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Aurora controls sister kinetochore mono-orientation and homolog bi-orientation in meiosis-I

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Aurora-B kinases are important regulators of mitotic chromosome segregation, where they are required for the faithful bi-orientation of sister chromatids. In contrast to mitosis, sister chromatids have to be oriented toward the same spindle pole in meiosis-I, while homologous chromosomes are bi-oriented. We find that the fission yeast Aurora kinase Ark1 is required for the faithful bi-orientation of sister chromatids in mitosis and of homologous chromosomes in meiosis-I. Unexpectedly, Ark1 is also necessary for the faithful mono-orientation of sister chromatids in meiosis-I, even though the canonical mono-orientation pathway, which depends on Moa1 and Rec8, seems intact. Our data suggest that Ark1 prevents unified sister kinetochores during metaphase-I from merotelic attachment to both spindle poles and thus from being torn apart during anaphase-I, revealing a novel mechanism promoting monopolar attachment. Furthermore, our results provide an explanation for the previously enigmatic observation that fission yeast Shugoshin Sgo2, which assists in loading Aurora to centromeres, and its regulator Bub1 are required for the mono-orientation of sister chromatids in meiosis-I.

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Introduction

For eukaryotic chromosome segregation to occur correctly, the two copies of the chromosome (sister chromatids) need to be physically connected. This is accomplished by the 'cohesin' complex (Haering and Nasmyth, 2003), which links sister chromatids from their generation in S-phase until the onset of anaphase. By metaphase, the two sister chromatids become attached to microtubules emanating from opposite spindle poles (bi-orientation). A central component ensuring the bi-orientation of sister kinetochores is the 'chromosomal passenger complex' (CPC) that is composed of Aurora-B, INCENP, Borealin/Dasra and Survivin (Vagnarelli and Earnshaw, 2004). In anaphase, cohesion between the sister chromatids is released, which is accomplished by cleavage of the Scc1/Rad21 subunit of cohesin by the protease separase (Uhlmann, 2003), a process that is under control of the mitotic spindle checkpoint (Musacchio and Salmon, 2007). Similar principles govern meiotic chromosome segregation (Lee and Orr-Weaver, 2001; Watanabe, 2004), but in contrast to mitosis, two rounds of chromosome segregation follow only one round of DNA replication in order to generate haploid gametes. Several modifications allow this two-step process (Petronczki *et al*, 2003): homologous chromosomes become connected via chiasmata, which result from crossover recombination during meiotic prophase, and sister kinetochores on each chromosome adopt a side-by-side rather than back-to-back conformation (Goldstein, 1981). These two mechanisms allow the recognition of homologous chromosomes as entities destined for opposite poles during anaphase-I. In addition, only cohesion between chromosome arms is lost during anaphase-I, which allows the separation of homologs; cohesion at the centromere is preserved depending on Shugoshin proteins (Watanabe, 2005), and sister chromatids therefore stay connected so that they can be properly segregated during meiosis-II. For meiosis-II, kinetochores are again in a back-to-back position, and chromosome segregation is very similar to mitosis.

In all model eukaryotes that have been studied, Aurora-B kinases are required for the proper bi-orientation of sister chromatids in mitosis. In the absence of Aurora-B, syntelic (both sister kinetochores attached to the same spindle pole) or merotelic (one kinetochore attached to two opposing spindle poles) attachment of chromosomes occurs with increased frequency (Tanaka *et al*, 2002; Hauf *et al*, 2003; Cimini *et al*, 2006; Knowlton *et al*, 2006). These malattachments escape the surveillance by the mitotic spindle checkpoint, indicating that Aurora-B kinases are also required for proper checkpoint function. Budding and fission yeast have a single Aurora kinase, Ipl1 and Ark1, respectively. These single Aurora kinases are thought to be homologous to Aurora-B and presumably interact with INCENP (*Schizosaccharomyces pombe* (*S.p.*) Pic1) and Survivin (*S.p.* Bir1) homologs (Kim *et al*, 1999; Leverson *et al*, 2002; Vanoosthuyse *et al*, 2007). Whereas Ipl1 has been shown to be required for the proper bi-orientation of chromosomes (Francisco and Chan, 1994; Biggins *et al*, 1999; Tanaka *et al*, 2002), a role for Ark1 in regulating chromosome attachment has not been demonstrated. Fission yeast cells that lack Ark1

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fail to divide the chromatin during anaphase, but nevertheless proceed to septation, resulting in a cut ('cell untimely torn') phenotype (Petersen and Hagan, 2003). Mutants in the condensin complex, which is required for compaction of chromatin during mitosis, display a similar phenotype (Saka *et al*, 1994), and Ark1 and Bir1 are indeed required for the correct intranuclear localization of condensin subunits during mitosis (Morishita *et al*, 2001; Petersen and Hagan, 2003).

In meiosis, Aurora-B kinases have been shown to regulate cohesion in *Caenorhabditis elegans* and *Drosophila melanogaster*. In the worm, the Aurora-B kinase AIR-2 promotes segregation of homologous chromosomes, presumably by phosphorylation-dependent removal of meiotic cohesin, which contains the meiosis-specific subunit Rec8 that replaces Rad21 (Kaitna *et al*, 2002; Rogers *et al*, 2002). In *Drosophila*, Aurora-B seems to be required to preserve centromeric cohesion beyond meiosis-I, depending on MEI-S332, a member of the Shugoshin family of proteins (Resnick *et al*, 2006). The only indication for a role of Aurora in controlling chromosome attachment in meiosis comes from budding yeast, where very recent work has shown that Ipl1 is required for the bi-orientation of homologous chromosomes (Monje-Casas *et al*, 2007; Yu and Koshland, 2007). Here, we examined the role of the fission yeast Aurora kinase Ark1 in chromosome segregation during mitosis and meiosis. We find that Ark1 is required to promote the bi-orientation of chromosomes in mitosis and to prevent or correct syntelic and merotelic attachment. Furthermore, Ark1 is necessary for the bi-orientation of homologs in meiosis-I. However, notably different from budding yeast (Monje-Casas *et al*, 2007), we describe that fission yeast Aurora is required for the monopolar attachment of sister chromatids in meiosis-I, and acts in a different pathway from the Moa1/Rec8 mono-orientation pathway that has been defined.

Results

Ark1 ensures faithful centromere segregation in mitosis

To address whether Ark1 has a role in the bi-orientation of chromosomes, we examined chromosome segregation in haploid fission yeast, in which chromosome 2 was marked with GFP close to the centromere (*cen2*-GFP; Yamamoto and Hiraoka, 2003) using a temperature-sensitive allele of *ark1* (*ark1-T7*; Kawashima *et al*, 2007) or analog-sensitive versions of Ark1 (*ark1-as2*, *ark1-as3*, see Materials and methods), which can be inactivated by specific inhibitors (4-amino-1-tert-butyl-3-(1′-napthyl)pyrazolo[3,4-d]pyrimidine (1NA-PP1) or 4-amino-1-tert-butyl-3-(1′-napthylmethyl)pyrazolo[3,4-d]pyrimidine (1NM-PP1)). When Ark1 was inactivated, centromere segregation was perturbed in half or more than half of the cells (Figure 1A). In about 40% of cells, one of the sister chromatids did not move entirely toward one pole in anaphase ('lagging'), and in 11–25% (depending on the allele), both sister centromeres moved to the same pole. This is indicative of merotelic and syntelic chromosome attachment, respectively. To obtain further insight into chromosome movement in *ark1*-mutant cells, we observed chromosome segregation in living *ark1-T7* cells at the restrictive temperature using *cen2*-GFP and the spindle pole body (SPB) marker Sid4-GFP (Figure 1B and C). We found that even before anaphase, alignment of chromosomes on the mitotic spindle was defective, since the *cen2*-GFP mark was found close to one of the spindle poles in about 60% of the *ark1-T7* cells at restrictive temperature (Figure 1C, and data not shown). In anaphase, sister chromatids of chromosome 2 missegregated in 28% of the cells. In a few cases (3% of all cells) the sister chromatids stayed entirely at one pole during anaphase. In the other cases, sister chromatids were lagging behind on the spindle during anaphase, with about two-thirds eventually moving to the correct and the remainder moving toward the incorrect pole (Figure 1B and C).

