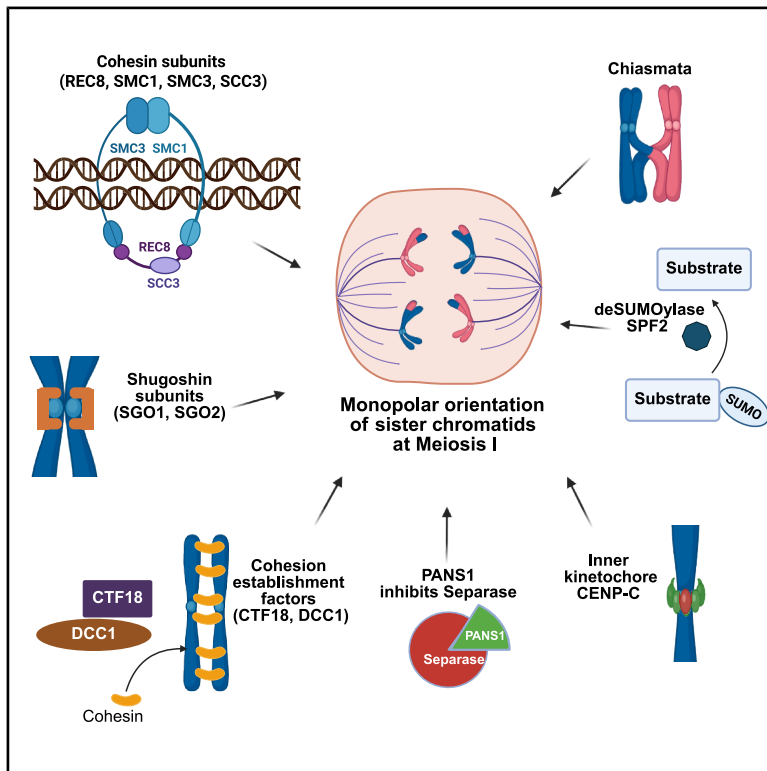


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Cohesin and its regulation promote monopolar kinetochore orientation at meiosis I in *Arabidopsis*

Graphical abstract



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In brief

Singh et al. used a forward genetic screen in *Arabidopsis* to identify ten proteins that promote monopolar kinetochore orientation during meiosis I. Cohesin subunits and cohesin regulators, CENP-C, and deSUMOylation act together with chiasmata to ensure the separation of homologs rather than sister chromatids at anaphase I.

Highlights

- A genetic screen identifies ten genes promoting monopolar orientation at meiosis I
- Monopolar orientation is supported by the cohesin complex and its regulators
- CENP-C and the deSUMOylase SPF2 also contribute to monopolar orientation
- Chiasmata and fusion of kinetochore synergistically support monopolar orientation



Article

Cohesin and its regulation promote monopolar kinetochore orientation at meiosis I in *Arabidopsis*

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SUMMARY

The first division of meiosis is unique in its capacity to halve the ploidy of future gametes. To this end, one key innovation compared with mitosis is the monopolar orientation of the pairs of sister kinetochores required for the proper separation of homologs at meiosis I. How monopolar orientation is imposed is unclear and seems to vary in eukaryotes. Here, we performed a forward genetic screen in *Arabidopsis thaliana*, specifically designed to identify the molecular components imposing monopolar orientation, based on mutants' ability to restore fertility in *spo11 osd1* haploid plants. We show that monopolar orientation involves all four cohesin subunits (REC8, SCC3, SMC1, and SMC3), the cohesion establishment factors CTF18 and DCC1, the cohesin protectors SGO1/2 and PATRONUS (PANS1), the inner kinetochore protein CENP-C, and the deSUMOylase SUMO PROTEASE RELATED TO FERTILITY 2 (SPF2). The mutants show bipolar orientation of achiasmatic chromosomes; however, monopolar orientation is maintained in the presence of crossovers, despite most of them displaying the splitting of sister kinetochores and reduced levels of cohesin at metaphase I. Taken together, the findings demonstrate that cohesion establishment and maintenance, kinetochore function, and deSUMOylation, together with crossovers, promote monopolar orientation in plants and support a cohesion-driven model of kinetochore orientation at meiosis I that is conserved across kingdoms.

INTRODUCTION

Meiosis halves the chromosome number by executing two rounds of division. Our understanding of how meiosis ensures that replicated chromosomes segregate together during one division and then divide during the next remains incomplete.^{1,2} The kinetochore is a large protein complex that enables microtubules to attach to chromosomes at the centromere. During meiosis I, the kinetochores of replicated sister chromatids act as a unified microtubule-binding face that “mono-oriens” toward one spindle pole, as opposed to “bi-orienting” during mitosis. This process is known as monopolar orientation, or sister kinetochore co-orientation. The outcome of monopolar orientation is the segregation of homologous chromosomes to opposing poles rather than chromatids, representing a reductional rather than equational division.

The ring-shaped cohesin complex ensures cohesion between replicated sister chromatids and regulates the packaging and organization of chromosomes during both mitosis and meiosis.^{3,4} During meiosis, a population of cohesin contains the meiosis-specific “kleisin” subunit REC8, and together with non-REC8-

containing cohesin, it entraps sister chromatids. While the rest of cohesins are cleaved by the Separase at anaphase I, cohesins in the vicinity of centromeres are protected by Shugoshin (SGO) proteins through the recruitment of the protein phosphatase 2A (PP2A) phosphatase. This protection is crucial for maintaining sister chromatid cohesion until anaphase II and ensuring balanced segregation.^{5–9} REC8 is also essential for monopolar orientation at metaphase I in fission yeast, vertebrates, and plants,^{10–13} supporting a model in which cohesins spatially organize centromeric chromatin to establishing monopolar orientation of sister kinetochores at metaphase I.^{1,14–16} Further, the depletion of core-centromeric REC8 causes bi-orientation in mice, suggesting that the cohesins at the core-centromere, but not at the peri-centromeres, are crucial for co-orientation.^{17,18} In *S. cerevisiae*, centromeric cohesion also promotes monopolar orientation, even if Rec8 is not essential for this function.^{18,19} In addition, point centromeres in *S. cerevisiae* harbor a protein complex called Monopolin containing a meiosis-specific subunit, Mam1, required for monopolar orientation.²⁰ Monopolin directly cross-links sister kinetochores and drives co-orientation. Functional homologs of Mam1 have not been identified outside of *S. cerevisiae*.^{20–23}



Notably, meiosis-specific proteins, Meikin in mouse, Moa1 in fission yeast, and Spo13 in budding yeast, localize to kinetochores and drive monopolar orientation in a cohesin-dependent manner by localizing the Polo-like kinase 1 (PLK1) to the kinetochore.^{13,18,24,25} MEIKIN/Moa1 is recruited by CENP-C, a core kinetochore subunit.^{13,26} No functional homologs of Meikin/Moa1, nor any monopolar orientation factor beyond the cohesin subunits Rec8 and SCC3, have been identified in plants, raising the question of whether such a mechanism is broadly conserved.

Here, a genetic screening was undertaken in *Arabidopsis* to identify factors involved in monopolar orientation. Mutant alleles identified in cohesin subunits, the cohesin regulators CTF18-DCC1 and SGO1/SGO2, the kinetochore protein CENP-C, and the SUMO PROTEASE RELATED TO FERTILITY 2 (SPF2) deSUMOylase disturb the co-orientation of kinetochores. Weakened monopolar orientation is associated with physical separation of sister kinetochores, reduced levels of chromosomal cohesins, and premature loss of cohesion at meiosis II. These findings support a model in which centromeric cohesins play parallel roles in monopolar orientation at meiosis I and sister chromatid cohesion at meiosis II.

RESULTS

A forward genetic screen for monopolar orientation

We designed a specific genetic screen to identify factors promoting the monopolar orientation of kinetochores at meiosis I, based on the restoration of the fertility of haploid plants (Figures 1 and S1). Similar screens previously identified factors promoting monopolar orientation in *S. cerevisiae* and *S. pombe*.^{23,25} In haploid *Arabidopsis* plants, meiosis still occurs, but chromosomes cannot recombine because of the absence of homologs (Figure S1).^{27,28} At anaphase I, the resulting five univalents do not separate into chromatids like at mitosis (i.e., 5:5) but rather segregate as single units at anaphase I (e.g., 3:2), demonstrating that the monopolar orientation of the sister chromatid kinetochores is maintained in haploid meiosis. The resulting unbalanced first division leads to low fertility. However, turning meiosis into mitosis in haploids with the *MiMe* mutations bypasses the meiotic defects and restores fertility²⁸ (Figure S1). *MiMe* is composed of three mutations—*spo11*, *rec8*, and *osd1*—that abolish recombination, the monopolar orientation of kinetochores, and the occurrence of the second division, respectively (Figure S1).²⁹ Note that cohesin complexes containing an α -kleisin subunit other than REC8 (SYN2-4,³⁰) must be active in the *MiMe* background to ensure sister chromatid cohesion at metaphase I but do not impose monopolar orientation. Of particular interest here, the double mutant *spo11 osd1* produced unbalanced spores and is sterile in both haploids and diploids because the monopolar orientation of kinetochores prevents balanced segregation of sister chromatids (Figures 1 and S1). We thus performed a forward genetic screen in haploid *spo11 osd1* mutants with the rationale that any mutation that would affect the monopolar orientation of kinetochores would restore fertility, as the *rec8* mutation does.

