The *Tetrahymena* meiotic chromosome bouquet is organized by centromeres and promotes interhomolog recombination

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Summary

In order to form crossovers and to undergo reductional segregation during meiosis, homologous chromosomes must pair. In *Tetrahymena*, meiotic prophase nuclei elongate immensely, and, within the elongated nucleus, chromosomes are arranged with telomeres assembled at one pole and centromeres at the opposite pole. This organisation is an exaggerated form of the bouquet, a meiotic chromosome arrangement that is widely conserved among eukaryotes. We show that centromere function is crucial for the formation of *Tetrahymena*'s stretched bouquet and, thereby, for homologue pairing. This finding adds to previous reports of the importance of centromeres in chromosome pairing in budding yeast and in *Drosophila*. *Tetrahymena*'s bouquet is an ataxia telangiectasia- and RAD3-related (ATR)-dependent meiotic DNA damage response that is triggered by meiotic DNA double-strand breaks (DSBs), suggesting that the bouquet is needed for DSB repair. However, in the present study we show that although homologous pairing is impeded in the absence of the bouquet, DSB repair takes place nevertheless. Moreover, recombinational DSB repair, as monitored by bromodeoxyuridine incorporation, takes place only after exit from the bouquet stage. Therefore, we conclude that the bouquet is not required for DSB repair *per se*, but may be necessary for the alignment of homologous loci in order to promote homologous crossovers over alternative repair pathways.

Key words: Meiosis, Centromere, Bouquet, Chromosome pairing, Recombination

Introduction

The bouquet is a chromosomal arrangement in meiotic prophase, in which all telomeres cluster in a limited area at the nuclear periphery (for a review, see Scherthan, 2001). This arrangement is conserved in a wide range of fungal, plant and animal species, with only a few deviations from the canonical appearance. For example, in *Caenorhabditis elegans* only one end of each chromosome participates in the cluster (Phillips et al., 2009), and in *Arabidopsis*, telomeres cluster at the nucleolus (Armstrong et al., 2001). In the cases studied so far, telomere clustering is accompanied by telomere-assisted chromosomal movements. The conventional view is that the bouquet configuration, or chromosome movements that accompany its formation, contribute to the coming-together of homologous chromosomes, but alternative explanations have been provided as well (see Koszul and Kleckner, 2009). In budding yeast and in maize, it was found that the bouquet facilitates, but is not absolutely required for, homologous pairing and recombination (Lee et al., 2012; see Harper et al., 2004; Koszul and Kleckner, 2009). On the other hand, fission yeast possesses a sophisticated form of the bouquet, which includes nuclear elongation and movements (see Yamamoto and Hiraoka, 2001), and pairing and recombination depend on it to a large extent (Davis and Smith, 2006).

There is increasing evidence for a previously disregarded role of centromeres in the initiation of chromosome pairing (see Stewart and Dawson, 2008; Moore and Shaw, 2009; Tsai and McKee, 2011). Particularly, in the meiosis of *Drosophila*, which does not feature a bouquet, centromere clustering has been proposed to substitute for the bouquet (Subramanian and Hochwagen, 2011). To better understand the meiotic pairing function of centromeres and to investigate its prevalence among eukaryotes, we undertook a study of meiotic centromere behaviour in *Tetrahymena thermophila*.

Tetrahymena thermophila is a unicellular ciliated protist. It possesses two nuclei, a polyploid vegetative macronucleus and a diploid generative micronucleus. During cell division, the macronucleus (whose chromosomes do not possess centromeres) splits by an amitotic process, whereas the micronucleus, which represents the germline, divides mitotically. Only the latter performs meiosis, which is induced when two cells of different mating types fuse (see Karrer, 2000; Collins and Gorovsky, 2005). During meiotic prophase, the nucleus undergoes a dramatic elongation, which is propagated by the polymerization of intranuclear microtubules (Ray, 1956; Wolfe et al., 1976; Kaczanowski et al., 1985) (Fig. 1A–I). Telomeres are clustered near one end of the elongated meiotic nucleus (Loidl and Scherthan, 2004) (Fig. 1J). Notably, centromeres assemble at the

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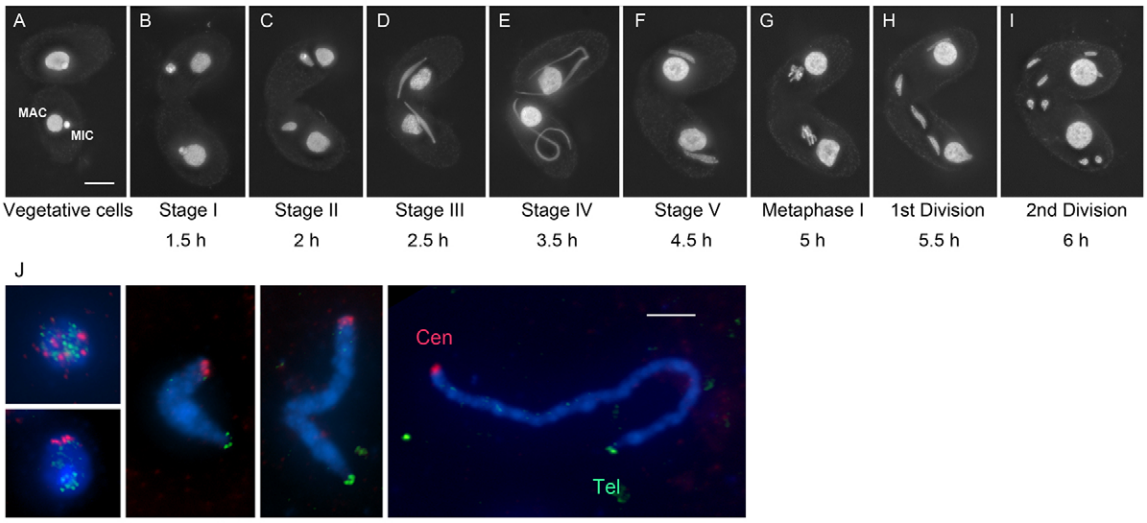