Cells with mutations in condensin fail to segregate the bulk of chromatin very similar to *ark1*-mutant cells, and we therefore wanted to exclude that the chromosome segregation defect observed after Ark1 inhibition is merely a consequence of the condensation defect. When we observed *cen2*-GFP segregation in temperature-sensitive condensin-mutant cells (*cut3-477*), we also found some failure in centromere segregation (Supplementary Figure S1), which might be attributed to the disturbed structure of the chromosomes (Hirano, 2005). However, this defect in centromere segregation was less pronounced than in *ark1*-mutant cells, although the chromatin condensation defect in these two strains was similar (Supplementary Figure S1). Furthermore, in the presence of low concentrations of the microtubule-destabilizing substance TBZ, *ark1-T7* cells exhibited considerable missegregation (about 35%) even at the permissive temperature when chromosome condensation was largely normal (Supplementary Figure S1). In contrast, in wild-type or *cut3*-mutant cells, TBZ only caused a very minor increase in missegregation (Supplementary Figure S1). This suggests that Ark1 is needed to establish proper microtubule–kinetochore attachment independent of its role in chromosome condensation. Ark1 also functions in the mitotic spindle checkpoint (Petersen and Hagan, 2003; data not shown). However, even the additional deletion of the checkpoint gene *mad2+* in *cut3-477* cells leads to a weaker defect in chromosome segregation than the one observed in *ark1-T7* cells (Supplementary Figure S1). Taken together, these results indicate that Ark1 plays a specific role in promoting proper chromosome segregation beyond its role in the mitotic checkpoint and in chromosome condensation.

Ark1 is required for the correction of malattachment

In both budding yeast and metazoans, Aurora kinases act in the bi-orientation of chromosomes by correcting improper, syntelic attachment of chromosomes to the same spindle pole (Tanaka *et al*, 2002; Hauf *et al*, 2003; Lampson *et al*, 2004). When we inactivated Ark1 in an otherwise unperturbed mitosis, segregation of both sisters to one spindle pole was rare (Figure 1). This could be because in an unperturbed mitosis, initial syntelic attachment is rare or because syntelic attachment can still be corrected in *ark1*-mutant cells. We therefore increased the frequency of misattachment by first arresting cells in mitosis without microtubules, using a cold-sensitive tubulin mutant *nda3-KM311* and then releasing to permissive temperature (Trautmann *et al*, 2004; Grishchuk and McIntosh, 2006; Kawashima *et al*, 2007). Because *ark1*-mutant cells do not arrest in mitosis under these conditions (Petersen and Hagan, 2003; data not shown), we could only perform the experiment with the *ark1-as3* allele, which is functional when cells are grown without inhibitor and thus allows arrest by *nda3-KM311*. Shortly before release, we

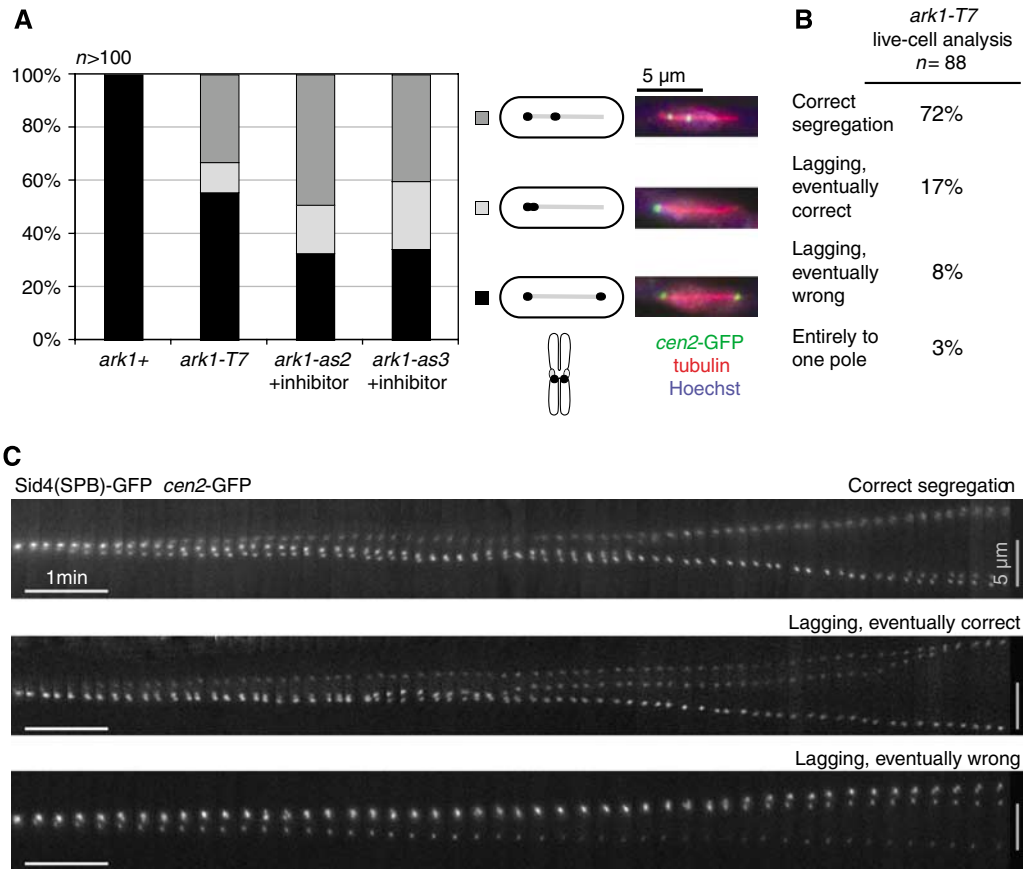

Figure 1 Ark1 is required for the bi-orientation of sister chromatids in mitosis. (**A**) Wild-type (*ark1+*), *ark1-T7*, *ark1-as2* or *ark1-as3* cells having *cen2*-GFP were synchronized in S-phase using hydroxyurea. Wild-type and *ark1-T7* cells were shifted to the restrictive temperature for *ark1-T7* (34°C) when being released, whereas *ark1-as2* and *ark1-as3* were grown at 30°C, but 5 μM 1NM-PP1 was added to the cultures at release. Eighty to hundred minutes after the release, the cells were fixed. Tubulin was visualized by TAT-1 antibody staining or mCherry-Atb2 (tubulin). *cen2* segregation was determined in a minimum of 100 anaphase cells. (**B, C**) *Ark1-T7* mutants having *cen2*-GFP and expressing Sid4-GFP to label the spindle pole bodies (SPB) were followed by live-cell microscopy at the restrictive temperature (34°C). Exemplary kymographs are shown in panel C.

inhibited Ark1-as3 by the specific inhibitor 1NM-PP1, which inactivates the kinase within 10 min (data not shown and Figure 2A). When checking anaphase spindles 10 min after the release, both *cen2*-GFP marks were found close to one edge of the spindle in about 20% of cells. No such missegregation was observed in wild-type cells treated with inhibitor or in *ark1-as3* cells grown without inhibitor (Figure 2A), indicating that its occurrence depends on the inactivation of Ark1. To determine whether this indeed represents initial misattachment that fails to correct, we filmed Ark1-inactivated cells being released from the *nda3* arrest. Among those cells that we could image from the start of mitosis, about half had both sister centromeres located entirely at one pole throughout prometaphase and anaphase (Figure 2B and C; Supplementary Figure S2). These data indicate that Ark1 is required to correct syntelic misattachment of chromosomes (also see Supplementary Note 1).