In practice (Figure 1A), we crossed the TailSwap haploid inducer line³¹ as female with *spo11-1+/- osd1+/-* plants and applied moderate ethyl methanesulfonate (EMS) mutagenesis on the obtained seeds. The *spo11-1+/- osd1+/-* plants were

also homozygous for the *glabra1* mutation, which gives the leaves a glabrous appearance that was used for visual selection of haploids (Figure 1A). One advantage of the screen in a haploid context is the phenotypic expression of recessive mutations in the first generation. As mutagenesis is applied to seeds that contain several pluripotent cells, the resulting plants are chimeric for the induced mutations, as exemplified by chlorotic mutations observed in sectors of some plants (Figure 1B). We then screened ~9,000 plants, looking for branches with longer fruits (Figure 1C), which were genotyped for *osd1* and *spo11* and tested for ploidy through chromosome spreads on terminal buds. In many cases, these were diploid, despite having the *glabra* phenotype. This noise could result either from the spontaneous doubling of somatic cells²⁷ or from more complex genetic events associated with genome elimination.³² In total, we retained thirteen lines derived from haploid branches with increased fertility. A combination of whole-genome sequencing, genetic mapping, and candidate testing with independent alleles led to the identification of all thirteen causal mutations, corresponding to ten genes (Table 1).

Quantification of monopolar orientation

We tested the effect of the identified mutations on monopolar orientation through (1) their ability to restore fertility in diploid *spo11 osd1* plants (Figure 1D). The *spo11 osd1* double mutant has very low fertility, and a monopolar mutation is expected to improve it (Figure S1). (2) Second, we analyzed the ability of the mutations to modify meiotic chromosome segregation in diploid *spo11* mutants (Figure 2). In diploid *spo11*, the ten univalents resulting from the absence of chiasmata fail to align at metaphase I (Figure 2D) and segregate erratically as single units at anaphase I, without separation of the sister chromatids, resulting in their unbalanced distribution on metaphase II plates (e.g., 7:3; Figure 2E). This confirms that monopolar orientation is still imposed in the absence of chiasmata.³³ Occasionally, we observed the separation of one or two pairs of sister chromatids, indicating that univalents can infrequently orient in a bipolar manner at metaphase I in *spo11* mutants, resulting in a “mixed segregation” with a majority of univalent distribution rather than pure “distribution of univalents.” A mutation that weakens monopolar orientation is expected to increase the frequency of bi-orientation of univalents at metaphase I and distribution of sisters at anaphase I. We confirmed both predictions for the mutations identified in the genetic screen (Figures 1 and 2; see below).

The four subunits of the cohesin complex promote monopolar orientation

The cohesin subunits REC8 and SCC3 are involved in the monopolar orientation of kinetochores in *Arabidopsis*.¹¹ Consistently, our screen identified two mutations in REC8 and one in SCC3 (Table 1). In addition, causal mutations were found in the two other cohesin subunits, SMC1 and SMC3 (*smc1-C624Y* and *smc3-G129E*; Table 1). SMC1-C624Y falls within the conserved hinge domain required for stable association with chromosomes.³⁴ As SCC3, SMC1, and SMC3 are each encoded by a single gene and are essential for plant development,^{11,30,35} the mutations identified here must represent partial or separation-of-function alleles. Like *rec8-3*, the *rec8-P60S*, *smc1-C624Y*,

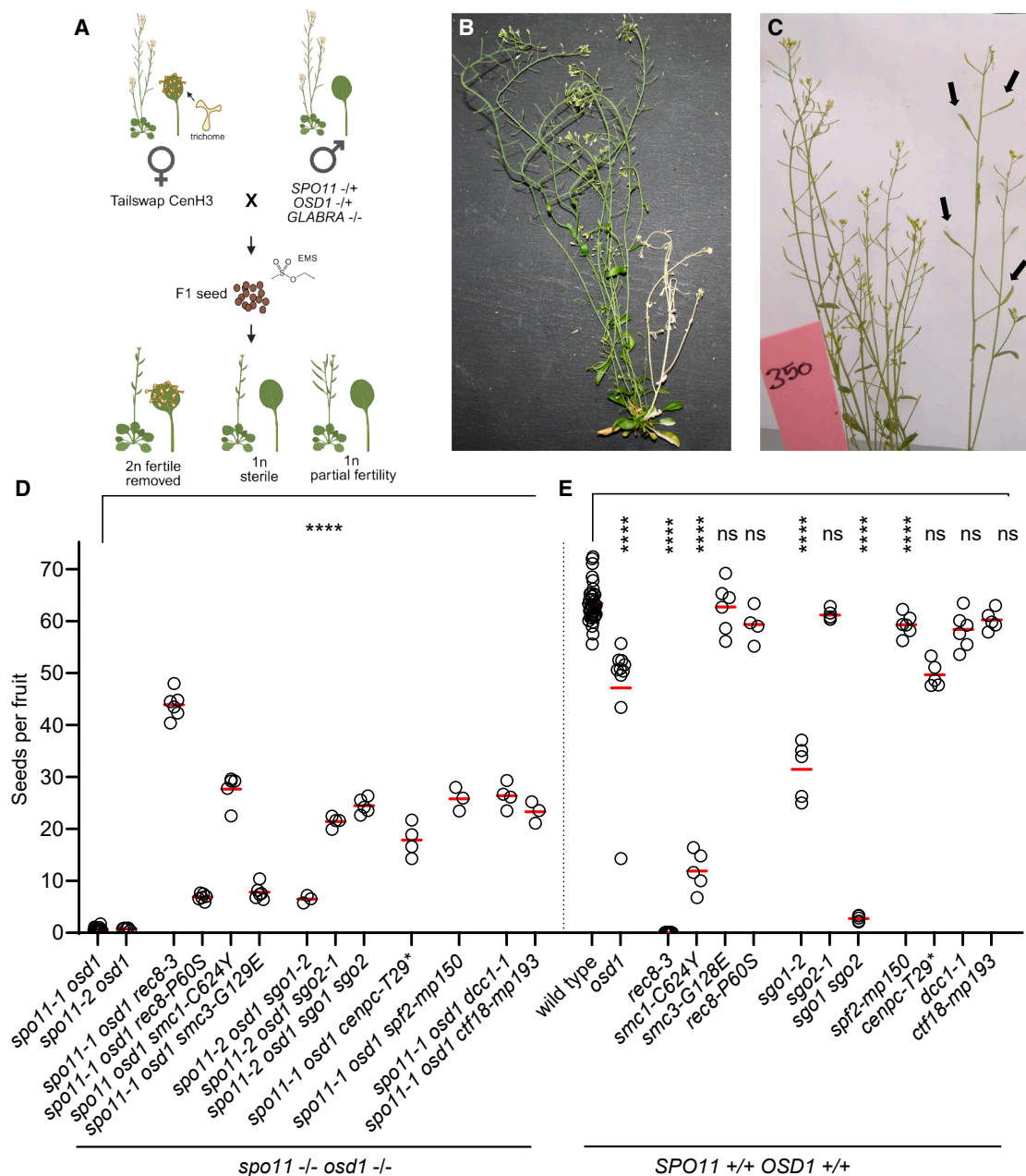


Figure 1. A forward genetic screen for monopolar orientation

(A) Principle of the genetic screen. See also Figure S1. Created in BioRender. Mahlandt, A. (2026) <https://BioRender.com/enlesfz>.

(B) An M1 mutant displaying chlorosis in a single branch.

(C) An M1 mutant showing restored fertility (mp350). Arrows point to elongated fruits on a single branch, while the rest of the plant has short fruits.

(D) Fertility of the identified mutants in the *spo11 osd1* diploid context. Each dot represents the fertility of one plant, measured as the average number of seeds per fruit counted on 10 fruits. The red line indicates the mean.

(E) Fertility of the identified mutants in the *spo11 osd1* diploid contexts. Legend, as in (D).

Tests are one-way ANOVA with Dunnett's correction for multiple testing. ns, $p > 0.05$; **** $p < 10^{-4}$. Source data are provided in Data S1A.

and *smc3-G129E* mutations significantly increased the fertility of *spo11 osd1* but to a lesser extent (Figure 1D). This suggests different penetrance in monopolar orientation, with *rec8-3* having the strongest effect, *smc1-C624Y* being intermediate, and *smc3-G129E* having the mildest effect. We then observed chromosome behavior on male meiotic chromosome spreads (Figure 2). In

spo11-1, the ten univalents fail to align on the metaphase I plate and mostly segregate at anaphase I as a single unit in an erratic manner (Figures 2D–2F and 2S). By contrast, when *spo11-1* was combined with *rec8-3*, *smc1-C624Y*, or *smc3-G129E*, we observed metaphase I with aligned univalents (Figures 2G, 2J, and 2A). Further, we observed metaphase II cells that contained

Table 1. Mutations recovered from the “monopolin” forward genetic screen

Protein	Function	Allele	Gene	Position (TAIR10)		Effect on the protein	Method of confirmation	Comment
REC8	cohesin subunit	mp257	AT5G05490	Chr5:1624739	C>T	R20>STOP	independent alleles	meiosis specific
		mp305		Chr5:1625214	C>T	P60>S		
SMC1	cohesin subunit	mp172	AT3G54670	Chr3:20239572	G>A	C624>Y	complementation test with smc1-1	null mutation is lethal
SMC3	cohesin subunit	mp226	AT2G27170	Chr2:11615684	C>T	G129>E	mapping to a single mutation	null mutation is lethal
SCC3	cohesin subunit	mp225	AT2G47980	Chr2:19633111	A>G	R326>G	independent alleles	null mutation is lethal
CTF18	cohesin loading	mp193	AT1G04730	Chr1:1327905	C>T	splice site	independent alleles	–
		mp274		Chr1:1326791	C>T	splice site		
DCC1	cohesin loading	mp270	AT2G44580	Chr2:18403182	C>T	splice site	independent alleles	–
SGO2	cohesin protection	mp267	AT5G04320	Chr5:1210471	G>A	splice site	independent alleles	–
SGO1	cohesin protection	mp350	AT3G10440	Chr3:3248542	G>A	splice site	independent alleles	–
CENP-C	kinetochore	mp221	AT1G15660	Chr1:5381313	ins C	T29 frameshift, ATG at codon 56	mapping to a single mutation	null mutation is lethal
SPF2	desumoylase	mp150	AT4G33620	Chr4:16150537	del C	L385 frameshift	independent alleles	–
		mp268		Chr4:16150107	G>A	D303>N		