Fig. 1. *Tetrahymena* meiosis. (**A–I**) DAPI-stained stages. (A) Growing cells prior to conjugation. (B) Starving cells of different mating type associate and induce synchronous meioses of their generative nuclei. During prophase, the generative micronucleus (MIC) elongates (C–E), then shortens (F) and forms bivalents (G), which are separated in a first (H) and second (I) division. The vegetative macronucleus (MAC) does not participate in the process. Meiotic stages I–V are classified according to Sugai and Hiwatashi (Sugai and Hiwatashi, 1974). Numbers underneath the panels indicate the time, after induction of meiosis, when the respective stage is most abundant (see Mochizuki et al., 2008; Lukaszewicz et al., 2010). Scale bar in A: 10 μm in A–I. (**J**) Examples of the distribution of centromeres (Cen, red) and telomeres (Tel, green) in elongating micronuclei. Centromeres were detected by immunostaining of the centromere-specific histone Cna1, and telomeres by FISH with a telomere probe. Scale bar: 10 μm.

opposite pole (Fig. 1J), leading to an extreme bouquet organisation with chromosome arms stretched in parallel between the two poles and homologous regions juxtaposed (Cui and Gorovsky, 2006; Mochizuki et al., 2008). The clustering of centromeres at the tip of the elongated nucleus suggests that either a centromere-proximal region or the centromere itself either drives elongation or attaches chromosomes to the elongating nucleus.

The *Tetrahymena* bouquet is triggered by meiotic DNA double-strand breaks (DSBs), because nuclei fail to elongate and centromeres do not cluster in the absence of DSBs in a *spo11*Δ mutant. However, nuclear elongation and centromere clustering are restored by artificially induced DNA lesions (Mochizuki et al., 2008). The bouquet is also dependent on the DNA damage sensor kinase ATR (Loidl and Mochizuki, 2009). Hence, it was hypothesized that nuclear elongation is a DNA damage response that helps or is required to repair DSBs. Here, we show that contrary to this expectation, DSBs are repaired in nuclei that do not form a bouquet. We also find that the *Tetrahymena* bouquet depends on centromere function, and that centromeres play an active role in homologous chromosome pairing.

Results

Depletion of centromeric protein Cna1 prevents centromere clustering

A time course of meiotic nuclear dynamics is shown in Fig. 1. In nonmeiotic cells, the germline nucleus is spherical. Upon mixing of starved cells of different mating types, pairs of cells will conjugate and initiate synchronous meioses. The meiotic nuclei will first become drop-shaped, then spindle-shaped, before elongation culminates in the so-called crescent. During elongation, DSBs are formed, recombination protein Dmc1 forms foci on chromatin, and homologous chromosomes become juxtaposed (Loidl and Mochizuki, 2009; Lukaszewicz et al., 2010; Howard-Till et al., 2011). Later, nuclei shorten and chromatin condenses into threads. Five bivalents become visible during the next step, which is followed by a first and second meiotic division.

To study a possible role of centromeres in nuclear elongation, we constructed strains for conditional RNAi depletion of the centromere-specific H3 histone Cna1p (Cervantes et al., 2006; Cui and Gorovsky, 2006). A hairpin construct targeting *CNA1* was placed under the *MTT1* metallothionein promoter, and expression of dsRNA was induced by the addition of $CdCl_2$ to cell cultures. To determine if RNAi was effective, we first applied it to vegetative cells (supplementary material Fig. S1). After 30 h (corresponding to 10–12 mitotic cycles) in the presence of cadmium, 70% of cells had lost the generative nucleus, in 14.5% it was small, and in 5.5% it was enlarged (n=200 cells). In the non-induced control, 100% of cells (n=100) had a normal-looking nucleus. This RNAi phenotype resembles the one reported for partial *CNA1* deletion (Cervantes et al., 2006; Cui and Gorovsky, 2006) and indicates chromosomal disjunction problems due to the depletion of Cna1p. Efficient Cna1p depletion was further confirmed by immunostaining of Cna1p (antibody kindly provided by Harmit Malik). In the wild type, clustered signals were detected at the periphery of nuclei (cf. Cervantes et al., 2006) whereas in CNA1p-depleted cells, only a diffuse and unspecific background staining was seen (supplementary material Fig. S1E,F).

For studying nuclear behaviour in meiosis, we mated wild-type cells to cells that had been depleted of Cna1p for ~30 h. In the resulting meiotic pairs, the wild-type partners displayed the characteristic elongated nucleus, whereas most of the *cna1*RNAi partners had lost the generative nucleus during premeiotic growth. Importantly, in the minority of cells where the generative nucleus was still present, its chromatin always remained spherical (Fig. 2A). Immunostaining of Cna1p highlighted the centromeric cluster at the tip of the elongated wild-type nucleus (Fig. 2B). Cna1p was also present in the