To confirm in a different setting that malattachment of chromosomes cannot be corrected in *ark1*-mutant cells, we used a temperature-sensitive mutant of fission yeast cohesin, *psc3-1T* (Nonaka *et al*, 2002). Sister chromatids precociously detach from each other in the absence of cohesin, but remain competent to attach to microtubules. However, in the absence of cohesion between sister chromatids, kinetochore–microtubule attachment cannot be stabilized because of a lack of tension. Therefore, attachment remains unstable, which in budding yeast depends on Ipl1 (Biggins and Murray, 2001). In live-cell microscopy experiments, the instability of sister chromatid attachment in *psc3-1T* cells was exemplified by an occasional switching of at least one sister chromatid from one spindle pole to the other (Figure 2D, arrows). When Ark1 was additionally inactivated, such switching was abolished (Figure 2D), indicating that Ark1 is required to keep sister chromatid attachment unstable in *psc3-1T* cells. This result is consistent with Ark1 being needed to correct kinetochore–microtubule attachments that fail to generate tension through bi-orientation. However, the fact that sister chromatids were not very motile when Ark1 is inhibited (Figure 2D) could also indicate a more profound defect in kinetochore–microtubule dynamics. Inactivation of condensin did not seem to have the same effect as Ark1 inactivation (Supplementary Figure S2B), which again suggests that Ark1 regulates chromosome attachment independent of condensin.

Ark1 has a role in nuclear division during meiosis-I

To study the requirement of Ark1 in meiosis, we used an '*ark1* shut-off' (*ark1 s.o.*) strain, where the *ark1+* gene is

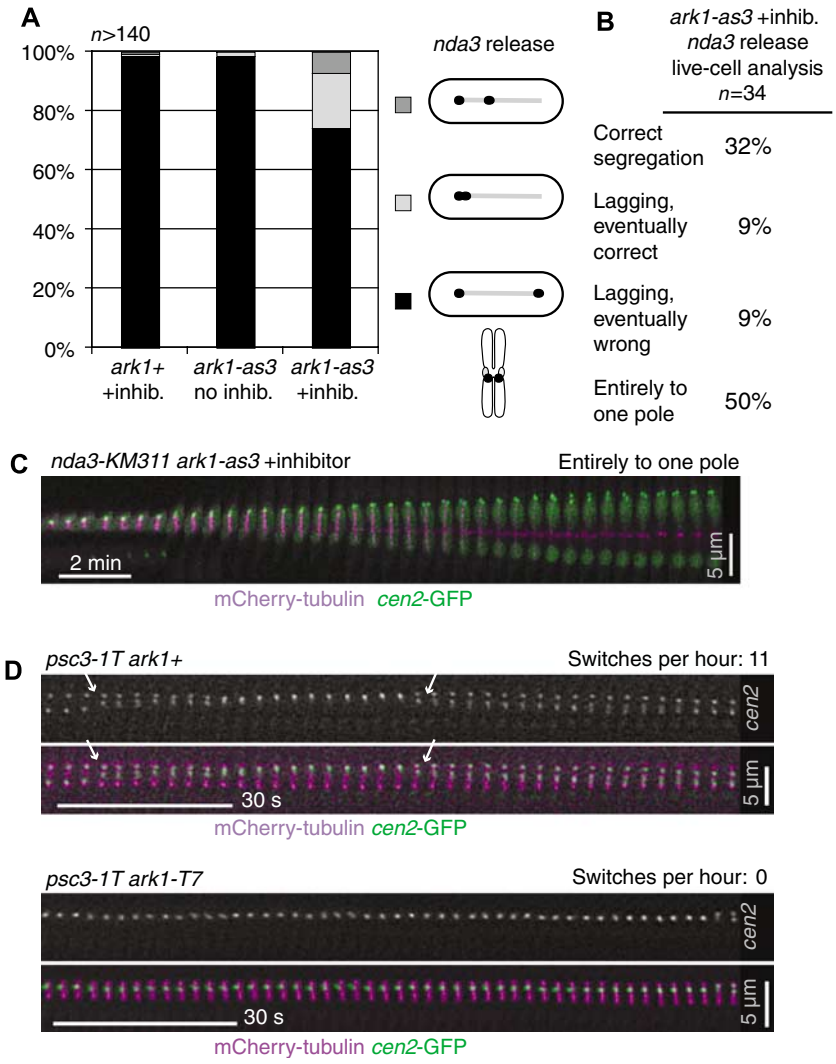

Figure 2 Ark1 is required for the correction of malattachment. (**A**) The indicated strains carrying the *nda3-KM311* mutation were arrested in mitosis by incubation at 19°C for 6 h, and released by transfer to 32°C. Where indicated (+inhib.) 5 μM 1NM-PP1 were added to the culture 10 min before release. Cells were fixed with methanol 10 min after upshift to 32°C. Chromosome segregation was assessed by determining *cen2*-GFP localization on anaphase spindles, which were labeled by mCherry-Atb2 (tubulin). (**B, C**) *nda3-KM311 ark1-as3* cells were arrested in mitosis as in panel A and released by transferring cells to a microscope stage kept at 32°C. 1NM-PP1 (5 μM) was added 5 min before release. Only cells that could be followed through mitosis starting from very short spindle length were considered. Cells in which the spindle was defective and those in which centromere 2 did not attach to the spindle were excluded from the analysis. A kymograph of a cell that showed chromosome 2 segregating with one SPB is shown in panel C. Example kymographs for all phenotypes as well as example kymographs from *ark1-as3* cells released from the *nda3-KM311* arrest without any inhibitor are shown in Supplementary Figure S2A. (**D**) The indicated strains marked with *cen2*-GFP and mCherry-Atb2 (tubulin) were followed by live-cell microscopy at the restrictive temperature (34°C). A centromere was considered to have 'switched' when the trajectory of *cen2*-GFP from one pole to the other could be clearly observed (arrows). The number of 'switches' per hour in a total of 2 h prometaphase observation time is given.

under control of the mitosis-specific promoter of the *rad21+* gene. In this strain, the level of Ark1 protein in meiosis was largely reduced, but minor amounts of Ark1 were still observed at centromeres and midspindles (Supplementary Figure S4). For some experiments, we therefore either additionally or alternatively used the *ark1-as2* allele together with the specific inhibitor.