10 chromatids in each plate, showing that the pairs of chromatids segregated at the first division in a balanced manner and contrasting sharply with *spo11-1* metaphase II (Figures 2H, 2I, and 2K; compare with 2E). This modification of chromosome segregation was almost fully penetrant in *spo11-1 rec8-3* and *spo11-1 smc1-C624Y*, with a majority of the cells showing a 10:10 segregation and a minority showing a mixed segregation resulting in unbalanced segregation (Figure 2S). For example, Figure 2L shows an 11:9 segregation of chromatids, which is interpreted as the chromatid pairs from nine univalents having been distributed in a bipolar manner, while one univalent was segregated in a monopolar fashion. In *spo11-1 smc3-G129E*, a similar modification of the segregation pattern was observed with a larger proportion of mixed segregation (Figures 2S and 2J), which is consistent with the lower restoration of fertility observed in *spo11-1 osd1 smc3-G129E* (Figure 1D). Altogether, this shows that all core cohesin subunits promote the monopolar orientation of sister kinetochores at meiosis I. The central role of REC8 suggests that cohesin complexes containing alternative α -kleisin subunits (SYN2-4³⁰) cannot sustain monopolar orientation at meiosis. SMC1, SMC3, and SCC3, which are single-copy and essential cohesin subunits, may be more crucial than suggested by the effects of the inevitable partial alleles analyzed here. The *smc1-C624Y* mutation, which has a strong effect on monopolar orientation, also slightly affects plant growth. Altogether, this strongly reinforces the conclusion that the entire cohesin complex is crucial in imposing monopolar orientation at meiosis I in plants.

SHUGOSHINS promote monopolar orientation, with SGO2 playing a dominant role

In *Arabidopsis*, two SHUGOSHIN homologs, SGO1 and SGO2, protect centromeric cohesion at anaphase in a partially redundant manner, with SGO1 having a more important role than SGO2.^{7,8,36} In the screen described here, mutations affecting

a splicing site in both SGO1 and SGO2 were identified (Table 1). The role of SGO1 and SGO2 in monopolar orientation was confirmed by introducing the independent alleles *sgo1-2* and *sgo2-1*⁸ into *spo11-2 osd1*. Interestingly, *sgo2* provoked a stronger restoration of fertility than *sgo1* (*t* test, $p = 5.10^{-6}$; Figure 1D). Consistently, chromosome spreads of male meiotic cells in *spo11-2 sgo1* and *spo11-2 sgo2* showed that *sgo2* provoked a stronger shift to bipolar segregation (chi-square, $p = 7.10^{-5}$; Figures 2 and S1D–S1I). Combining *sgo1* and *sgo2* mutations further restored the fertility of *spo11-2 osd1* (Figure 1D; $p = 0.011$). Further, *spo11-2 sgo1 sgo2* showed more penetrant bipolar segregation of sister chromatids than *spo11-2 sgo2*, with an almost systematic 10:10 distribution (Figures 2S and S1; $p < 10^{-6}$). Thus, both SGO1 and SGO2 promote monopolar orientation in a partially redundant manner, with SGO2 playing a more important role. The observation that SGO1 and SGO2 have opposite relative importance for monopolar orientation and cohesin protection advocates that these two proteins have distinct functions and could suggest that different pools of cohesins promote monopolar orientation and centromeric cohesion.

PANS1 promotes monopolar orientation

As cohesins and cohesin protectors appear to promote monopolar orientation, we reasoned that PATRONUS (PANS1), which regulates cohesin cleavage, could also promote monopolar orientation. PANS1 is one of the two *Arabidopsis* homologs of securin, which inhibits the cleavage of cohesins by separase. In the *pans1* mutant, hyperactive separase leads to premature cohesion loss at meiosis.^{8,37} Indeed, the *pans1* mutation was able to promote the bipolar distribution of sister chromatids in *spo11-1* (Figures 2 and S2), further supporting the role of cohesins in monopolar orientation. Note that the *pans1* mutation cannot be found in our forward screen, as *pans1* is synthetically lethal in combination with *osd1*.³⁸

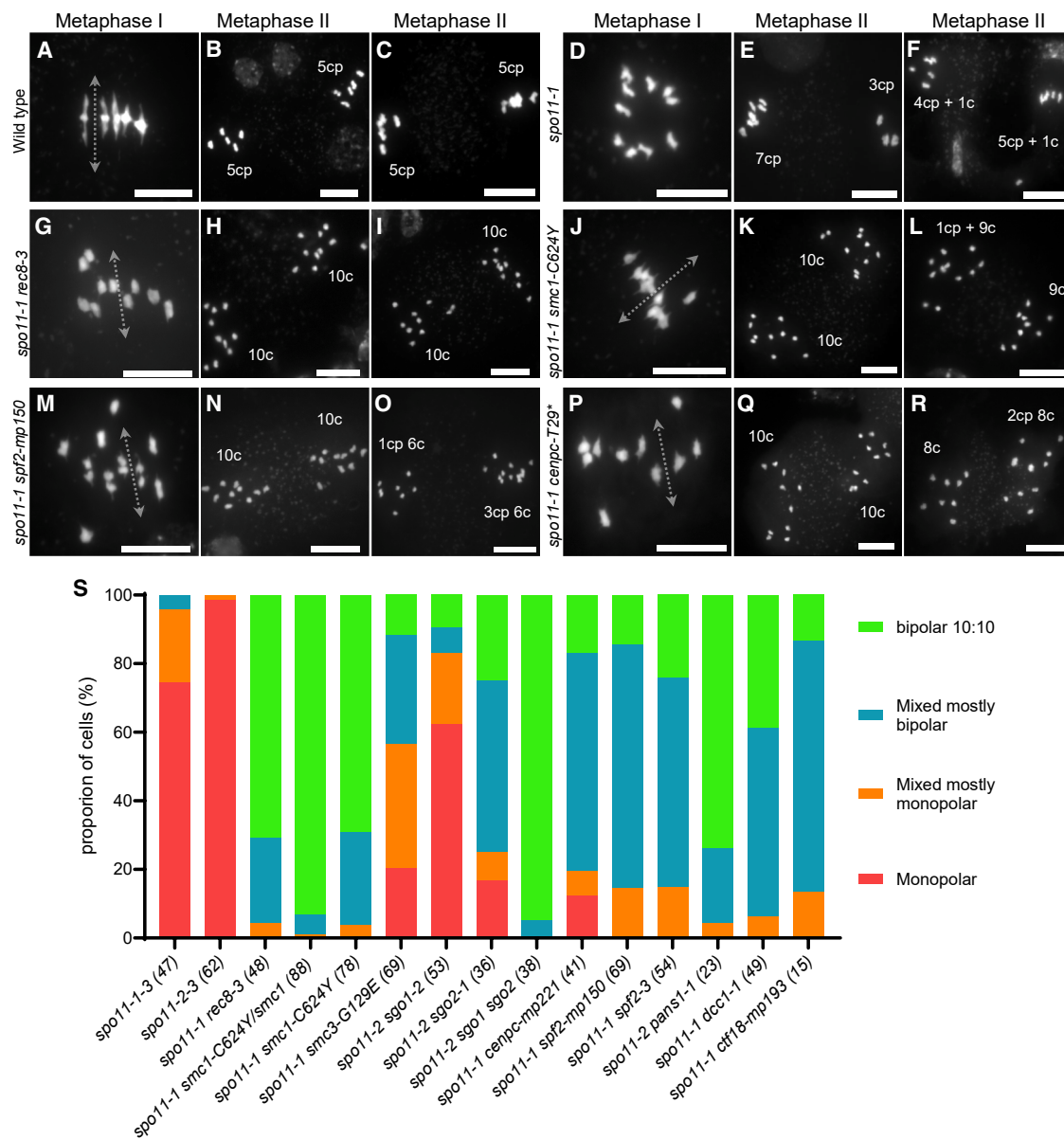


Figure 2. Mutation in *rec8*, *smc1*, *spf2*, or *cenpc* leads to the bipolar orientation of univalents and distribution of sister chromatids

Chromosome spreads of male meiocytes. For each genotype, a metaphase I and two representative metaphase II cells are shown. The direction of the poles at metaphase I is indicated by a dashed arrow. The number of chromatid pairs (cp) and free chromatids (c) is indicated on each metaphase II plate.

(A) Wild-type metaphase I, with five bivalents aligned on the metaphase plate.

(B and C) Wild-type metaphase II. Five chromosomes (chromatid pairs, cp) are aligned on each of the two metaphase II plates.

(D) *spo11-1* metaphase I. Ten univalents are scattered without proper alignment.

(E) *spo11-1* metaphase II with an unbalanced number of chromatid pairs (7:3), resulting from the distribution of univalents at meiosis I.

(F) *spo11-1* metaphase II plate showing the distribution of 9 univalents (4:5) and the splitting of two chromatids.

(G) *spo11-1 rec8-3* metaphase I. Univalents align, suggesting bipolar orientation.

(H and I) *spo11-1 rec8-3* metaphase II. Ten free chromatids are present on each plate, resulting from the balanced distribution of sister chromatids at meiosis I.

(J) *spo11-1 smc1-C624Y* metaphase I.

(K and L) *spo11-1 smc1-C624Y/scmc1* metaphase II.

(K) Ten free chromatids are present on each plate.

(L) 9 free chromatids are present on each plate and a chromatid pair on one side, suggesting a mixed segregation.

(M) *spo11-1 spf2-mp150* metaphase I/early anaphase I. Most bivalents show bipolar orientation.

(N) a *spo11-1 spf2-mp150* metaphase II with 10 chromatids on each plate.

(O) a *spo11-1 spf2-mp150* metaphase II with mixed segregation.

(P) *spo11-1 cenpc-T29** metaphase I.