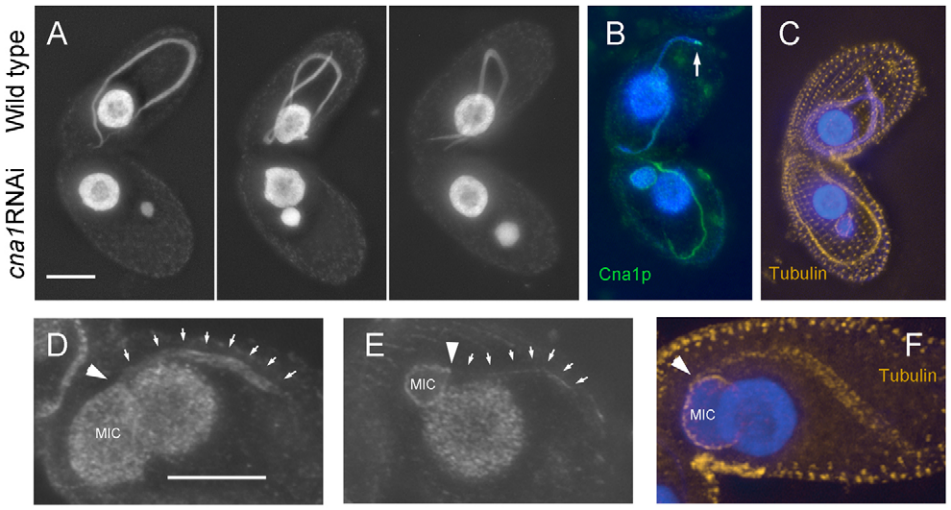

Fig. 2. Conjugating wild-type and *cna1*RNAi cells. (**A–C**) In the wild-type partner (top) the chromatin in the meiotic nucleus is elongated, whereas in the *cna1*RNAi cell (bottom) it stays round. (A) Examples of *cna1*RNAi cells with differently sized meiotic nuclei are shown (DAPI staining). (B) Immunostaining of Cna1p (green) reveals clustered centromeres in the tip of the wild-type nucleus (arrow). In the *cna1*RNAi partner, Cna1p delineates the contours of the empty elongated nucleus, while the chromatin remains clustered at one end. (For an explanation of Cna1p expression in *cna1*RNAi cells, see the main text.) (C) α-tubulin staining (orange) outlines the elongated nucleus in the wild-type partner, as well as the chromatin-containing and chromatin-free nuclear portions in the *cna1*RNAi partner. Scale bar in A: 10 μm in A–C. (**D–F**) Details of *cna1*RNAi nuclei. Arrowheads indicate the boundary between the chromatin-containing and the empty part (arrows) of the nucleus. (D,E) Cna1p staining. There is a strong background staining of chromatin. (F) α-tubulin staining (orange). Chromatin is stained with DAPI (blue) in B, C and F. Scale bar in D: 10 μm in D–F.

Cna1p-depleted partner, which is due to the transfer of free protein from the wild-type partner (McDonald, 1966). However, it was not incorporated into centromeres, since this only occurs during S phase (see Cui and Gorovsky, 2006). Instead, ectopic Cna1p deposited along a thread-like structure projecting from the vegetative nucleus (Fig. 2B,D,E). This appendix probably represents a chromatin-free elongated portion of the nucleus. Therefore, it seems that *cna1*RNAi nuclei do elongate, but that chromosomes with disabled centromeres do not stretch between the two poles, and a mass of chromatin is left behind at one end. To add support for this interpretation, we immunostained against α-tubulin, since microtubules are known to line elongated meiotic nuclei (Davidson and LaFountain, 1975; Wolfe et al., 1976; Gaertig and Fleury, 1992). Indeed, tubulin also delineated an elongated chromatin-free extension of *cna1*RNAi nuclei (Fig. 2C,F). Therefore, centromeres are required for connecting chromosomes to the tip of the elongating nucleus, but not for driving elongation.

Centromere clustering is dependent on microtubules and promotes pairing of intercalary chromosome regions

The centromere assembles a protein complex, the kinetochore, which links the chromosome to microtubules (see Santaguida and Musacchio, 2009). Therefore, it is possible that the failure of Cen1p-depleted centromeres to attach to the nuclear tip is due to their inability to capture microtubules. We tested this possibility by treating cells with the microtubule inhibitor nocodazole (cf. Kaczanowski et al., 1985). In untreated cells, centromeres are clustered prior to the onset of meiosis. This probably reflects the chromosomal Rabl orientation, a consequence of the poleward centromere orientation during preceding telophase (Mochizuki et al., 2008, and references therein; supplementary material Fig. S1E). At the beginning of nuclear elongation, centromeres become transiently dispersed and re-assemble in the fully elongated nucleus (Mochizuki et al., 2008; Loidl and Mochizuki, 2009). As expected, nocodazole efficiently prevented nuclear elongation. When nocodazole was added 110 min after meiosis induction, elongation was completely suppressed (no elongated nuclei in 200 conjugating cells scored 3.5 h after meiosis induction, as compared to 59% in the wild type at the same stage). Moreover, centromeres failed to cluster (Loidl and Mochizuki, 2009) (Fig. 3A). This indicates that microtubules are not only required for nuclear elongation but also for the clustering of centromeres. Therefore, centromere clustering probably depends on the ability of centromeres to associate with microtubules. Notably, telomere clustering was also abolished in nocodazole-treated cells (Fig. 3B).

It was shown previously by treatment with the microtubule inhibitor benomyl that pairing of a fluorescence *in situ* hybridization (FISH)-marked intercalary chromosome region is reduced in non-elongating MICs (Loidl and Mochizuki, 2009). Here, we confirmed and extended this result using nocodazole, which has a stronger effect on disrupting centromere clusters (Loidl and Mochizuki, 2009). We found that pairing in non-elongated nuclei was reduced to 10.0% of the frequency in elongated bouquet nuclei (Fig. 3C,D). This confirms that the clustering of centromeres and telomeres at opposite ends of elongated nuclei and the resulting parallel arrangement of chromosome arms supports the homologous pairing of intercalary regions.