The most prominent phenotype observed after Ark1 depletion in mitosis is a failure to condense chromosomes, which leads to a defect in nuclear division (Petersen *et al*, 2001). We similarly found a defect in chromosome condensation during meiosis in *ark1 s.o.* cells (Supplementary Figure S5). Nevertheless, four distinct, albeit often unequally sized, nuclei formed after meiosis-II in *ark1 s.o.* cells (Supplementary Figure S5). However, in about 50% of *ark1 s.o.* cells, the two meiosis-II spindles formed extremely close to each other and often in what seemed to be one nucleus (Figure 3A), indicating a failure of nuclear division during meiosis-I. To assess this phenotype better, we arrested cells after meiosis-I using the *mes1* mutation (Izawa *et al*, 2005). Under these conditions, more than 80% of *ark1+* cells but only 30% of *ark1 s.o.* cells formed two nuclei (Figure 3B and C). Nevertheless, the mononucleated *ark1 s.o.* cells seemed to have undergone anaphase-I, because Rec8-GFP was largely removed from chromatin (Supplementary Figure S6). The failure in nuclear segregation after Ark1 depletion

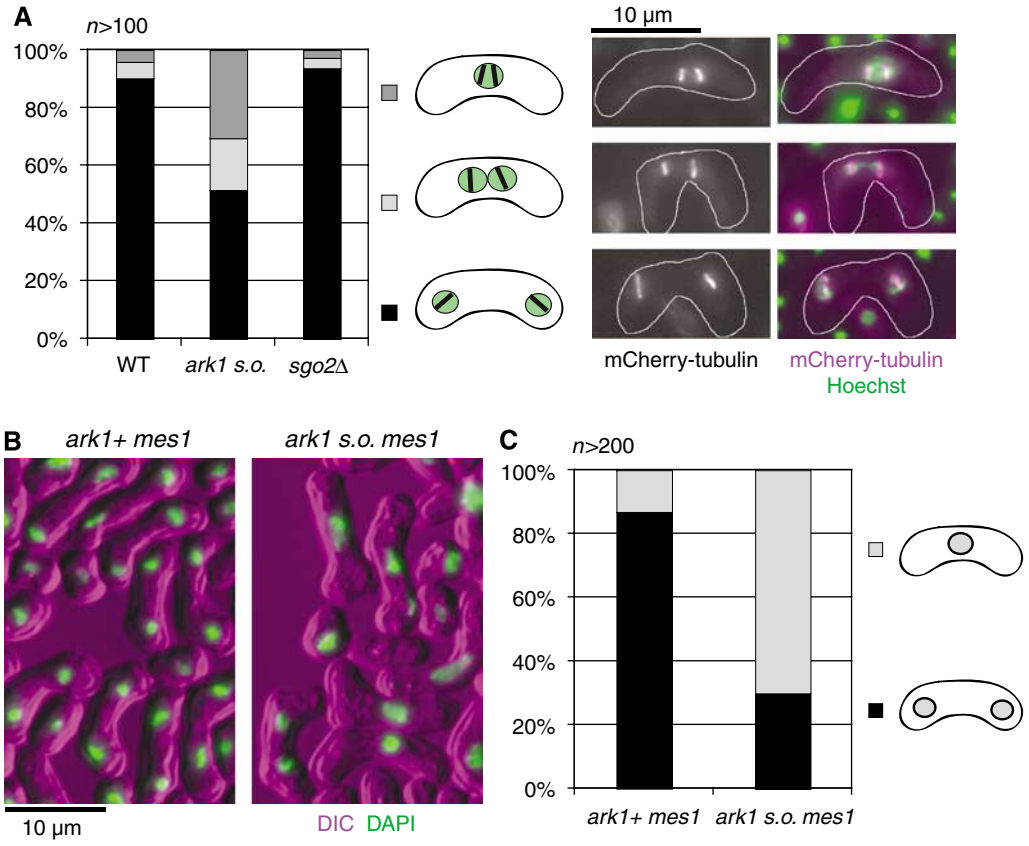

Figure 3 Ark1 is required for nuclear division during meiosis-I. (**A**) The position of metaphase-II spindles was evaluated in fixed cells. DNA was stained with Hoechst 33342. (**B, C**) The indicated strains were arrested after anaphase-I by the *mes1* mutation. Cells were fixed and DNA was stained with DAPI. The number of nuclei was determined in at least 200 asci (C); exemplary cells are shown in panel B.

presumably reflects a failure in condensin function, since mutants in the condensin subunit *cut3* also show a slightly increased number of cells with one nucleus when arrested by the *mes1* mutation at a semi-permissive temperature (data not shown).

Ark1 promotes homolog bi-orientation in meiosis-I

To address whether Ark1 has any role in meiotic chromosome segregation, we examined the segregation of *cen2*-GFP in *ark1 s.o.* and *ark1-as2* cells during anaphase-I. When Ark1 was inhibited or depleted, both homologous chromosomes 2 segregated to the same pole in about 30–40% of anaphase-I cells (Figure 4A). In another 30% of cells, at least one of the homologs was lagging on the anaphase-I spindle. These segregation problems could be caused either by a failure to bi-orient bivalents or by a failure to join homologous chromosomes through chiasmata. Since the intergenic recombination frequency between the *lys3+* and *ura1+* locus on chromosome 1 was similar in *ark1+* and *ark1 s.o.* strains (data not shown), the latter is unlikely. Therefore, these data suggest that Ark1 is required for the bi-orientation of connected homologs during meiosis-I (also see Supplementary Note 2).

The role of Ark1 in bi-orientation is independent of kinetochore geometry

Paliulis and Nicklas (2000) have shown that specific features of the chromosome and not of the spindle determine the special chromosome segregation of meiosis-I. In fission yeast,

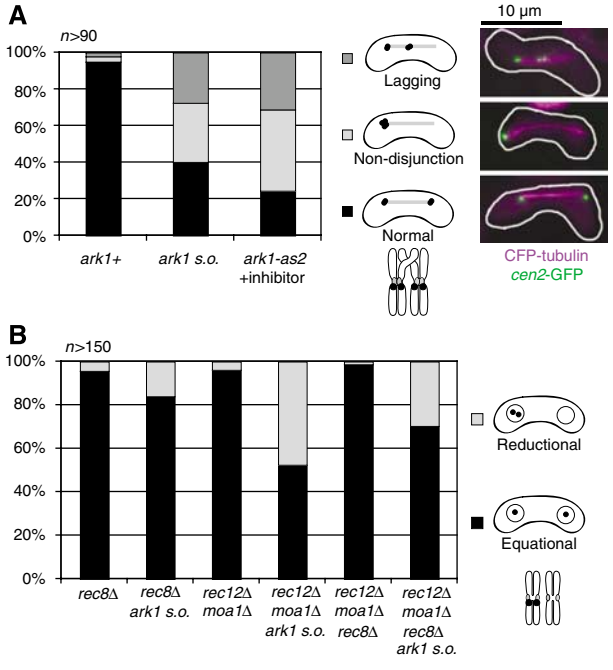

Figure 4 Ark1 is required for proper homolog bi-orientation in meiosis-I. (**A**) The indicated strains with both homologous chromosomes 2 marked by GFP were observed during anaphase-I using CFP-Atb2 (tubulin) as a marker. (**B**) The indicated strains, in which one of the homologous chromosomes 2 was marked by GFP, were fixed during meiosis by methanol. DNA was stained by Hoechst 33342 and the segregation of sister centromeres was determined in binucleated cells.

genetic tricks can be used to create mitosis-like chromosomes during meiosis-I. Deletion of the gene for the meiosis-specific cohesin subunit Rec8 causes a failure in recombination and therefore chiasmata generation. Additionally, sister kinetochores in each homolog are faithfully bi-oriented presumably because geometry at the centromeric region is mitosis-like and sister chromatids remain cohered by mitotic cohesin complexes, which persist into meiosis (Yokobayashi *et al*, 2003). Similarly, mitosis-like chromosomes can be generated by deleting *rec12+*, which is required for recombination, and *moa1+*, the gene product of which is required for the mono-orientation of sister kinetochores in concert with Rec8 (Yokobayashi and Watanabe, 2005). In both these genetic backgrounds, Ark1 shut-off caused mis-segregation of sister centromeres in meiosis-I (Figure 4B), with the effect being greater in a *rec12*Δ *moa1*Δ strain than in a *rec8*Δ strain (see Supplementary Note 3). Thus, during meiosis-I, Ark1 can promote the equational segregation of sister chromatids (Figure 4B), or the bipolar segregation of homologs (Figure 4A), depending on the chromosome structure. We suggest that Ark1 promotes the bi-orientation of any two kinetochore-containing entities that are connected. This is in accordance with findings by Dewar *et al* (2004), who showed that budding yeast Ipl1 ensures the bi-orientation of two separate kinetochores on an unreplicated plasmid.

