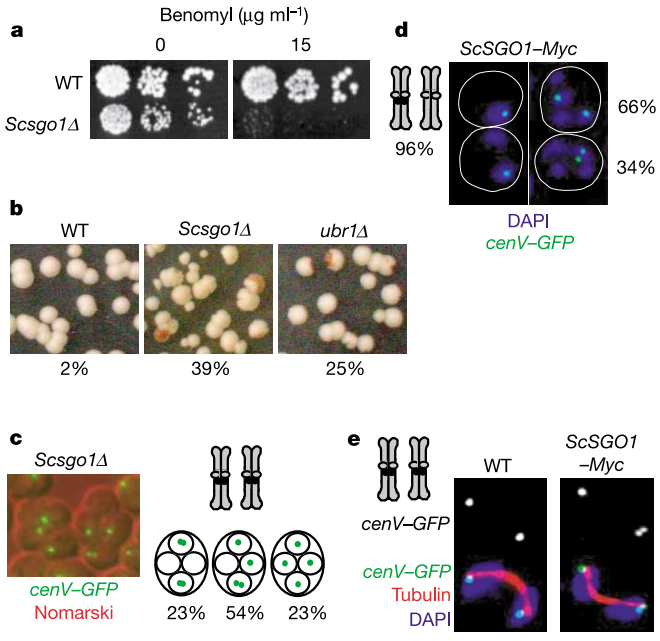

Figure 5 Analysis of budding yeast shugoshin ScSgo1. **a**, Serial dilutions of the indicated cultures were spotted onto YPD plates containing 0 or 15 μg ml^{-1} benomyl. **b**, Chromosome loss was analysed in wild-type (WT) and *Scsgo1*Δ mutants by a colony-sectoring assay. The *ubr1*Δ mutant was used as a positive control[40]. The frequency of sectoring colonies is shown at the bottom ($n > 120$). **c**, Examples of segregation of *cenV–GFP* in *Scsgo1*Δ tetrads. The segregation patterns in tetrads were classified mostly as one of the three shown at the right ($n = 200$). **d**, *ScSGO1–Myc* diploids were induced to synchronous meiosis and examined for the segregation of *cenV–GFP* marked on one of two homologues at meiosis I and meiosis II. The cells mostly underwent a reductional segregation pattern at meiosis I (96%, $n = 207$), whereas there was a high incidence of non-disjunction at meiosis II (34%, $n = 322$). **e**, Cells marked with *cenV–GFP* on both homologues were induced to meiosis and counter-stained with anti-tubulin antibody and DAPI. Cells at late anaphase I were examined for dots of *cenV–GFP* expression. *ScSGO1–Myc* cells frequently showed split *cenV–GFP* expression dots at either pair of sister chromatids (72%, $n = 138$), whereas control wild-type cells did not (<2%, $n = 106$).

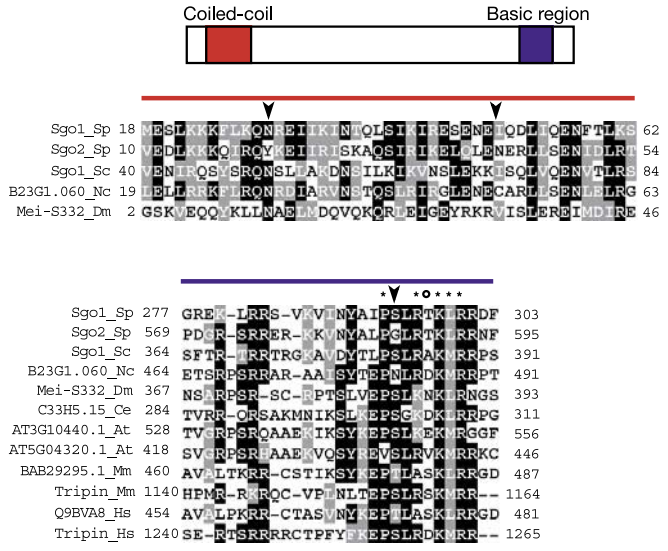

Figure 6 Alignment of the amino-terminal coiled-coil regions and C-terminal basic regions of shugoshin-like proteins in various organisms. The primary sequences of the N-terminal regions of Sgo1 are conserved among *S. pombe* (Sp; Sgo1 and Sgo2), *S. cerevisiae* (Sc; ScSgo1) and *N. crassa* (Nc; B23G1.060), whereas the sequences in other species, including MEI-S332, are not conserved, although all carry the putative coiled-coil motif (predicted by COILS program[44]). Dm, *Drosophila melanogaster*; Ce, *Caenorhabditis elegans*; At, *Arabidopsis thaliana*; Mm, *Mus musculus*; Hs, *Homo sapiens*. See text for definition of arrowheads, asterisks and circle.