(legend continued on next page)

Cohesion establishment factors CTF18 and DCC1 promote monopolar orientation

The screen yielded additional losses of function in genes that interact with cohesin: two alleles of *CTF18* and one of *DCC1* (Table 1). These proteins function together as part of the RFC^{CTF18} complex to facilitate the establishment of sister chromatid cohesion during replication.^{39–41} A CRISPR allele was generated in *DCC1* for independent confirmation, *dcc1-2*, which carries a 1,163-bp deletion and encodes only the first 29 of 388 amino acids. The *dcc1-2* and *ctf18-mp193* mutations restored the fertility of *spo11-1 osd1* to intermediate levels (Figure 1D). In *spo11-1 dcc1-1* and *spo11-1 ctf18*, segregation patterns corresponded to a clear reduction of monopolar orientation (Figures 2S and S2). This implicates both CTF18 and DCC1 in imposing monopolar orientation, likely via facilitation of cohesion establishment, although at an importance below that of the cohesin meiotic subunit REC8.

The inner kinetochore protein CENP-C promotes monopolar orientation

Among the mutants recovered in the screen, one had an insertion of one base pair in the coding sequence of the kinetochore protein CENP-C. Genetic mapping confirmed that this mutation was causal in restoring fertility of *spo11-1 osd1* (Table 1; Figure 1D). The *cenpc-T29** mutation induces a frameshift at codon T29, encoding then a histidine residue immediately followed by a STOP codon. Intriguingly, the *cenpc-T29** mutant is viable, while the CENP-C protein is presumably essential in *Arabidopsis*, as in other eukaryotes.⁴² However, an ATG codon at position 56 may serve as an alternative translation start codon that would generate a CENP-C protein truncated of 55 amino acids at its N terminus. The *cenpc-T29** mutation improves the fertility of *spo11-1 osd1* and promotes bipolarity of sister chromatids in the *spo11-1* (Figures 1D and 2P–2S). These findings demonstrate that the kinetochore itself plays a role in monopolar orientation in *Arabidopsis* and suggest that the N terminus of CENP-C is implicated. Note that the N terminus of CENP-C is involved in monopolar orientation in other species, as it recruits Moa1/Meikin.^{13,26}

The deSUMOylase SPF2 promotes monopolar orientation

Finally, we identified two independent mutations in the deSUMOylase *SPF2* (At4G33620; Table 1). The At4G33620 gene encodes for one of the two *Arabidopsis* homologs of the yeast deSUMOylase ULP2.⁴³ It is known not only as SPF2 but also as ULP2-like1 and ASP2 (*Arabidopsis* SUMO protease 2).^{43–45} The *spf2-mp150* mutation provokes a frameshift upstream of the protease domain of SPF2, presumably abolishing its function. This allele significantly restored the fertility of *spo11-1 osd1* to levels similar to those obtained with *smc1-C624Y*, *sgo2*, *ctf18*, or

dcc1, yet less than those obtained with *rec8* (Figure 1D). Introducing the *spf2-mp150* or the *spf2-3* mutation in *spo11-1* provokes alignment of univalents at metaphase I and a clear shift toward equational segregation of sister chromatids (Figures 2M–2O and 2S). This result implicates SPF2 in monopolar orientation, suggesting that the deSUMOylation of one or several targets favors monopolar orientation.

Chiasmata promote monopolar orientation

Next, we examined the phenotype of the mutant identified in the screen in the presence of active SPO11 and OSD1, i.e., in single mutants. REC8 is required for efficient double-strand break repair at meiosis, leading to chromosome fragmentation and full sterility of the *rec8* null mutant (i.e., in the presence of DNA double-strand breaks generated by SPO11-1/SPO11-2).^{11,46} By contrast, *rec8-P60S*, *smc3-G129E*, *spf2-mp150*, *cenpc-T29**, *sgo2-1*, *ctf18-mp193*, and *dcc1-1* single mutants undergo largely normal meiosis (Figures 3 and S3)⁸ and complete fertility, except for *cenpc-T29**, which has a fertility reduction of 18% relative to the wild type despite apparent normal meiosis (Figure 1E). In all of these mutants, five bivalents aligned at metaphase I with a stretched-diamond arrangement similar to the wild type, indicating normal mono-orientation of sisters in the presence of chiasmata. Mono-orientation at metaphase I is confirmed by the observation of normal meiosis II, with five chromatid pairs aligned at metaphase II plates (Figures 3 and S3). Thus, the identified mutations that promote bipolar orientation of sister kinetochores in a univalent context do not weaken monopolar orientation enough to provoke their bipolar orientation in a bivalent context. This shows that the presence of chiasmata promotes monopolar orientation, as shown in other organisms.^{13,47,48}

The *smc1-C624Y* single mutant showed strongly reduced fertility and meiotic defects (Figures 1E and 3D–3I). The null *smc1* mutant is not viable.³⁰ In most *smc1-C624Y* metaphase I cells, five bivalents aligned with an arrangement similar to the wild type, suggesting efficient DNA double-strand breaks and crossover formation and normal mono-orientation of sisters in the presence of chiasmata. However, pairs of univalents were infrequently observed (one pair of univalents in 4/43 metaphase I cells), suggesting an incomplete implementation of crossovers (Figure 3E, asterisks). These univalents aligned at metaphase I in a bipolar manner, consistent with a defect in maintaining monopolar orientation of univalents. Bivalents, including those in the same cells, showed a shape similar to the wild type, strongly suggesting a maintained monopolar orientation. This confirms that chiasmata themselves promote monopolar orientation and not upstream events such as the presence of SPO11 or the formation of double-strand breaks. On rare occasions, bivalents adopt a shape suggestive of bipolar orientation of pairs of sisters despite the presence of chiasmata (asterisk in Figure 3F) (see also below).

(Q and R) Most bivalents show bipolar orientation: (Q) a *spo11-1 cenpc-T29** metaphase II with 10 chromatids on each plate and (R) a *spo11-1 cenpc-T29** metaphase II with 8 chromatids on each plate and a 0:2 distribution of univalents. Scale bar, 10 μ m. See also Figure S2.

(S) To quantify the mode of chromosome distribution, metaphase II cells were sorted into four categories with a gradient from pure monopolar to pure bipolar: (i) distribution of univalents/monopolar (e.g., Figure 3E), (ii) mixed segregation with a majority of univalent distribution and a minority of chromatid distribution/mixed mostly monopolar (e.g., Figure 3F), (iii) mixed segregation with a majority of distribution of chromatids/mixed mostly bipolar (e.g., Figures 3L and 3O), and (iv) distribution of sisters resulting in a 10:10 segregation/bipolar 10:10 (e.g., Figure 3H). The proportion of cells in each category is shown, and the number of cells analyzed is in parentheses. Supporting data are provided in Data S1B.

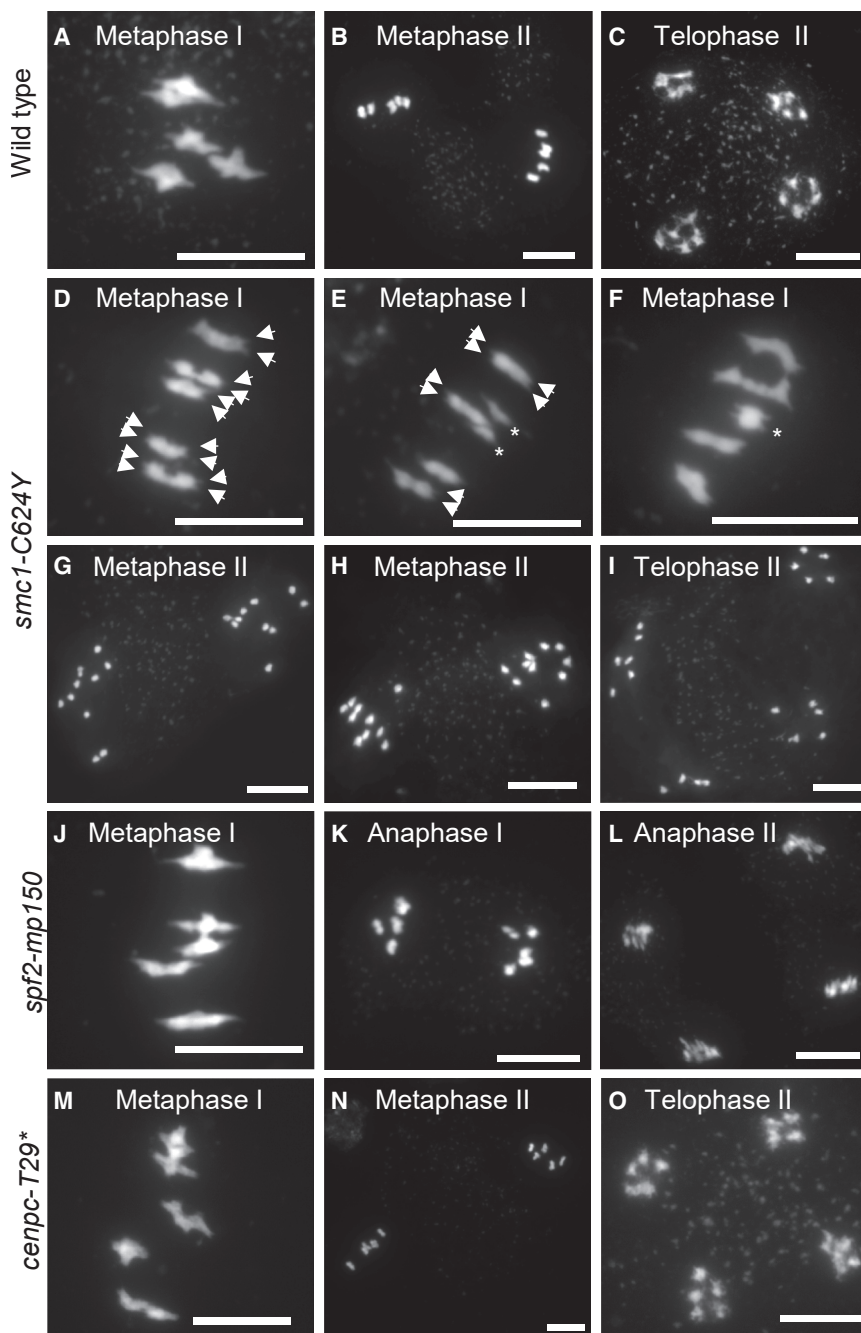


Figure 3. Chromosome spreads of single mutant male meiocytes

The meiotic stage is indicated for each panel.