Ark1 is required for the mono-orientation of sister chromatids in meiosis-I

When we tested the segregation of homologs during anaphase-I, we found that frequently at least one of the homologs was lagging and often the *cen2*-GFP signal of lagging homologs split in two (Figure 4A). We therefore hypothesized that the sister kinetochores on one homolog, which normally attach to only one spindle pole, were pulled in opposite direction. To verify this assumption, we labeled only one of the homologous chromosomes 2 with GFP and determined the segregation pattern. Indeed, in about 12% of anaphase-I cells, the GFP signal split in two when Ark1 was depleted (Figure 5A), indicating that sister chromatids were pulled to opposite poles and separated precociously. These data suggested that in the absence of Ark1, sister kinetochores erroneously become attached to opposite spindle poles during meiosis-I. In accordance, we found that a visible separation of sister centromeres could already be observed during metaphase-I (Supplementary Figure S7). We therefore considered the possibility that Ark1 is required for the localization and function of Moa1, which prevents bi-orientation of sister chromatids at meiosis-I (Yokobayashi and Watanabe, 2005). However, visualization of Moa1-GFP did not reveal any difference between wild-type and *ark1 s.o.* cells during meiosis-I (Figure 5B). Similarly, no significant difference in Moa1 localization was observed by chromatin

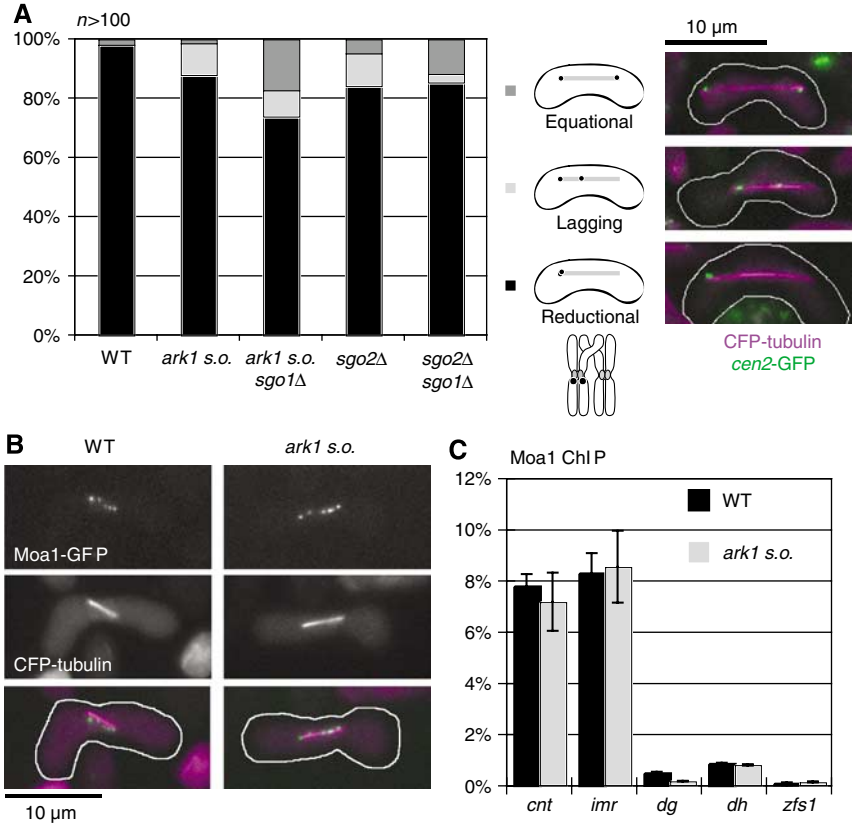

Figure 5 Ark1 is required for proper sister chromatid mono-orientation in meiosis-I independent of Moa1. (**A**) One of the homologous chromosomes 2 was marked by GFP in the indicated strains and the segregation pattern was determined during anaphase-I using CFP- or mCherry-Atb2 (tubulin) as a marker. (**B**) Cells from the indicated strains expressing Moa1-GFP and CFP-Atb2 (tubulin) were observed during metaphase-I. (**C**) Diploid strains of the indicated genotype were arrested in prophase-I by deletion of *mei4+*, and the amount of Moa1 at centromeres was determined by ChIP using a Moa1 antibody.

immunoprecipitation (ChIP; Figure 5C). Furthermore, the localization of Rec8 at the centromeric central core, which is important for mono-orientation of kinetochores (Watanabe *et al*, 2001; Yokobayashi *et al*, 2003), was intact in the *ark1 s.o.* cells (Supplementary Figure S8). Thus, the mono-orientation defect caused by the reduction of Ark1 is likely different from the one caused by the absence of Moa1 or Rec8, suggesting that Ark1 and Moa1/Rec8 influence sister kinetochore mono-orientation in meiosis-I through distinct mechanisms.

Sister centromeres separate precociously after Ark1 depletion because of merotelic attachment rather than complete bi-orientation

Cells in which *moa1+* is deleted show entirely equational segregation of sister chromatids in meiosis-I if recombination is abolished by *rec12*Δ (see Figure 4B). In this situation lagging chromatids can be observed in anaphase-I. Their appearance is completely suppressed by deleting *sgo1+*, the meiosis-I-specific protector of centromeric cohesion (Yokobayashi and Watanabe, 2005). In contrast, neither deletion of *rec12+* nor the additional deletion of *sgo1+* leads to completely equational segregation in *ark1 s.o.* cells (Supplementary Figure S8C, and data not shown). When contemplating the reason for the mono-orientation defect in *ark1 s.o.* cells, we noticed the high frequency of lagging chromosomes or lagging sister chromatids during anaphase-I (Figures 4A and 5A). Given that lagging chromosomes may originate from merotelic attachment and Ark1 is involved in its correction (Figure 1), we envisaged that the primary defect in *ark1 s.o.* cells preventing monopolar attachment could be the inability to correct merotelic attachment of a unified pair of sister kinetochores (Figure 6B). In *rec12*Δ *moa1*Δ cells, all sister centromeres eventually segregated at anaphase-I even in the presence of Sgo1, implying that microtubule-mediated pulling on bi-oriented sister kinetochores can overcome Sgo1-mediated protection (Vaur *et al*, 2005; Yokobayashi and Watanabe, 2005; Figure 6B). The tension on homologs that are attached in a merotelic manner is expected to be less, because some microtubules on both sister kinetochores likely attach to the same pole (Figure 6B, '*sgo1+*'). This reduced tension might not be sufficient to overcome protection by Sgo1. This hypothesis makes the key prediction that deprotection of sister chromatid cohesion by *sgo1*Δ would increase the equational segregation of sister centromeres in *ark1 s.o.* cells, different from *rec12*Δ *moa1*Δ cells (see Figure 6B). Consistent with this scenario, the ratio of cells in which sister centromeres were moving entirely to opposite poles in anaphase-I increased from ~1% in *ark1 s.o.* to 17% in *ark1 s.o. sgo1*Δ cells (Figure 5A). Furthermore, lagging sister chromatids could be observed in *ark1 s.o.* cells even after *sgo1+* deletion, indicating that single chromatids were attached in a merotelic manner (Figures 5A and 6B, '*sgo1*Δ').