A search for bouquet-related proteins

Telomere-associated proteins such as Rap1, Ndj1/Tam1, Mps3, Taz1 and related proteins have been found to play a crucial role in meiotic telomere attachment to the nuclear periphery and bouquet formation in a range of organisms (see Scherthan, 2007;

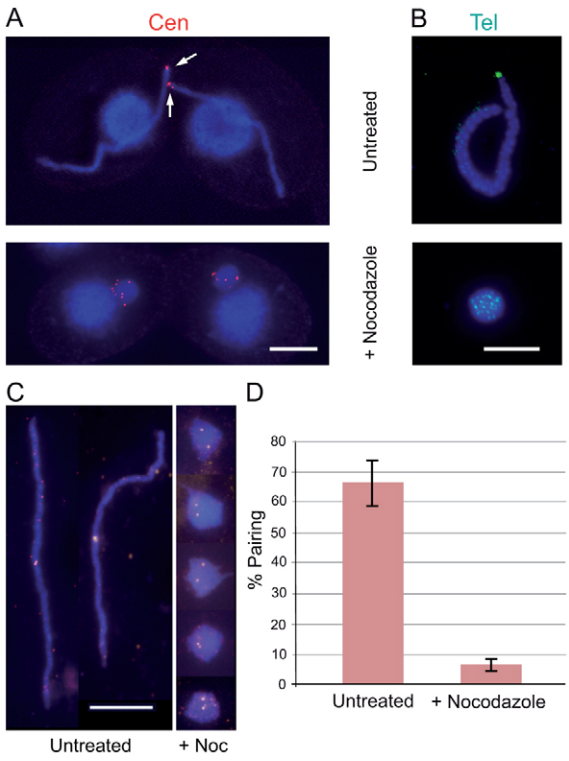

Fig. 3. Inhibition of microtubules prevents bouquet formation and pairing. (**A**) Immunostaining of protein Cna1p (red) and (**B**) telomere FISH (green) highlight centromere (Cen, arrows) and telomere (Tel) clusters at the ends of elongated bouquet nuclei in untreated cells (upper panels). Nocodazole treatment prevents centromere and telomere clustering and nuclear elongation (lower panels). (**C**,**D**) Pairing of a FISH-labelled intercalary chromosomal region (pink) in elongated and non-elongating (nocodazole-treated) meiotic nuclei. (C) Left: Examples of paired signals in elongated nuclei. Right: Examples of unpaired signals in non-elongating nuclei. These are discriminated from non-meiotic nuclei by their larger size. Cells prepared for FISH (see the Materials and Methods section) burst, hence isolated nuclei are seen in B and C. Scale bars in A, B and C: 10 μm. (D) Quantification of pairing. Each column represents the average from three experiments with 25 nuclei evaluated per experiment. Error bars indicate s.d.

Schmitt et al., 2007; Bupp et al., 2007; Conrad et al., 2008; Hiraoka and Dernburg, 2009). Since in *Tetrahymena*, telomeres are clustered at the centromere-distal end of the nucleus, we wondered if they are attached to the periphery by similar telomere-associated proteins. Therefore, we BLAST-searched the proteome for homologues of *S. pombe* Taz1 or Rap1 and *S. cerevisiae* Ndj1 or Rap1 or human Trf proteins (Altschul et al., 1997). However, we failed to detect any. Since the sequence conservation of telomere-associated proteins is generally poor (Linger and Price, 2009), we also compared protein domains using the Feature Architecture Comparison Tool (FACT; http://fact.cibiv.univie.ac.at; Koestler et al., 2010). However, this search also produced only hits with a distant similarity to known telomere-associated proteins.

DSBs are repaired even when bouquet formation is prevented

We have previously shown that bouquet formation is a response to DSBs, and we hypothesized that the parallel arrangement of chromosomes within the elongated nucleus facilitates recombinational repair of DSBs (Mochizuki et al., 2008). Therefore, we investigated whether the bouquet is indeed required for DSB repair. Again we suppressed bouquet formation by exposure to nocodazole. We then immunostained Dmc1p as a marker for DSBs (see Howard-Till et al., 2011). The number of Dmc1p foci in non-elongated nuclei (179±18 s.d., $n=10$) at t=3.5 h after meiotic induction was similar to the number of foci in elongated nuclei of the control (170±32 s.d., $n=14$) (Fig. 4A) (for the method of counting foci, see Howard-Till et al., 2011). This indicates that DSBs are efficiently induced when no bouquet is formed. We next assayed for the presence of Dmc1p foci 5.5 h after meiotic induction, and found that they were reduced both in the untreated control and in the nocodazole-treated culture (Fig. 4A). This is a strong indication that DSBs are repaired even when a bouquet does not form.

The loss of Dmc1 signal could, however, be due to the detachment of recombination proteins from unduly long persisting DSBs. Therefore, as an independent confirmation, the dynamics of DSB-formation and repair were monitored by pulsed-field gel electrophoresis (PFGE). We have previously found that in PFGE, intact chromosomes stay in the loading well, whereas chromosomes that are fragmented by meiotic DSBs enter the gel (Lukaszewicz et al., 2010). Here we found that, similar to untreated meiosis, bands diagnostic of DSBs disappear at late time points in nocodazole-treated meiosis (Fig. 4B). This is additional proof that DSBs are repaired in the absence of bouquet formation.