(A–C) Wild type.

(D–I) *smc1-C624Y*.

(D) Metaphase I with five aligned bivalents. Arrows indicate centromere splitting.

(E) Metaphase I with two univalents aligned in a bipolar manner, indicated by asterisks.

(F) Metaphase I with a bivalent showing bi-orientation of its chromatid pairs, indicated by an asterisk.

(G) Metaphase II with complete cohesion loss following meiosis I (left, 10 chromatids; right, 10 chromatids).

(H) Metaphase II with incomplete cohesion loss following meiosis I (left, three aligned chromatid pairs and four free chromatids; right, 10 chromatids).

(I) Telophase II with unbalanced segregation.

(J–L) *spf2-mp150* showing wild-type-like meiosis.

(M–O) *cenpc-T29** with wild-type-like meiosis. Scale bar, 10 μ m.

See also Figure S3.

sgo2, both monopolar orientation and centromeric cohesion are affected.

Splitting of sister kinetochores and reduced levels of cohesins

We noticed that the edge of bivalents on chromosome spreads tended to appear split into two tips at metaphase I in several of the mutants (e.g., arrows in Figures 3D and 3E). To test whether this reflects separation of kinetochores, we performed immunostaining of the inner kinetochore component CENH3, using a protocol that maintains the cellular 3D organization⁴⁹ (Figure 4). In wild type, the sister kinetochores appear as adjacent, with an average distance between the two CENH3 signal peaks of 390 nm (Figures 4A and 4B). In *smc1-C624Y*, the sister kinetochores appear split, with the distance between the two CENH3 signals being almost doubled. In *dcc1-1*, *ctf18*, *cpc-T29**, and *spf2*, kinetochores also revealed a larger separation than in the wild type (Figures 4A and 4B). These observations correlated the weakening of monopolar orientation with the loosening of the physical link between sister kinetochores. However, we did not observe a significant increase in inter-kinetochore distances in *sgo2*.

Next, we performed immunostaining of the meiosis-specific cohesin subunit REC8 on metaphase I bivalents (Figure 5). Bivalents in *smc1-C624Y* showed a drastic reduction of REC8 intensity compared with the wild type, both at centromeres (colocalization with CENH3) and broadly on chromosomes (DNA stained by DAPI), showing that cohesin complexes fail to be properly

Centromeric cohesion is impaired in *smc1-C624Y* and in *sgo1 sgo2*

At metaphase II, *smc1-C624Y* meiocytes showed separated sister chromatids, with up to ten free chromatids on each plate (Figures 3G and 3H), rather than five aligned chromatid pairs as in the wild type (Figure 3B). This reveals a severe premature loss of sister chromatid cohesion and suggests a defect in maintaining peri-centromeric cohesins, likely associated with a global weakening of cohesins in *smc1-C624Y* (see also below). As previously shown, a premature loss of cohesion is also observed in *sgo1 sgo2*.⁸ This shows that in *smc1-C624Y* and in *sgo1*

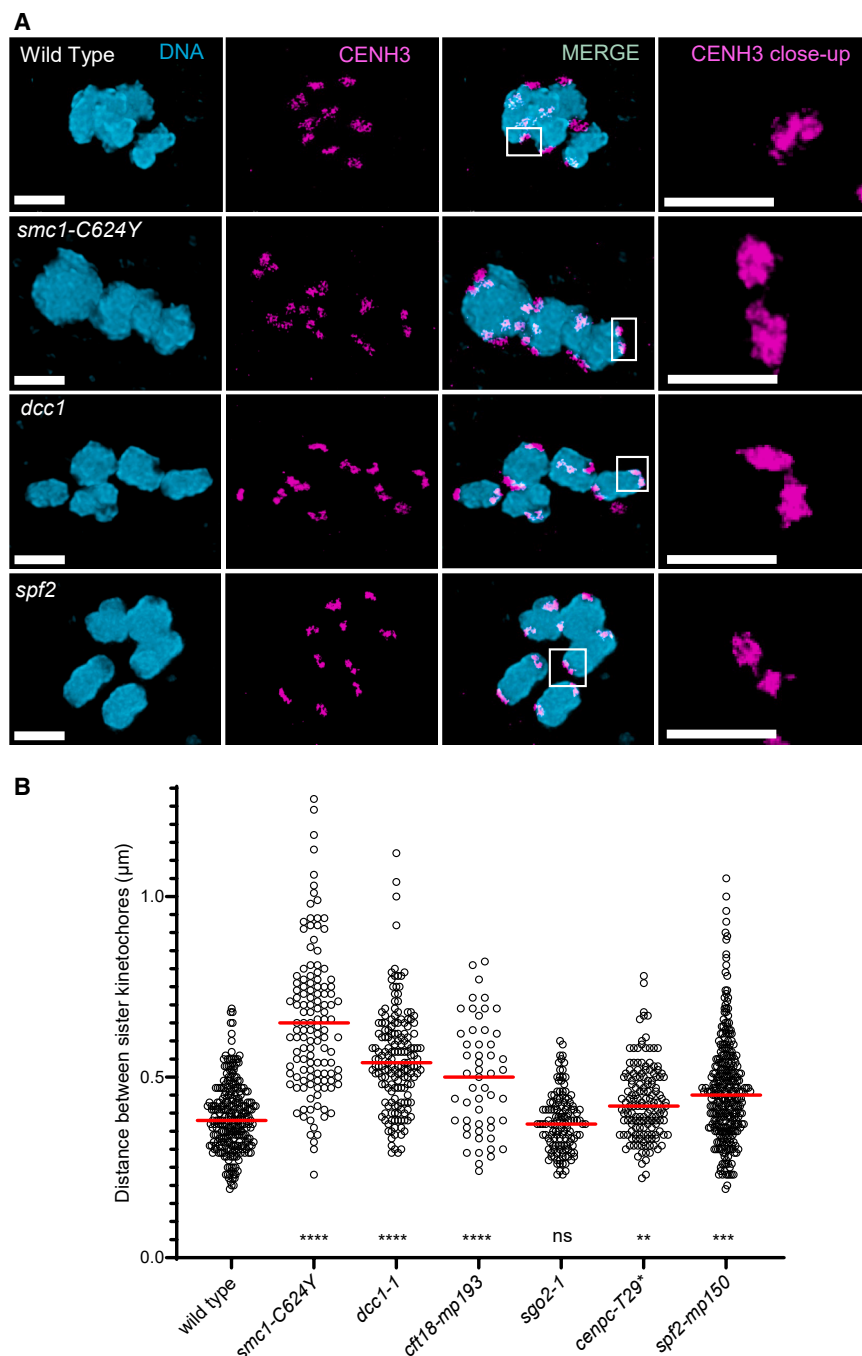


Figure 4. Inter-sister kinetochore distances at metaphase I

(A) Confocal/STED images of metaphase I bivalents in the wild type, *smc1-C624Y*, *spf2-mp150*, and *dcc1-1*; from left to right: DNA stained with DAPI (blue, confocal); CENH3 immunolabeling (purple, STED); merge of DNA and CENH3; close-up of one sister kinetochore pair. A box on the merge panel indicates the position of the close-up. Scale bar, 2 μ m.

(B) Quantification of the distance between the two peaks of STED CENH3 signals in the wild-type and monopolar orientation mutants.

Each data point represents a pair of CENH3 signals. Red bars indicate the median. Kruskal-Wallis and uncorrected Dunn's tests compared with the wild type; ns, $p > 0.05$; ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided in [Data S1C](#).

This implicates the deSUMOylase SPF2 in cohesion maintenance, which in turn would support the close association of kinetochores and monopolar orientation.

Epistasis analysis supports a cohesion-dependent mechanism of monopolar orientation

We next combined monopolar orientation-affected alleles and assessed their meiotic behavior ([Figure 6](#)). The *sgo2* single mutant does not show any visible meiotic defect but enhances the cohesion defects of *sgo1*, provoking a complete loss of sister chromatid cohesion after anaphase I.⁸ Strikingly, combining *sgo2* with *cenpc-T29** also provoked a premature loss of sister chromatid cohesion ([Figure 6C](#)), while neither of the single mutants showed this defect. Similarly, the *spf2-4 cenpc-T29** double mutant showed a lack of sister chromatid cohesion at metaphase II ([Figure 6F](#)), while neither of the single mutants showed this defect. These genetic interactions implicate both SPF2 and CENP-C in centromeric cohesion maintenance at meiosis. Together, the findings implicate CENP-C, SPF2, and SGOs in both mo-

nopolar orientation at meiosis I and in the maintenance of centromeric cohesion until metaphase II.

The *spf2-4 smc1-C624Y* double mutant displays a stretched and aberrant bivalent structure at metaphase I ([Figures 6G and 6H](#)), reminiscent of mutants with DNA double-strand-break repair defects, notably null *rec8*.¹¹ This suggests that in this double mutant, cohesins are decreased to a level that affects the recombination process. Loss of sister chromatid cohesion at metaphase II is also observed in *spf2-4 smc1-C624Y* ([Figure 6I](#)) and in *cenpc-T29* smc1-C624Y* ([Figure 6L](#)), like in the single

loaded or maintained on chromosomes in this hypomorphic *smc1* mutant ([Figures 5A–5C](#)). The *dcc1* and *ctf18* mutants also showed a global reduction of the REC8 signal, which is consistent with the described role of the DCC1-CTF18 complex in facilitating cohesion establishment during replication. We did not observe reduced levels of REC8 on *sgo2* metaphase chromosomes. REC8 levels appeared reduced in *cenpc-T29**, suggesting that CENP-C also promotes cohesin levels at meiosis I. Bivalents in *spf2* similarly showed a significant reduction in meiotic cohesin broadly on chromosomes and at centromeres.