Thus, we suggest that Ark1 promotes monopolar attachment of sister kinetochores at meiosis-I most likely by correcting merotelic attachment of paired sister kinetochores, a mechanism that is fundamentally different from that of Moa1 and Rec8, which are thought to promote the side-by-side orientation of sister kinetochores by fostering cohesion in the central core region of the centromere (Figure 6B).

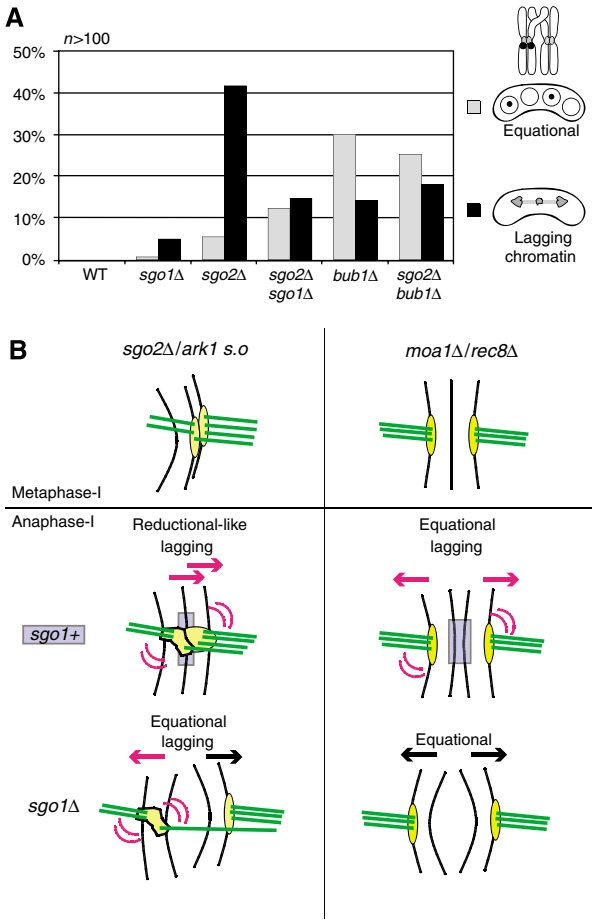

Figure 6 Sgo2 promotes mono-orientation similar to Ark1. (**A**) To assess the percentage of equational segregation (light gray bars), one of the homologous chromosomes 2 was marked by GFP in the indicated strains and the segregation pattern was judged in asci that had formed four spores. The percentage of lagging chromosomes or chromatids (dark gray bars) was determined by staining fixed cells with DAPI and the TAT-1 antibody recognizing tubulin. (**B**) Model for the chromosome segregation defects after Sgo2/Ark1 versus Moa1/Rec8 depletion. The centromeric region of one homolog is shown. In *sgo2*Δ or *ark1 s.o.* cells, the pair of sister kinetochores on one homolog tends to attach in a merotelic manner, that is, to both spindle poles, during metaphase-I. In anaphase, lagging homologs are common because of merotelic attachment. Eventually, both sister centromeres segregate to the same pole in most cases. If the protector Sgo1 is removed, sister centromeres segregate to opposite poles more frequently. Lagging chromatids are still observed after deletion of *sgo1+*, indicating merotelic attachment of single sister kinetochores. When *moa1+* or *rec8+* is deleted, sister kinetochores on one homolog lose their intimate connection. When *rec12+* is deleted in addition to *moa1+*, sister chromatids segregate completely equationally in anaphase-I. Nevertheless, lagging chromosomes occur, but deleting *sgo1+* abolishes their appearance.

The Bub1–Sgo2–Ark1 pathway operates for proper chromosome segregation in meiosis

Recent reports indicated that the Shugoshin protein Sgo2 interacts with the CPC protein Bir1 and is required for the full recruitment of the CPC including Ark1 to centromeres (Kawashima *et al*, 2007; Vanoosthuyse *et al*, 2007). Cells depleted of Sgo2 exhibit non-disjunction of homologs in about 20% and equational segregation of sister centromeres in ~5% at meiosis-I (Kitajima *et al*, 2004; Rabitsch *et al*,

2004; Riedel *et al*, 2006; Kawashima *et al*, 2007; Figures 5A and 6A, and data not shown). Since localization of Ark1 at centromeres is reduced in *sgo2*Δ cells at meiosis-I (Supplementary Figure S9; Kawashima *et al*, 2007), the non-disjunction of homologs in *sgo2*Δ cells was attributed to defects in Ark1 function. Based on the results described above, we hypothesized that the mono-orientation defect in *sgo2*Δ cells originates from the inability to correct merotelic attachment of paired sister kinetochores, like in *ark1 s.o.* cells. Supporting this assumption, an attempted separation of sister centromeres at anaphase-I was observed in about 16% of *sgo2*Δ cells, which is very similar to that in *ark1 s.o.* cells (Figure 5A). Moreover, deletion of *sgo1+* increased the equational segregation of sister centromeres of chromosome 2 at meiosis-I to 12% (Figure 5A; also see Figure 6A), which is again very similar to that in *ark1 s.o.* cells. Thus, we conclude that defects in meiotic chromosome segregation in *sgo2*Δ cells are mostly caused by reduced Ark1 function. In contrast to the chromosome segregation function, the role of Ark1 in promoting chromosome condensation is not shared by Sgo2 (Supplementary Figure S5).

Previous data supported the view that Bub1 acts upstream of Sgo1 and Sgo2 (Kitajima *et al*, 2004; Supplementary Figure S9). Fittingly, Ark1-GFP localization was perturbed during metaphase-I to a similar extent as in *sgo2*Δ cells by deletion of *bub1+* (Supplementary Figure S9E and F). Furthermore, we found that deletion of *sgo2+* did not enhance the mono-orientation defect of *bub1*Δ cells (Figure 6A), indicating that Bub1 and Sgo2 work in a single pathway. These results suggest that the defects in mono-orientation after Bub1 depletion might be partly caused by the inability to correct merotelic attachment of paired sister kinetochores, similar to the situation in *ark1 s.o.* or *sgo2*Δ cells (see Discussion).

Discussion

Mitotic functions of Aurora are conserved in fission yeast

Aurora kinases are highly conserved throughout eukaryotes, and have been implicated in proper chromosome segregation in several organisms (Vagnarelli and Earnshaw, 2004). Noticeably, however, in fission yeast, a well-studied model in mitosis research, it was unknown whether the single Aurora kinase, Ark1, has any role in regulating the proper attachment of chromosomes during mitosis. Here, we demonstrate that Ark1 inhibition causes misattachment of chromosomes to the mitotic spindle (Figure 1). As in budding yeast (Tanaka *et al*, 2002), Aurora seems necessary to destabilize syntelic attachment that fails to create tension (Figure 2). In addition, lagging chromatids occurred with high frequency when Ark1 was inhibited (Figure 1). Those might arise because attachment to microtubules is weak or dysfunctional, or because the corresponding kinetochore is attached to both spindle poles (merotelic attachment). We favor the latter hypothesis, since most sister chromatids in *ark1-T7* cells move to the spindle poles in anaphase-A with velocities comparable to those observed in wild-type cells (data not shown), indicating that there is no general problem with attachment or microtubule-dependent anaphase movement. In vertebrate cells, where kinetochore–microtubule attachment can be visualized directly, it has been shown that Aurora-B is required to suppress merotelic attachment, possibly by destabilizing the faulty attachment (Cimini *et al*, 2006; Knowlton *et al*, 2006). We consider it an additional possibility that Aurora is required to build the kinetochore in a way that favors attachment of all microtubules on one kinetochore to the same pole. It has been proposed that fission yeast Pcs1, which is a homolog of one of the components of the budding yeast monopolin complex, is required to clamp together single microtubule-binding sites on one kinetochore, thus favoring their attachment to one pole (Rabitsch *et al*, 2003). Indeed, deletion of *pcs1+* causes kinetochores to attach in a merotelic manner (Rabitsch *et al*, 2003; Gregan *et al*, 2007). Thus, Ark1 might be required for Pcs1 function. Our preliminary experiments nevertheless failed to detect an influence of Ark1 on Pcs1 localization (data not shown).