Recombination-related DNA synthesis takes place during the collapse of the bouquet

Since we recognized that bouquet formation is not required for the recombinational repair of DSBs and thus, that the repair of DSBs is not likely the signal for exiting the bouquet, we wondered when DSB repair takes place. Previous observations have shown that markers of DSBs, Dmc1p and g-H2A.X foci, are most abundant in maximally elongated nuclei and virtually gone when distinct bivalents emerge (Howard-Till et al., 2011). To pinpoint repair within this time window, we assayed for DNA synthesis that accompanies recombinational repair. DNA synthesis can be detected *in situ* by the incorporation and (immuno)labelling of base analogues (cf. Wimber and Prensky, 1963; Terasawa et al., 2007; and references therein). In *Tetrahymena*, meiotic DNA synthesis was observed by Allis et al. (Allis et al., 1987), but these authors were vague about the stage when it takes place.

To detect recombination-related DNA synthesis, we exposed cells to bromodeoxyuridine (BrdU) 2–2.5 h after the initiation of meiosis (at a time when nuclei just start to elongate) and fixed cells 4.5 h or 5 h after initiation. We observed heavily labelled nuclei in a minority of unconjugated or early conjugating cells, which undergo delayed S-phase (see Allis et al., 1987). In cells at later stages of conjugation, we observed BrdU foci in shortening nuclei (corresponding to Stage V in Fig. 1) and subsequent stages, but extremely rarely in maximally elongated nuclei (Fig. 4C). *spo11*Δ strains served as a negative control and, as expected, meiotic stages were not labelled in the absence of DSBs (Fig. 4C). This experiment demonstrates that DSBs are repaired only after nuclei have started shortening, therefore completed repair can not be the signal to exit the bouquet stage.

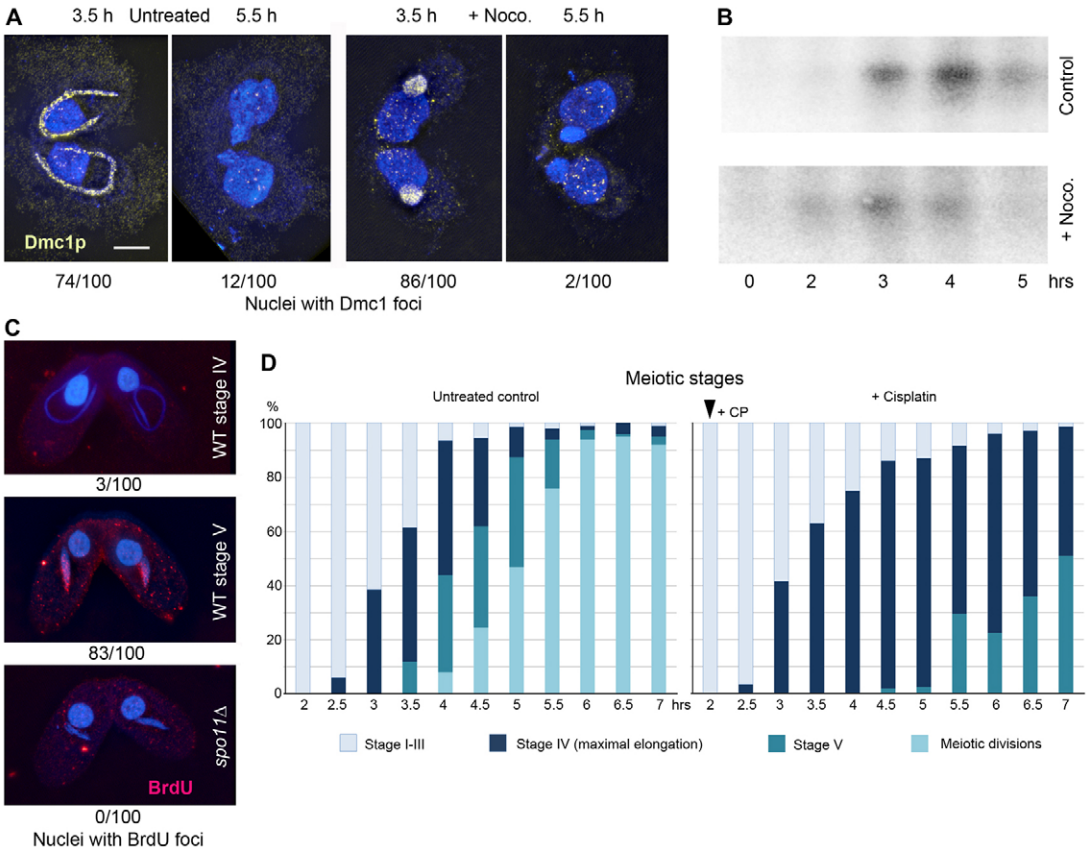

Fig. 4. DSB repair is independent of nuclear elongation and takes place after the fully elongated stage. (**A**) DSB-dependent foci of the recombination protein Dmc1 (yellow) similarly appear and disappear in the nuclei of untreated control cells and in nocodazole-treated (+ Noco.) cells where nuclei do not elongate. Stray foci in the macronuclei are due to the presence of the related Rad51p, which is recognized by the same antibody (see Howard-Till et al., 2011). Cells are prepared by a spreading method that only preserves chromatin-associated nuclear protein (see the Materials and Methods section). (**B**) PFGE shows the timely appearance and disappearance of DSB-generated chromosome fragments in non-elongating meiotic nuclei. DNAs from untreated and nocodazole-treated cells sampled during different times in meiosis are compared. Under short-run conditions, DSB-generated fragments migrate as a distinct band rather than a smear (Lukaszewicz et al., 2010). The generally weaker appearance of DSB-dependent bands in the nocodazole-treated samples is due to the fact that a high percentage of cells abort conjugation and meiosis in the presence of the drug (data not shown). (**C**) Incorporation of BrdU (red) indicates recombination-related DNA synthesis. DNA synthesis does not take place in maximally elongated nuclei (top; compare with stage IV in Fig. 1) but only when nuclei shorten again (middle; compare with stage V in Fig. 1). In *spo11*Δ meiosis, no BrdU is incorporated during the corresponding stage (bottom). Scale bar: 10 μm (A and C at same scale). (**D**) Continued DSB induction [by exposure to cisplatin (+CP)] prevents exit from the bouquet. Nuclei were scored as being at the maximally elongated bouquet stage if they were longer than the cell (compare with Fig. 1E).