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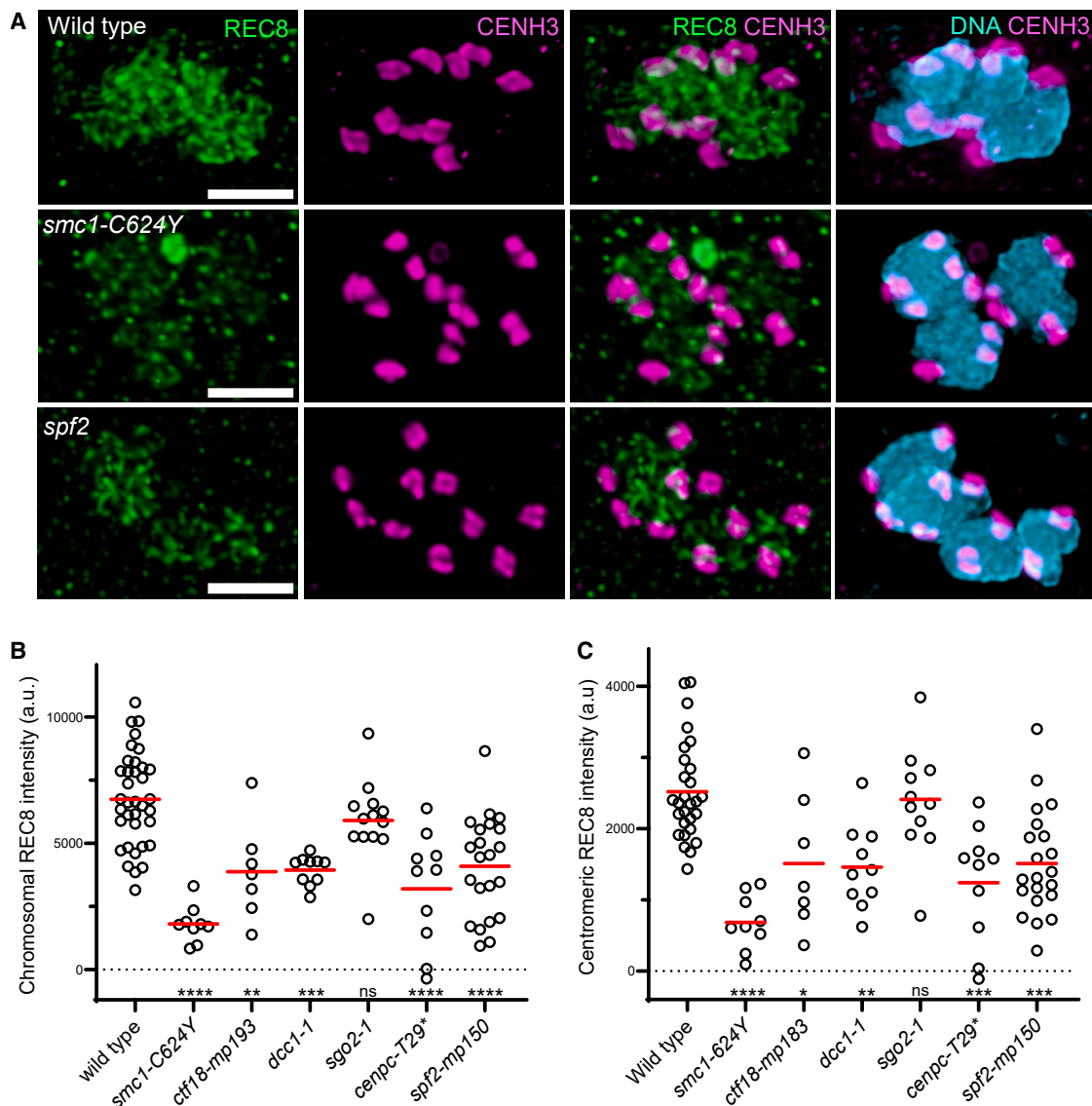


Figure 5. REC8 signal intensity at metaphase I

(A) Confocal images of metaphase I bivalents in the wild type, *smc1-C624Y*, and *spf2-mp150*. From left to right: REC8 immunolabeling (green), CENH3 immunolabeling (purple), merge REC8 (green) and CENH3 (purple), and merge DNA stained with DAPI (blue) and CENH3 immunolabeling (purple). Scale bar, 2 μ m. (B) Quantification of the REC8 signal intensity on chromosomes as defined by the DAPI signal. Supporting data are provided in [Data S1D](#).

(C) Quantification of REC8 intensity on centromeric regions as defined by the CENH3 signal.

Each data point represents a cell. Red bars indicate the mean. Kruskal-Wallis and uncorrected Dunn's tests compared with the wild type; ns, $p > 0.05$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Source data are provided in [Data S1E](#).

smc1-C624Y. Similarly, complete loss of sister chromatid cohesion at metaphase II is observed in *cenpc-T29* sgo1 sgo2* (Figure 6O), like in *sgo1 sgo2*.⁸

At metaphase I, distinct shapes were observed for bivalents in *cenpc-T29* sgo1-2 sgo2-1*, *cenpc-T29* smc1-C624Y*, and *cenpc-T29* spf2-3* double mutants (Figure 6). The shape of these bivalents diverges from that of the wild type typical stretched-diamond shape and appears instead as rounded ovals or squares. Some bivalents with a square shape are strongly suggestive of a bipolar orientation of the two pairs of sister chromatids (Figures 6D, 6J, and 6N, asterisks). This is reminiscent of the

shape of bivalents with bi-orientation in human oocytes.⁵⁰ Thus, in *cenpc-T29* sgo1 sgo2*, *cenpc-T29* smc1-C624Y*, and *cenpc-T29* spf2* mutants, monopolar orientation appears to be sufficiently affected to allow bi-orientation of chromatid pairs within a bivalent, overcoming the pro-monopolar effect of chiasmata. This is also consistent with the complete loss of cohesion observed at metaphase II in these three double mutants. Taken together, this supports a model in which SPF2 and CENP-C—together with cohesins and their regulators DCC1/CTF18 and SGOs—promote the monopolar orientation of kinetochores at meiosis in a cohesion-dependent manner.

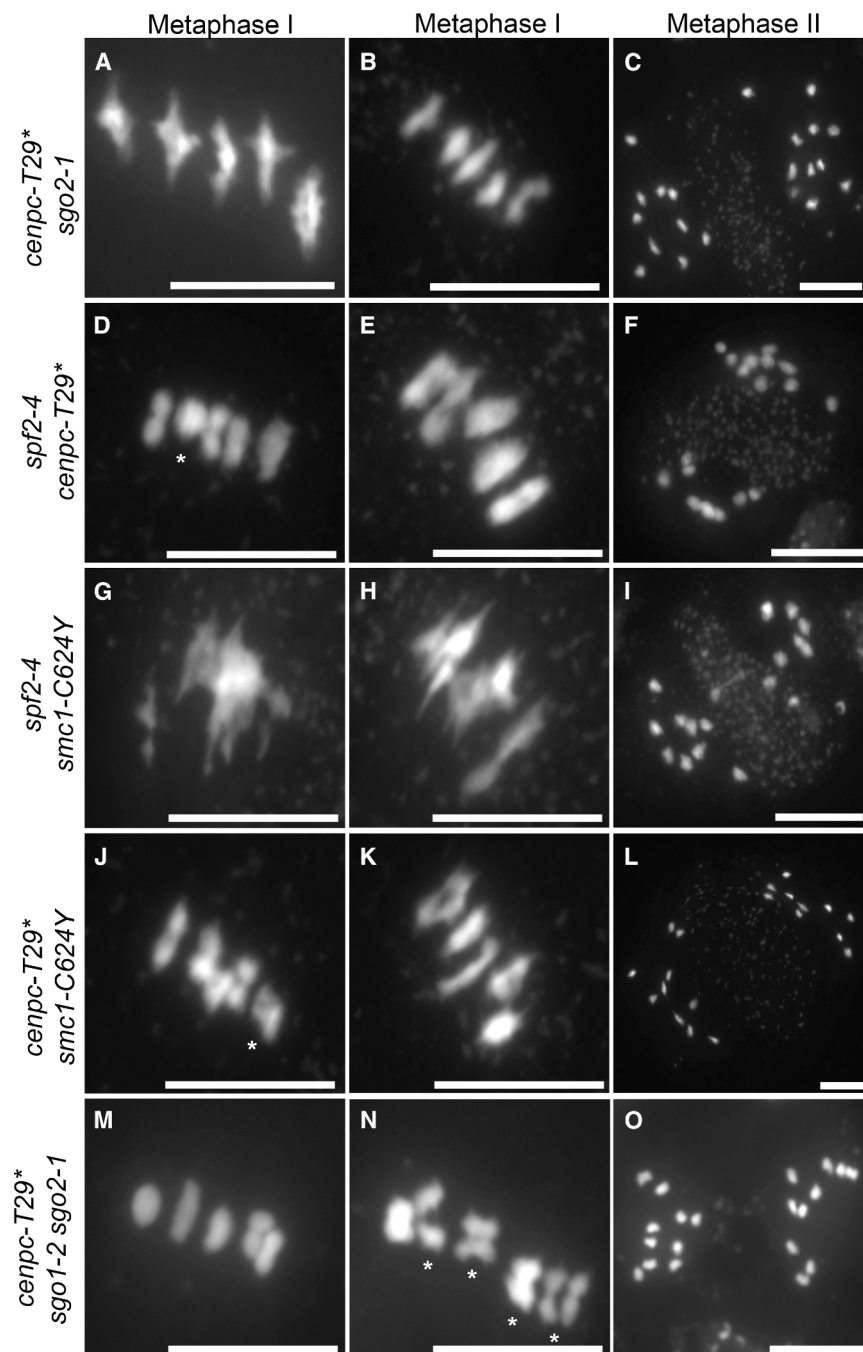


Figure 6. Chromosome spreads of monopolar orientation double mutants

(A–C) *cenpc-T29* sgo2-1*.
(D–F) *spf2-4 cenpc-T29**.
(G–I) *spf2-4 smc1-C624Y*.
(J–L) *cenpc-T29* smc1-C624Y*.
(M–O) *cenpc-T29* sgo1-2 sgo2-1*.
(A, B, D, E, G, H, J, K, M, and N) Metaphase I.
(C, F, I, L, and O) Metaphase II.
Asterisks indicate bivalents suggestive of a bipolar orientation of the two pairs of sister chromatids.
Scale bar, 10 μ m.

yet remain viable. We further find that CTF18 and DCC1, which play a role in establishing sister chromatid cohesion at replication, also support monopolar orientation. Accordingly, we observed a reduced level of cohesins on *smc1-C624Y*, *ctf18*, and *dcc1* meiotic metaphase I chromosomes. Altogether, this suggests that monopolar orientation requires a level of cohesion fidelity beyond that of other functions of cohesins at mitosis and meiosis.