In addition to its functions in regulating kinetochore attachment, budding yeast Ipl1 is a component of the 'NoCut' pathway, which prevents abscission in the presence of spindle-midzone defects (Norden *et al*, 2006). In our experiments it was evident that an equatorial microtubule ring, which normally forms during mid or late anaphase-B in the plane of cell division (Pichova *et al*, 1995; Heitz *et al*, 2001), was formed precociously when Ark1 was inhibited (Supplementary Figure S2A), thus also implying Ark1 in the regulation of cytokinesis.

Ark1 acts on chromosome attachment in a similar way during mitosis and meiosis

Since Ark1 promotes sister chromatid bi-orientation in mitosis, but sister chromatids have to mono-orient during meiosis-I, it was unclear how Ark1 would influence chromosome segregation in meiosis-I. We found that Ark1 normally promotes the bi-orientation of homologs in meiosis-I, but if the morphology of the bivalent is disrupted and mitosis-like chromosomes are created, Ark1 promotes the bi-orientation of sister chromatids (Figure 4). This indicates that the molecular mechanism of Ark1 function is the same in mitosis and meiosis, and the different outcome is determined by the structure of the bivalent.

In contrast to mitosis, where Ark1 inhibition causes only about 10–20% sister chromatid co-segregation, depletion of Ark1 in meiosis causes a more pronounced co-segregation of homologous chromosomes (30–40%). The most likely explanation is that tension-controlled correction of attachment is more important in meiosis, because the two pairs of sister kinetochores on a bivalent are not as tightly coupled as the sister kinetochores on a mitotic chromosome, which might favor their syntelic attachment (Shonn *et al*, 2000).

Very recently it has been demonstrated that the budding yeast Aurora kinase, Ipl1, is similarly required for homolog bi-orientation and sister chromatid bi-orientation of artificial mitosis-like chromosomes in meiosis-I (Monje-Casas *et al*, 2007). Because of the high conservation of Aurora functions in all eukaryotes, we expect that this will also hold true for metazoans.

Does Ark1 facilitate cleavage of Rec8?

In *C. elegans*, the Aurora-B kinase AIR-2 seems to be required to efficiently remove cohesin complexes containing Rec8 from chromosome arms (Kaitna *et al*, 2002; Rogers *et al*, 2002). In contrast, we find that Ark1 is not essential for Rec8 removal during meiosis-I in fission yeast (Supplementary Figure S10). In time-lapse movies of fission yeast expressing

Rec8-GFP, one can clearly observe the solubilization of Rec8 at the onset of anaphase-I, presumably at the moment when it is cleaved and removed from chromatin (Supplementary Figure S10). Although this step is not as easy to discern when Ark1 is inhibited, because chromosomes are less condensed, it is clear that this solubilization also happens fairly efficiently if Ark1 is inhibited and Rec8 is subsequently degraded with kinetics similar to wild-type cells (Supplementary Figure S10). Nevertheless, it is possible that Ark1 facilitates but is not essential for Rec8 cleavage (see Supplementary Note 3).

A novel mechanism to promote mono-orientation of sister chromatids during meiosis-I

We show that fission yeast Ark1 is required for the faithful mono-orientation of sister chromatids in meiosis-I (Figure 5). In fission yeast, mono-orientation of sister kinetochores is regulated by Moa1 and Rec8, which may cooperatively promote the formation of a side-by-side structure of sister kinetochores through cohesion of the centromeric central core region (Yokobayashi and Watanabe, 2005). The depletion of Moa1 together with Rec12 or of Rec8 entirely disrupts the mono-orientation of sister chromatids at meiosis-I. The mono-orientation defect in *ark1 s.o.* cells is less pronounced, and Moa1- or Rec8-localization is not disrupted when Ark1 is depleted (Figure 5; Supplementary Figure S8). This indicates that Ark1 and Moa1 act in separate pathways to promote mono-orientation. Our experiments suggest that in the absence of Ark1, sister kinetochores on one homolog become attached in a merotelic manner so that they are torn apart at anaphase-I, even though they have the proper side-by-side configuration that favors mono-orientation (Figure 6B). Thus, the complicated chromosome segregation defects in meiotic cells depleted of Ark1 can be explained by the well-recognized role of Aurora in correcting malattachment of chromosomes. Syntelic and merotelic attachment of bivalents in meiosis-I provokes non-disjunction of homologs and precocious sister separation, respectively.

In budding yeast, it has been proposed that the two pairs of sister kinetochores on a bivalent only attach to one microtubule each (Winey *et al*, 2005) and one sister kinetochore may thus be inactivated. Consequently, merotelic attachment might not be possible, which would explain the unperturbed mono-orientation in Ipl1-depleted cells (Monje-Casas *et al*, 2007) despite the otherwise similar function of Ipl1 and Ark1. There is, however, controversy in the literature whether the depletion of Ipl1 causes a mono-orientation defect (Yu and Koshland, 2007). In any case, our data clearly indicate that in fission yeast, both sister kinetochores are active in meiosis-I and can attach to microtubules. As attachment of both kinetochores to microtubules at meiosis-I is observed in several organisms (Lee *et al*, 2000; Parra *et al*, 2004), the mechanism we identified here may be functional in other eukaryotes as well.

The Bub1–Sgo2–Ark1 pathway ensures meiosis-I chromosome segregation by correcting malattachment of homologs

Our finding that Ark1 is needed for the bi-orientation of homologs and the mono-orientation of sister kinetochores in meiosis-I provides a crucial clue to solve the enigma why Sgo2 and Bub1 are required for monopolar attachment as well as proper homolog disjunction in meiosis-I. In either *sgo2*Δ or *bub1*Δ cells, centromeric localization of Ark1 is reduced at meiosis-I (Supplementary Figure S9). This is consistent with previous findings that Bub1 acts upstream of Sgo2, which in turn plays a crucial role to load the CPC to centromeres (Kitajima *et al*, 2004; Kawashima *et al*, 2007; Vanoosthuyse *et al*, 2007). Since Moa1 localization is intact in either *sgo2*Δ or *bub1*Δ cells (data not shown), it is reasonable to assume that perturbation of mono-orientation in these cells may originate from the reduced Ark1 activity at centromeres. Indeed, the defects of *sgo2*Δ cells in monopolar attachment at meiosis-I resemble those of *ark1 s.o.* cells (Figure 5A). Because *bub1*Δ cells are defective in both Sgo1 and Sgo2 localization to centromeres (Kitajima *et al*, 2004; Supplementary Figure S9), one would expect that *bub1*Δ phenocopies the *sgo1*+ *sgo2*+ double deletion. However, the frequency of equational segregation is significantly higher in *bub1*Δ cells (~30%) than *sgo2*Δ *sgo1*Δ cells (12%) (Figure 6A; Bernard *et al*, 2001). Since in mitosis *bub1*Δ cells show a higher number of lagging chromosomes than *sgo2*Δ cells (Bernard *et al*, 1998; S Kawashima and Y Watanabe, unpublished results), we suggest that Bub1 has functions that go beyond Sgo2 regulation both in mitosis and meiosis. Whatever the nature of these additional functions is, our results argue that Bub1 depletion perturbs monopolar attachment by generating merotelic attachment of paired sister kinetochores, like Sgo2 or Ark1 depletion.