Exit from the bouquet is prevented by ongoing DSB production

The above experiments demonstrate that nuclei exit the bouquet stage before DSB repair is completed. Thus, while DSBs trigger bouquet formation, the bouquet is not upheld in the presence of unrepaired DSBs. To obtain more insight into how the bouquet is regulated by DSBs, we created a situation where DSBs are continuously produced. For this, we treated wild-type meiotic cells with cisplatin, which induces DSBs (Mochizuki et al., 2008; and references therein). When we administered cisplatin 120 min after initiation of meiosis, i.e. at a time when DSB begin to form naturally, nuclei elongated as usual. However, in the continued presence of cisplatin, and hence ongoing DSB production, they were unable to exit the elongated bouquet stage (Fig. 4D). This suggests that some initial DSB processing rather than completed DSB repair is sufficient for exit from the bouquet.

Discussion

Centromere function is required for the stretched bouquet arrangement of chromosomes

It has been suggested previously that the parallel arrangement of chromosome arms within the enormously elongated meiotic prophase nucleus of *Tetrahymena* promotes the juxtapositioning of homologous regions (Kaczanowski et al., 1985; Loidl and Scherthan, 2004; Loidl and Mochizuki, 2009). Here we confirm that centromeres and telomeres cluster at opposite ends of the nucleus, causing the chromosome arms to adopt a stretched bouquet arrangement. This ultimate bouquet arrangement is abolished if Cna1, a centromeric histone H3, is depleted. This demonstrates that the kinetochore, rather than a centromere-associated chromosomal region, is required for centromere clustering.

In principle, centromeres could have an active role in nuclear elongation by capturing and moving along elongating

microtubules, thereby stretching chromatin and the nucleus. However, in the absence of Cna1p, chromatin-free parts of nuclei, probably comprising the nuclear envelope, intranuclear microtubules, and perhaps other elements of the nuclear skeleton, were still able to elongate. Therefore, centromeres do not drive nuclear elongation but are passively moved with the elongating nucleus. The attachment of centromeres to the nuclear periphery is probably mediated by microtubules, because treatment with the microtubule inhibitor nocodazole disrupts centromere clustering. The role of microtubules is, therefore, twofold. They stretch the nucleus in a centromere-independent manner, and they anchor centromeres to one end of the elongated nucleus.

Both centromeres and telomeres contribute to the stretched bouquet arrangement of chromosome arms, and telomere clustering also seems to be dependent on microtubules. We have failed to find homologues of telomere-associated proteins, which are known from a number of organisms to anchor telomeres to the nuclear periphery and, through the nuclear membrane, to components of the cytoskeleton (Morimoto et al., 2012; see Hiraoka and Dernburg, 2009; Ding et al., 2010). These connections allow telomere movements, which promote homology searching (see Koszul and Kleckner, 2009; Rao et al., 2011). In *Tetrahymena*, other factors may contribute to a simpler, direct interaction with intranuclear microtubules. Because the regulation of induction and exit is different for the canonical bouquet and *Tetrahymena*'s bouquet version (Loidl and Mochizuki, 2009), and they probably depend on different anchor proteins, it is currently difficult to determine if they are homologous devices.

DSB repair is independent of bouquet formation and is completed after maximal nuclear elongation

We have shown previously that bouquet formation is an ATR-dependent meiotic DNA damage response in *Tetrahymena* (Loidl and Mochizuki, 2009). Therefore, it would be plausible to assume that DSB repair is facilitated by the elongated state, and only successful DSB repair allows the release from this state. However, this is not the case. Mutants unable to repair DSBs, such as *rad51*(RNAi), *mre11*Δ and *sae2*/*com1*Δ exit the elongated state and arrest at metaphase with fragmented chromosomes and other signs of persisting DSBs (Lukaszewicz et al., 2010; Howard-Till et al., 2011). Moreover, here we show that DSB repair synthesis takes place only after nuclei have exited the bouquet. On the other hand, persistent artificial generation of DSBs delays or prevents exit from the bouquet. Therefore, we propose that DSBs processed to some intermediate step in meiotic DSB repair no longer activate the DNA damage sensing machinery.

In the absence of the bouquet, and hence close homologous alignment, repair probably occurs not via homologous recombination, but by some alternative mechanism such as nonhomologous end joining or intersister recombination. Efficient repair via the sister chromatid is indeed likely, as exemplified by a *dmc1*Δ mutant, where DSB repair was found to result in metaphase univalents (Howard-Till et al., 2011). Intersister repair would even be the preferred DSB repair pathway, if special measures to promote interhomolog repair were not in place (Howard-Till et al., 2011, and references therein). Therefore, we propose that the stretched bouquet is important to ensure that a sufficient proportion of repair events are channelled towards interhomolog recombination, thus enforcing crossover events, which are essential for proper homologue segregation.