We show that the two plant SGOs, SGO1 and SGO2, support monopolar orientation. SGO is a conserved family of proteins that protect cohesins during meiosis by shuttling PP2A to pericentromeres to dephosphorylate REC8 and prevent its cleavage by Separase.^{6,51,52} This protection of cohesin in the centromeric region is essential for balanced distribution of chromatids at the second division. Both Arabidopsis SGO1 and SGO2 function in centromeric cohesion protection during meiosis, in a partially redundant manner.⁸ Mutation of SGO1 leads to a partial premature loss of centromeric cohesion, mutation of SGO2 does not, and a complete premature loss of cohesion is observed in the double mutant. Similarly, we show here that both SGO1 and SGO2 promote monopolar orientation in a partially redundant manner, but with SGO2 having a more prominent role than

SGO1. The observation that SGO1 and SGO2 have opposite relative importance for monopolar orientation and cohesin protection suggests that these two proteins have distinct targets. One attractive model is that SGO1 and SGO2 protect two distinct pools of cohesins, one promoting monopolar orientation at meiosis I and the other ensuring centromeric cohesion at meiosis II.

AtCENP-C promotes mono-orientation and cohesion maintenance

The inner kinetochore protein CENP-C is a crucial scaffold component that regulates kinetochore assembly during mitosis

DISCUSSION

Monopolar orientation is highly dependent on cohesins

Monopolar orientation in yeasts and metazoans appears to be largely promoted by the specific regulation of meiotic cohesin in the vicinity of centromeres.^{10,17,18} We demonstrate here that in addition to the meiotic-specific cohesin subunit REC8, the other cohesin subunits SMC1, SMC3, and SCC3 promote monopolar orientation in Arabidopsis. These three subunits are each encoded by a single gene and are essential, but we identified mutations in each of them that weaken monopolar orientation

and meiosis.⁵³ CENP-C promotes monopolar orientation by recruiting Moa/MEIKIN in yeast and mammals.^{13,26} We show here that CENP-C also has a role in imposing mono-orientation during meiosis in Arabidopsis, even if no equivalent of Moa/MEIKIN was identified (see also below). The *cenpc-T29** allele is able to promote equational segregation of univalents at anaphase I. REC8 levels are reduced on *cenpc-T29** metaphase I chromosomes, and combining the *cenpc-T29** with either *sgo2* or *spf2* triggers the early loss of sister chromatid cohesion after the first division. Taken together, CENP-C likely promotes monopolar orientation during meiosis I in a cohesin-dependent manner.

deSUMOylation promotes monopolar orientation

Our findings implicate SPF2, one of the Arabidopsis homologs of the yeast Ulp2, in promoting monopolar orientation. Reduced levels of cohesin and splitting of kinetochores at metaphase I, together with premature loss of cohesion in *spf2 cenpc-T29**, suggest that SPF2 also participates in cohesin maintenance. SUMOylation is known to have diverse roles in coordinating chromosome segregation in both mitosis and meiosis through means of kinetochore assembly, regulation of error correction, and cohesin establishment and maintenance.^{54–58} Ulp2 has been shown to prevent the buildup of SUMO chains on SMC complexes, including cohesin in budding yeast.⁵⁹ Here, we propose that SPF2-mediated deSUMOylation contributes to the maintenance of cohesins, supporting sister chromatid cohesion and monopolar orientation. Oppositely, SUMO was shown to protect centromeric cohesins in post-anaphase I meiosis in mice.⁵⁸ As SUMOylation is pervasive at meiosis,⁶⁰ it is possible that SUMOylation of different targets can have an opposite effect on cohesin stability. Further, one cannot exclude cohesin-independent roles of SPF2 at kinetochores. Notably, Ulp2 also prevents SUMOylation-mediated turnover of condensins, which also promote monopolar orientation in *S. cerevisiae*.^{59,61}

Split kinetochores, chiasmata, and monopolar orientation

All mutations that we describe here were able to suppress the monopolar orientation of sister kinetochores of achiastic univalents (*spo11* metaphase I). However, in all of them, monopolar orientation was restored by the presence of chiasmata (bivalents), strongly supporting the role of chiasmata in promoting monopolar orientation in Arabidopsis, as shown in other organisms.^{13,47,48} Although sister kinetochores were oriented to the same pole in bivalents, like in the wild type, they were more separated from each other than in the wild type in *smc1-S624Y*, *dcc1*, *ctf18*, *cenpc-T29**, and *spf2*. This was also associated with reduced cohesin levels. In addition, *smc1-C624Y* showed a premature loss of cohesion before metaphase II. This suggests a model in which these mutations reduce cohesin levels, loosening the normally tight association between the sister kinetochores. This loose association is still sufficient to promote monopolar orientation in the presence of chiasmata but not in univalents. This is consistent with observations in mouse oocytes that demonstrated that specific extinction of REC8 in the immediate vicinity of centromeres causes a significant increase in inter-sister kinetochore distance in metaphase I bivalents, and this separation alone is not sufficient to reverse

mono-orientation in bivalents but effectively does so when univalents are present at the metaphase I plate.¹⁷ Combining mutations and presumably further destabilizing cohesins led to a range of meiotic defects. In *spf2 smc1-C624Y*, we observed aberrant chromosomal structures, similar to those observed in *rec8*, which are caused by unrepaired recombination intermediates.^{11,62} This highlights the essential role of cohesin in homologous recombination. In several combinations, we also observed premature loss of cohesion before metaphase II, suggesting an insufficient maintenance of centromeric cohesin. Finally, in three mutant combinations (*cenpc-T29* sgo1 sgo2*, *cenpc-T29* smc1-C624Y*, and *cenpc-T29* spf2*), we observed rotated bivalents, strongly suggesting that monopolar orientation is sufficiently affected to allow bipolar orientation of the two sister chromatid pairs even in the presence of chiasmata within the bivalent structure.

Are MOKIRs specific to *Opisthokonta*?

While long recognized as an essential and unique feature of meiosis,⁶³ mechanisms of monopolar orientation have remained elusive, notably in the plant kingdom. In *Opisthokonta*, the eukaryotic branch that contains both fungi and animals, it appears that the meiotic-specific protein MOKIR plays a central role. MOKIR stands for meiosis one kinase regulator and is represented by Moa, Spo13, and MEIKIN in *S. pombe*, *S. cerevisiae*, and mammals, respectively.^{18,25,64–66} These mostly intrinsically disordered proteins are considered orthologs despite limited sequence conservation and act as a meiosis-specific shuttle for Polo kinase localization to the kinetochore.^{13,66} Mouse oocytes and spermatocytes lacking MEIKIN show frequent splitting of kinetochores prior to anaphase I and bi-orienting kinetochores in cells lacking chiasmata, reminiscent of the series of mutants described here. However, none of the Arabidopsis genes we identified here correspond to the Moa/Spo13/MEIKIN portrait. We confirmed that monopolar orientation in Arabidopsis depends on the regulation of cohesins, like in *Opisthokonta*, but did not find any meiosis-specific protein involved in the process, except the meiotic cohesin subunit REC8. Several reasons can explain how we could have missed a MOKIR plant representative, such as gene duplication or lack of mutation saturation, but this raises the possibility that it does not exist. We also failed to identify a MOKIR candidate in the plant kingdom using a sensitive homology search. It should be noted that the Polo kinase/PLK is absent in plants.⁶⁷ One possibility is that plants use a different kinase for this function and that a meiotic-specific shuttle equivalent to MOKIR exists. One may alternatively envisage that MOKIR arose in the *Opisthokonta* clade to reinforce the meiotic chromosomal architecture and that the cohesin REC8 might be sufficient to confer meiotic specificity in plants and possibly in the ancestral eukaryote.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled by the lead contact, Raphael Mercier (mercier@mpipz.mpg.de).

Materials availability

All plant lines generated in this study are available from the [lead contact](#) with a completed materials transfer agreement.

Data and code availability

- Source data for [Figures 1D, 1E, 2, 4, and 5](#) are provided in [Data S1](#).
- Imaging 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.