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kinetochore–microtubule attachments or tension and thus stabilizes Sgo2 to maintain cohesion at centromeres. However, we could not detect any decrease of the cohesin Rad21 from centromeres in *sgo2*Δ cells even when the spindles were destabilized by the *nda3* mutation so that all cells were arrested at prometaphase by spindle checkpoint (T.S.K and Y.W., unpublished observations). The detailed analysis of shugoshin function at mitotic kinetochores will be an intriguing subject of future study.

Our studies revealed that the conserved kinetochore kinase Bub1 is required for the centromeric localization of both mitotic and meiotic shugoshins, suggesting that Bub1 might regulate loading and/or maintenance of shugoshin at kinetochores. Although it is unclear whether shugoshin is a direct substrate of Bub1, the coincident localization of these proteins at metaphase kinetochores during mitosis and meiosis I suggests a possible close interaction between these proteins. Notably, *sgo1*Δ and *sgo2*Δ mutants share many if not all of the defects of *bub1*Δ cells in meiosis and mitosis, respectively (Figs 2d and 4b, c). Thus, we propose that shugoshin is a crucial target of Bub1 function at kinetochores. As mutations in human homologues of Bub1 have been found in subtypes of colorectal cancer that exhibit chromosome instability[36], our results suggest that human shugoshin may be a potential oncoprotein. In this regard, it is intriguing that the human shugoshin-like protein Q9BVA8 was recently identified as an antigen whose level is elevated in most breast cancers[37]. Chromosome segregation in meiosis is also clinically important, as failures in this process result in aneuploidy, a major cause of miscarriage and birth defects in humans[38]. In conclusion, we have identified a novel protein family, shugoshin, that protects sister chromatid cohesion proteins at centromeres during meiosis. Moreover, we have also established that shugoshin has a crucial role at kinetochores in guarding against chromosome instability during mitosis. This work provides a new model for considering the evolution of meiosis and an integrated understanding of eukaryotic chromosome segregation. □

Methods

Screening of the Rec8 protector

We searched for a gene that is toxic only when co-expressed with Rec8 in vegetative cells. The Rec8 coding sequence fused with GFP was cloned under the thiamine-repressible *nmt1* promoter into pREP82 (*ura4*$^+$ marker), to construct pREP82–*rec8*$^+$–*GFP*. We used an *S. pombe* complementary DNA library, which was constructed using messenger RNA prepared from meiotic cells, and pREP3 vector (*nmt1* promoter, *LEU2*$^+$ marker) (Y. Akiyoshi and Y.W., unpublished observations). The *leu1 ura4-D18* cells carrying pREP82– *rec8*$^+$–*GFP* were transformed with the cDNA library, spread on agar plates containing thiamine (promoter off) and incubated at 30 °C for 3 days. The colonies were then replicated onto two thiamine-free plates: one containing 5′-FOA (5′-fluoro-ortoic acid) and uracil where only cells that drop the plasmid pREP82–*rec8*$^+$–*GFP* can grow (thereby expressing a library clone alone), and the other without 5′-FOA and uracil (allowing co-expression of *rec8*$^+$–*GFP* and a library clone). We added phloxine B—a drug that stains dead cells red—to both plates, thereby highlighting dying colonies. After incubation for two days, the colonies exhibiting only dead cells on the co-expression plate were picked up, and the library-derived plasmids were recovered and analysed.