Diverse meiotic pairing roles of centromeres

Centromeres have been invoked in different events at meiotic prophase in a variety of organisms (see Stewart and Dawson, 2008). Commonly, centromeres are clustered at the onset of meiosis. In some cases, this may be a mere consequence of the poleward centromere orientation during the preceding telophase ('Rabl orientation'). However, often a centromere cluster is established *de novo* early in meiotic prophase, which suggests a dedicated mechanism. In the budding yeast, an initial centromere cluster dissociates to several smaller clusters or pairs, which seem to consist of nonhomologous centromeres (Tsubouchi and Roeder, 2005; Stewart and Dawson, 2008). Recently, a succession of centromere clustering and nonhomologous pairwise centromere coupling was also documented in *Arabidopsis* (Da Ines et al., 2012). The function of nonhomologous centromere coupling is unclear; it may reflect a transient state in the course of pairwise homology testing events.

Centromere clustering is followed, either directly, or by detour through nonhomologous centromere coupling, by homologous centromere pairing (Tsubouchi and Roeder, 2005; Stewart and Dawson, 2008; Da Ines et al., 2012). In contrast to centromere coupling, this is dependent on DSB formation (Obeso and Dawson, 2010; Falk et al., 2010). In most cases, the assortment of homologous centromeres seems to be driven by the homologous pairing of adjacent arm regions rather than by the recognition of the centromeres themselves (see Stewart and Dawson, 2008). Possible exceptions may be autonomous centromere pairing in *Drosophila* male meiosis (Tsai and McKee, 2011) and premeiotic centromere pairing in wheat (Moore and Shaw, 2009). In any case, clustering promotes the homologous pairing of centromeres and/or adjacent regions, and therefore, they are often among the earliest regions to synapse. In budding yeast, centromeres were consistently reported to be preferred synapsis initiation sites (Tsubouchi et al., 2008). An analogous situation exists in *Drosophila* oocytes, where centromere clusters form early in prophase and are synapsis initiation sites (Takeo et al., 2011; Tanneti et al., 2011). Often, pairing not only begins at the centromere, but is also most persistent in this region (Qiao et al., 2012; Bisig et al., 2012; for older reports see Stewart and Dawson, 2008). Enduring centromere pairing may support proper disjunction after the dissolution of the synaptonemal complex and/or serve as a backup connection in achiasmatic bivalents (Stewart and Dawson, 2008).

Here we add another facet to the involvement of centromeres in meiosis. In *Tetrahymena*, centromeres are actively involved in the homology searching and pairing process by creating a stretched bouquet arrangement. Whereas the canonical telomere bouquet contributes little to pairing (Harper et al., 2004; Koszul and Kleckner, 2009; Lee et al., 2012; Penfold et al., 2012), the stretched bouquet of *Tetrahymena* is indispensable for pairing. It is possible that this highly ordered chromosome arrangement has evolved to compensate for the lack of a synaptonemal complex in this organism.

Materials and Methods

Strains, cell growth and induction of meiosis

Tetrahymena thermophila strains B2086 and Cu428 served as wild types and as the source material for the construction of the *CNA1*hp strain. The *spo11* knockout (*spo11*Δ) line was described previously (Mochizuki et al., 2008). Cells were grown at 30°C to a density of ~2×10^5 cells/ml according to standard methods (see Orias

et al., 2000), and they were made competent for conjugation by starvation in 10 mM Tris–Cl (pH 7.4) for at least 16 h. Conjugation and meiosis were induced by mixing starved cultures of different mating types.

For preventing nuclear elongation, nocodazole was applied at a concentration of 10 µg/ml, 110 min after induction of meiosis. The DNA damaging agent cisplatin was prepared as a 2 mg/ml stock solution in starvation medium and administered at a final concentration of 100 µg/ml to conjugating cells.

RNAi knockdown of *CNA1*

To create a *cna1*RNAi construct, fragment bp 4 to bp 388 of the *CNA1* ORF was amplified from genomic DNA using PCR primers to add appropriate restriction sites for cloning back-to-back into the RNAi hairpin (hp) vector pD5H8-MTT-SerH3-HP (Howard-Till and Yao, 2006). This vector contains rDNA, which is amplified in the *Tetrahymena* genome by autonomous replication, ensuring high copy numbers of inserted transgenic DNA, a neomycin resistance gene, and the Cd^{2+}-inducible *MTT1* metallothionein promoter (see Howard-Till and Yao, 2006). The *CNA1* PCR fragments were used to replace the *SERH3* fragments in this vector, thereby creating JLTet3[CNA1-HP]. JLTet3 was introduced into mating cells by biolistic transformation at 10 hrs post-mixing to allow cells to process the rDNA vector. Transformants (*CNA1*hp) were selected initially in media containing 120 µg/ml paromomycin, and then were transferred to increasingly higher concentrations, up to 500 µg/ml, to increase the number of rDNA copies carrying the hairpin construct. Expression of dsRNA was induced by the addition of $CdCl_2$ to a final concentration of 0.1 µg/ml. For efficient depletion of Cna1p in meiotic cells, $CdCl_2$ was added to exponentially growing cells for ca. 30 h. (To minimize loss of CNA1hp-carrying rDNA copies due to random assortment during mitoses, cells were grown in the presence of 120 µg/ml paromomycin.) These cells were then starved in the absence of $CdCl_2$ and mated to wild type. Presumably because of the leakiness of the *MTT1* promoter, the *CNA1*hp strain was unstable and could not be maintained for longer than one month at room temperature. It had to be repeatedly re-created by transformation with pJLTet3.