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AUTHOR CONTRIBUTIONS

R.M. and D.K.S. led the project. S.J., C.T., M.D., V.S., and L.C. developed and performed the genetic screen. S.D., A.M., D.K.S., C.T., V.S., and L.C. performed and analyzed the microscopy experiments. D.K.S., A.M., S.D., V.S., C.T., B.W., and L.C. performed and analyzed the genetic experiments. I.S. and R.M. designed the project. D.K.S., A.M., and R.M. wrote the manuscript, incorporating contributions from all authors.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CENH3 (raised in chicken)	Singh et al. ⁶⁸	Mercier Lab PAK008
REC8 (raised in rabbit)	Cromer et al. ⁸	Mercier Lab PAK035
anti-chicken STAR orange	Abberior	Cat# STORANGE-1005-500UG; RRID: AB_2857377
anti-rabbit STAR green)	Abberior	Cat# STGREEN-1002-500UG; RRID: AB_2833016
Bacterial strains		
DH5α	Thermo Fisher	18265017
GV3101	Gold Biotechnology	CC-105
Biological samples		
<i>osd1-3</i>	Heyman et al. ⁶⁹	Koncz collection
<i>rec8-3</i>	d'Erfurth et al. ²⁹	SAIL_807_B08, N836037
<i>spo11-1-3</i>	Stacey et al. ⁷⁰	SALK_146172, N646172
<i>spo11-2-3</i>	Hartung et al. ⁷¹	GABI_749C12, N471844
<i>smc1-1</i>	Schubert et al. ³⁰	SALK_017437, N517437
<i>sgo1-2</i>	Cromer et al. ⁸	SK2556, CS1000416
<i>sgo2-1</i>	Cromer et al. ⁸	Koncz collection
<i>spf2-3/asp2-2</i>	Castro et al. ⁷²	SALK_140824, N640824
<i>spf2-4/asp2-3</i>	Sessions et al. ⁷³	SAIL_182_F02, N808743
<i>ctf18-3</i>	Alonso et al. ⁷⁴	SALK_043339, N543339
<i>glabra1-hc1</i>	Elmayan et al. ⁷⁵	N/A
GFP-tailswap	Ravi et al. ²⁷	N/A
<i>pans1-1</i>	Cromer et al. ⁸	Salk_070337, N570337
Chemicals, peptides, and recombinant proteins		
Cellulase	ducheffa-biochemie	Onozuka R-10; Cat# C8001
Pectolyase	ducheffa-biochemie	Cat# P8004; -Y-23
Driselase	Sigma-Aldrich	Cat# D8037
Experimental models: Organisms/strains		
<i>Arabidopsis thaliana</i>	N/A	N/A
Oligonucleotides		
Data S2	N/A	N/A
Recombinant DNA		
pICH86966	Grutzner et al. ⁷⁶	Addgene#48075
pICSL01009	Grutzner et al. ⁷⁶	Addgene #46968
pICH47751	Grutzner et al. ⁷⁶	Addgene #48002
pICH47761	Grutzner et al. ⁷⁶	Addgene #48003
pICH47772	Grutzner et al. ⁷⁶	Addgene #48004
pICH47781	Grutzner et al. ⁷⁶	Addgene #48005
pAGM4723	Grutzner et al. ⁷⁶	Addgene #48015
pICSL11015	Grutzner et al. ⁷⁶	Addgene # 117499
pAGM51323	Grutzner et al. ⁷⁶	Addgene #153224
pICH50927	Grutzner et al. ⁷⁶	Addgene #48049

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
imageJ 1.54p	National Institutes of Health	https://fiji.sc/
imaris 10.2.0	Bitplane, Oxford Instruments	https://imaris.oxinst.com/
huygens 25.10	Huygens Software Suite	https://svi.nl/Huygens-Software
Graphpad Prism 10.6.1	Graphpad	graphpad.com

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Arabidopsis thaliana plants were grown in greenhouses or growth chambers under 16-hour day/8-hour night conditions at 20 degrees C. In addition to the EMS and CRISPR alleles produced in this study, we used the following genetic material: *osd1-3*,⁶⁹ *rec8-3/SAIL_807_B08*,²⁹ *spo11-1-3/SALK_146172*,⁷⁰ *spo11-2-3/GABI_749C12*,⁷¹ *smc1-1/SALK_017437*,³⁰ *sgo1-2* and *sgo2-1*,⁸ *spf2-3/asp2-2/SALK_140824*,⁷² *spf2-4/asp2-3/SAIL_182_F02*,⁷³ *ctf18-3/SALK_043339*,⁷⁴ *glabra1-hc1*,⁷⁵ *GFP-tailswap*,²⁷ *pans1-1/Salk_070337*.⁸

METHOD DETAILS

EMS Mutagenesis

Seeds were incubated for 17 h at room temperature with gentle agitation in 5 mL of 0.1% (v/v) EMS. Neutralization was performed by adding 5 mL sodium thiosulfate 1M for 5 min. Three milliliter water was added to make the seeds sink. The supernatant was removed and the seeds were washed three times for 20 min with 15 mL of water before sowing. Haploid plants were identified by the absence of trichomes, indicative of the *glabra* mutation inherited from the paternal genome. Genotyping primers are provided in [Data S2A](#).

CRISPR-Cas9 mutagenesis of *AtDCC1*

To assemble a complete CRISPR-Cas9 binary vector, four guide RNAs were first designed spanning the genomic sequence of AT2G44580 using CRISPOR.^{77,78} Cloning of the guide RNAs into the final Cas9-containing binary vector was carried out using the MoClo Golden Gate cloning toolkit⁷⁹ and integrating an intron-containing Cas9.⁷⁶ we amplified the tracer RNA sequence from pICH86966 (Addgene#48075) as a template. The forward primer consists of 23 bp specific to clone pICH86966, 20-base pair guide RNA recognition sequence, and BsaI site, accompanied by a specific overhang tailored ([Data S2B](#)). The reverse primer remained constant for all guide RNA cloning. In the first level of cloning, we utilized a BsaI-based Golden Gate assembly reaction, employing the AtU6 promoter from clone pICSL01009 (Addgene #46968) and the amplified above-mentioned desired guide RNA into the chosen guide RNA vector. Four guide RNAs specific to *DCC1* were cloned into the vectors pICH47751 (Addgene #48002), pICH47761 (Addgene #48003), pICH47772 (Addgene #48004), and pICH47781 (Addgene #48005), respectively. The complete assembly into the final destination vector pAGM4723 (Addgene #48015) was performed using a BpiI-based Golden Gate reaction. The construct incorporated the FAST-Red clone (Addgene #117499; pICSL11015) as a seed coat selection marker at position 1, followed by the Cas9 clone (Addgene #153224; pAGM51323), carrying an enhanced SpCas9 driven by the AtRPS5 promoter, at position 2. The four guide RNA modules were assembled into positions 3, 4, 5, and 6, respectively. The final position contained the linker clone pICH50927 (Addgene #48049), which is specifically designed for four-guide assemblies. This completed construct was then transformed into *A. tumefaciens*, and harvested cells were used to transform Col-0 *A. thaliana* plants using the floral dip method.⁸⁰ Positive transformants were selected by screening seeds for FastRED fluorescence.

Chromosome preparations

Young inflorescences were harvested from *Arabidopsis* plants and immediately fixed in 3:1 ethanol:acetic acid. Flower buds approximately 0.5 mm in size were isolated from the inflorescences and washed in citrate buffer, followed by digestion in citrate buffer (pH 4.5) containing 0.3% cellulase, 0.3% pectolyase, and 0.3% driselase for 2 hours at 37 °C. Four to five digested buds were then transferred to a clean slide and macerated using a bent dissection needle. Approximately 15 µL of 60% acetic acid was added to the mixture and gently stirred at 45 °C on a hot plate for 1 minute. The slide was subsequently flushed with ice-cold 3:1 fixative, first around the droplet and then directly onto it. Slides were left to dry in a tilted position at room temperature, after which 10 µL of DAPI (1µg/ml) in mounting medium was applied and a coverslip was added. Chromosomes were visualized using a Zeiss Axio Observer epifluorescence microscope.

Immunocytology

Slides that preserve the 3D structure of freshly fixed meiocytes were prepared and used for immunocytology using published methods.⁸¹ Briefly, fresh inflorescences were collected from *Arabidopsis* plants, buds roughly 0.5mm in size were selected, dissected to expose anthers, and fixed in paraformaldehyde for 30 minutes under vacuum. Fixed buds were digested in citrate buffer

(pH 4.5) containing 0.3% cellulase, 0.3% pectolyase, and 0.3% driselase at 37 °C for 60 minutes to remove cell walls. Anthers were then isolated and slightly ruptured to release meiocytes, then squashed with a polyacrylamide gel layer onto coverslips. Coverslips were treated for 48 hours at 4 degrees C with primary antibodies for CENH3⁶⁸ (PAK008 raised in chicken, 1:500) or REC8 (PAK035 raised in rabbit, 1:250) prepared in blocking buffer. Secondary antibodies (Abberior anti-chicken STAR orange, anti-rabbit STAR green) were then incubated for 24 hours at 4 degrees C before the addition of mounting media (slow fade gold antifade) with DAPI and the sealing of slides with nail polish. Slides were imaged using an Abberior Facility Line STED microscope using a 2D STED depletion laser. The excitation laser power used was fixed at 10% for REC8 and 20% for CENH3 with 20nm x 20nm x 150nm voxel size. Images were deconvoluted with Huygens Essential. Distances between the STED CENH3 signal maxima were measured in 3D with Imaris using the Spot and Distance to nearest neighbor tools. To quantify the REC8 fluorescence, non-deconvolved confocal sum projection images were used in Fiji. Regions of interest (ROIs) were drawn over confocal DAPI signals for chromosomes and over the CENH3 signals for centromeric regions. Regions of “background” were defined outside the DAPI signal. The REC8 intensity of the background regions was subtracted from the REC8 signal of the regions of interest using the formula: (Corrected Intensity of ROI) = (Measured Intensity of ROI) - (Mean intensity of background) * (surface of ROI). Aggregates of REC8 protein (e.g. in the *smc1-C624* cell shown in [Figure 5](#)) that are observed in the nucleoplasm of both wild-type and mutants were excluded from the background regions.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed with Graphpad Prism 10.6.1. Details of the performed tests and nature of N are provided in the figure legends. N values and are indicated in the figures and the source numerical data are provided in [Data S1](#).