In summary, we have shown that the conserved functions of Aurora for correcting malattachment are acting in fission yeast mitosis and meiosis. Furthermore, we demonstrate that this activity of Aurora is required to ensure monopolar attachment at meiosis-I, and we expect that the same will hold true for metazoans.

Materials and methods

S. pombe strains

All strains used in this study are listed in Supplementary Table 1 in Supplementary data. To generate the *ark1-as2* allele (Ark1-Leu166Ala; Bishop *et al*, 2000), the *ark1* gene was PCR-mutagenized from a strain into which a hygromycin-resistance cassette (hygR) had been integrated 400 bp 5′ of the *ark1*+ open reading frame (ORF). The hygR-*ark1-as2* construct was integrated in a wild-type strain at the endogenous locus. The *ark1-as2* allele rendered the cells sensitive to 5 μM 1NA-PP1 or 5 μM 1NM-PP1 (both from TRC, North York, ON, Canada). The *ark1-as3* strain was created from *ark1-as2* by additionally mutating Ser229 to Ala. Both the *ark1-as2* and the *ark1-as3* strain used in this study contain the additional amino-acid mutations Gln28Arg and Gln176Arg, which were unintentionally inserted during the first PCR mutagenesis. Because the *ark1-as3* strain without addition of inhibitor grows similar to a wild-type strain and is not benomyl-sensitive like other *ark1*-mutants, it is unlikely that the two additional mutations affect the functionality of Ark1. The *ark1-as2* strain is benomyl-sensitive even when grown without inhibitor. To create the *ark1 s.o.* or *cut3 s.o.* allele (see Supplementary data), a kanamycin- or hygromycin-resistance cassette and about 1000 bp of promoter region from the *rad21*+ gene were integrated 5′ of the respective ORF by PCR-based gene targeting (Bahler *et al*, 1998). To visualize microtubules, CFP-Atb2 or mCherry-Atb2 were expressed from the *nmt81* promoter in the pREP81 plasmid (*LEU2*+), or mCherry-Atb2 was expressed from the endogenous locus by integration of the *nmt41* promoter and the mCherry-coding region upstream of the *atb2*+ ORF. To tag Rec8 with GFP, we modified plasmid pFA6a-GFP(S65T)-hphMX6 (Sato *et al*, 2005) by integrating 950 bp from the 3′-end of the *rec8*+ ORF upstream of GFP and 350-bp genomic sequence 3′ of the *rec8*+ ORF downstream of GFP. The plasmid was linearized by *Xcm*I digest and integrated in a wild-type strain. The strains or alleles not mentioned above have been described (Saka *et al*, 1994; Nonaka

et al, 2002; Yamamoto and Hiraoka, 2003; Grallert *et al*, 2004; Kitajima *et al*, 2004; Yokobayashi and Watanabe, 2005; Kawashima *et al*, 2007).

Culture conditions

Medium for mitotic cultures was YEA (YE with additional 50 mg/l adenine) or minimal medium (MM) containing 5 g/l NH_4Cl and additional nutrients if required (Alfa *et al*, 1993). To synchronize cells in mitosis (Figure 1A), we arrested cells in S-phase by incubation in 12 mM hydroxyurea for 4.5 h at 25 or 30°C depending on the strain. The arrest was released by washing the cells twice with fresh, warm medium before reculturing. Cells carrying the *nda3-KM311* mutation (Figure 2A and B) were arrested in mitosis by incubation at 19°C for 6 h and subsequently released by shifting to 32°C. To observe cells in meiosis, cells were first grown to logarithmic phase. If the *nmt41* or *nmt81* promoter should be induced by thiamine depletion, cells were grown in MM containing 5 g/l NH_4Cl and, if necessary, 200 mg/l leucine and 50 mg/l adenine for about 14 h at 30°C. Cells were washed, collected and spotted on sporulation agar (SPA; Gutz *et al*, 1974) to which leucine or adenine had been added if necessary. After a further 7–8 h of incubation at 30°C, cells were observed directly or fixed by methanol at −80°C. To observe *ark1-as2* cells in meiosis, the cells were first incubated in MM with 5 g/l NH_4Cl for 8–9 h, and then washed and incubated in MM without NH_4Cl for 4–5 h before spotting on a plate with synthetic sporulation agar (SSA; SSL with agar; Egel, 1971) containing 5 μM 1NA-PP1. Cells were observed after 7–10 h of incubation at 30°C.

Immunostaining and DNA staining

For immunostaining, cells were fixed with paraformaldehyde. To stain microtubules, we used the mouse anti-tubulin TAT1 antibody (kind gift from K Gull) at a dilution of 1:200, followed by an Alexa568-coupled anti-mouse secondary antibody (Invitrogen) at 2 μg/ml. To stain DNA, methanol-fixed cells were washed, resuspended in PEM buffer (100 mM PIPES, 5 mM EGTA, 5 mM $MgCl_2$, pH 6.9) and stained by 1 μg/ml Hoechst 33342 or 1 μg/ml DAPI.

Image acquisition

Images were acquired on a Zeiss AxioImager microscope (Zeiss, Jena, Germany) with MetaMorph software (Molecular Devices Corporation, Downingtown, PA). Typically, a Z-stack of about 4-μm thickness, with single planes spaced by 0.25–0.4 μm, was acquired and subsequently projected to a single image. To compare signal intensities, all images were taken with the same exposure conditions and processed similarly.

Time-lapse imaging

Live-cell recordings were performed on a DeltaVision RT system (Applied Precision, Issaquah, WA) equipped with a heating chamber. For imaging mitosis in *ark1*-mutants, cells were grown in liquid medium at permissive temperature, transferred to a glass-bottom culture dish (MatTek, Ashland, MA) coated with lectin and incubated on the microscope stage at the restrictive temperature (34°C) for at least 1 h, before starting image acquisition. To image cells in an *nda3-KM311* release, cells were transferred from a liquid culture at 19°C to a glass-bottom culture dish coated with lectin, which was placed in the microscope chamber heated to 32°C. Image acquisition was started immediately. Images usually were acquired with the Z-sweep acquisition (OAI) feature and deconvolved using softWoRx software. Kymographs were assembled with Adobe Photoshop and Image Ready software.

Chromatin immunoprecipitation

The procedure was carried out essentially as described previously (Yokobayashi *et al*, 2003; Kawashima *et al*, 2007). Anti-Moa1 polyclonal antibodies, anti-GFP polyclonal antibodies (Living Colors Full-length A.v. Polyclonal Antibody, Clontech) and anti-Cnp1 polyclonal antibodies were used for IP (Yokobayashi and Watanabe, 2005). DNA prepared from whole-cell extracts or immunoprecipitated fractions was analyzed by quantitative PCR with ABI PRISM7000 (Applied Biosystems) using SYBR Premix Ex Taq (Perfect Real Time; Takara). The primers used for PCR were described previously (Yokobayashi *et al*, 2003; Kawashima *et al*, 2007).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions

YW and SH conceived the project; SH and ML designed and performed the mitosis experiments; AB and SH designed and performed most meiosis experiments; SAK contributed the data in Figure 6A; Supplementary Figure S9A, B, E and F; TT performed the ChIP experiments and SH and YW wrote the paper.

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