Schizosaccharomyces pombe strains

All strains used are listed in Supplementary Table 1. Deletion and GFP- or Flag-tagging of endogenous *sgo1*$^+$ and *sgo2*$^+$ were performed by a polymerase chain reaction (PCR)-based gene-targeting method[39]. *sgo1*$^+$–*Flag–GFP* was created by inserting GFP at the C terminus of the PCR-amplified *sgo1*$^+$–*Flag*, and integrated at the endogenous *sgo1* locus. We further replaced the endogenous promoter of *sgo1*$^+$ with the *nmt1* promoter to generate P*nmt–sgo1*$^+$ or P*nmt–sgo1*$^+$*–Flag–GFP* by the PCR-based gene targeting method[39]. We abbreviate the tagged protein to Sgo1–GFP or Sgo1–Flag, depending on the purpose. We used a *mei4*Δ mutation to arrest meiotic cells before meiosis I (close to late prophase in meiosis I) and a *mes1*Δ mutation to arrest cells after meiosis I, as described[8].

Chromatin immunoprecipitation assays

We used diploid *sgo1*$^+$*–Flag–GFP* cells for ChIP assays with Sgo1. To achieve a highly synchronous culture, we replaced the endogenous *slp1*$^+$ (*CDC20* homologue) promoter with the *rad21*$^+$ promoter, which is not active during meiosis, to arrest the cells at metaphase I. The cells were incubated in nitrogen-depleted medium for 12 h at 30 °C with the result that approximately 60% of the cells were arrested at metaphase I. For ChIP with Sgo2, *nda3-KM311 sgo2*$^+$*–GFP* cells were grown at 30 °C, and then shifted to 18 °C. After incubation for 8 h most of the cells were arrested at prometaphase. The cells were fixed with 3% paraformaldehyde at 18 °C for 30 min and extracts were prepared. ChIP assays were carried out as described previously[35]. The sequences of primers used have been described previously[20] except for the *mat* primers 5′-GTATGTGGAACAAGAGAAG-3′ and 5′-CTCGCCTGCTTACATTTTAAGG-3′.

Preparation of anti-Sgo1 antibodies

The *sgo1*$^+$ ORF was amplified by PCR from an *S. pombe* cDNA library, and inserted into plasmids pGEX4T-2 (Pharmacia Biotech) and pET-19b (Novagen) for the production of recombinant proteins glutathione *S*-transferase (GST)–Sgo1 and His-tagged Sgo1, respectively. GST–Sgo1 was used to immunize a rabbit, and the raised antibodies were purified by His-tagged Sgo1 as previously described[13].

Immunostaining

To stain endogenous Sgo1, wild-type diploid cells cultured for 5 h in MM-N medium were fixed with 3% formaldehyde and stained by the method described previously[13]. Sgo1 was detected using rabbit anti-Sgo1 antibodies and Alexa-488-conjugated anti-rabbit antibody (Molecular Probes). Tubulin was detected using the mouse anti-tubulin antibody TAT-1 (a gift from K. Gull) and Cy3-tagged anti-mouse antibody (Chemicon). For detecting GFP-tagged proteins, we used mouse anti-GFP antibody (Roche) and BODIPY FL-conjugated anti-mouse antibody (Molecular Probes). Mis6–haemagglutinin (HA) was detected with rabbit anti-HA antibody Y-11 (Santa Cruz) and Alexa-488-conjugated anti-rabbit antibody. Cells were counterstained with DAPI (4,6-diamidino-2-phenylindole) to visualize DNA.

Co-immunoprecipitation

Cells of strain P*adh–rec8*$^+$*–3HA* P*nmt41–sgo1*$^+$*–FLAG–GFP* and control P*adh–rec8*$^+$*–3HA* cells were cultured without thiamine for 15 h at 30 °C, collected, and extracts were prepared. To liberate chromatin-bound proteins, we treated the extracts with DNase I. After clarifying the extracts by centrifugation, the Sgo1–Flag–GFP protein was immunoprecipitated with anti-Flag antibody M2 (Sigma). Rec8–3HA and Sgo1–Flag–GFP were detected by anti-HA antibody Y-11 and anti-Flag antibody M2, respectively.

Analysis of budding yeast

All strains used are listed in Supplementary Table 1. The chromosome loss assay was carried out as described previously[40]. The *ScSGO1* gene was deleted or epitope-tagged using PCR-generated cassettes[41]. Correct gene targeting was checked by PCR. *URA3–GFP* dots marking chromosome V (*cenV–GFP*) were described previously[6]. Sporulation was induced by incubating cultures of diploid cells at 30 °C as described previously[42]. *In situ* immunofluorescence was performed as described[42].

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