Pulsed-field gel electrophoresis (PFGE)

Chromosome-sized DNA was prepared in agarose plugs as described previously (Lukaszewicz et al., 2010). DNA was separated by PFGE under conditions where intact chromosomes do not enter the gel, whereas DSB-dependent chromosome fragments of different sizes appear as a distinct band. Chromosomes of the generative nucleus were distinguished from vegetative nucleus-borne minichromosomes (which are scattered all along the gel) by Southern hybridization with a probe specific to the generative nucleus (Lukaszewicz et al., 2010).

Fixation, cytological preparation and staining

Depending on the further use of slides for different immunostainings or FISH, cells were fixed and prepared according to one of the following methods. (A) 5 ml aliquots of (conjugating) cell suspension were transferred to a centrifuge tube, and 250 µl of 10% Triton X-100 and 500 µl of 37% formaldehyde were added. After 30 min, the fixed cell suspension was centrifuged for 2 min at 1,000 ***g***, and the pellet was resuspended in 500 µl of 4% paraformaldehyde + 3.4% sucrose solution. A drop of this mixture was spread onto a slide and air-dried. (B) 5 ml aliquots of (conjugating) cell suspension were transferred to a centrifuge tube and 500 µl of a mixture (450 µl 10% Triton+50 µl 37% formaldehyde) were added. After 25–30 min on ice, another 450 µl of 37% formaldehyde were added. After 5 min, the cell pellet was centrifuged, and the pellet was resuspended in 500 µl of 4% paraformaldehyde + 3.4% sucrose solution. A drop of this mixture was spread onto a slide and air-dried. (C) 5 ml aliquots of (conjugating) cell suspension were transferred to a centrifuge tube and 20 µl of partial Schaudinn's fixative (saturated $HgCl_2$, ethanol 2:1) were added. After 5 min, cells were washed two times with methanol, resuspended in 50 µl of methanol, and drops of this suspension were applied to a slide and air-dried. (D) 5 ml aliquots of (conjugating) cell suspension were transferred to a centrifuge tube and the cell pellet was collected by centrifugation. The pellet was resuspended in 1 ml of fixative (methanol, chloroform, acetic acid 6:3:2). After 1 h the cells were washed and resuspended in 1 ml of 70% ethanol, and drops of this suspension were applied to a slide and air-dried.

DAPI staining and immunostaining of α-tubulin (1:300, mouse monoclonal, Clone DM1A, Lab Vision, Fremont, CA) was performed on slides prepared by procedure (A). For immunostaining with an antibody against Dmc1p and Rad51p (1:50 mouse monoclonal, Clone 51RAD01, NeoMarkers, Fremont, CA), slides prepared by procedure (B) were used. Immunostaining of Cna1p with rabbit polyclonal antibodies (1:200; gift from Harmit Malik) worked on slides prepared by procedure (C). In all cases, slides were rinsed in 1×PBS (2×5 min) and 1×PBS + 0.05% Triton X-100 (5 min). Primary and fluorescence-labelled secondary antibodies were applied as described previously and the preparations were mounted under a coverslip in Vectashield anti-fading agent (Vector Laboratories Inc., Burlingame, CA,) supplemented with 0.5 mg/ml DAPI (Howard-Till et al., 2011).

For FISH, slides made by procedure (D) were pretreated with 1 M sodium thiocyanate and denatured in 70% formamide. DNA probes were denatured separately and hybridized to the slides. For testing pairing, a PCR-generated probe against a 22.1 kb intercalary chromosomal region was Cy3-labelled (for details see Loidl and Mochizuki, 2009). For detecting telomeres, biotinylated oligonucleotides of telomeric and subtelomeric repeats were produced, hybridized and detected with FITC-labelled avidin (see Loidl and Scherthan, 2004). For simultaneous Cna1 staining and telomere FISH, slides were prepared as for FISH, then immunostained, and subsequently subjected to the FISH procedure.

Slides were evaluated under a fluorescence microscope with the appropriate filters. For documentation, 3-D stacks of images were recorded, deconvolved and projected as previously described (e.g. Loidl and Mochizuki, 2009; Howard-Till et al., 2011).

Detection of recombination-related DNA synthesis

BrdU (from a 2×10^{-1} M stock in DMSO) was added to a final concentration of 2×10^{-4} M to conjugating cells 2 or 3 h after induction of meiosis. All manipulations with cultures exposed to BrdU were done under red darkroom illumination. Cells were harvested 4 h 15 min after induction of meiosis. Slides of BrdU-fed cells prepared by procedure (A) were washed with water (5 min) and incubated with 1 M sodium thiocyanate at 90℃ for 15 minutes. The slides were rinsed with 2×SSC and denatured in 70% formamide for 2 minutes at 65℃ to expose labelled nucleotides to the antibody (cf. Loidl and Scherthan, 2004). Denaturation treatment was stopped by transfer to ice-cold water, followed by 1×PBS (2×5 min) and 1×PBS + 0.05% Triton X-100 (5 min). Rat anti-BrdU antibody (1:40; Abcam, Cambridge, UK) was applied at 4℃ o/n followed by fluorescence-labelled secondary antibody on the next day.

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References

Allis, C. D., Colavito-Shepanski, M. and Gorovsky, M. A. (1987). Scheduled and unscheduled DNA synthesis during development in conjugating Tetrahymena. *Dev. Biol.* **124**, 469-480.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

Armstrong, S. J., Franklin, F. C. H. and Jones, G. H. (2001). Nucleolus-associated telomere clustering and pairing precede meiotic chromosome synapsis in Arabidopsis thaliana. *J. Cell Sci.* **114**, 4207-4217.

Bisig, C. G., Guiraldelli, M. F., Kouznetsova, A., Scherthan, H., Höög, C., Dawson, D. S. and Pezza, R. J. (2012). Synaptonemal complex components persist at centromeres and are required for homologous centromere pairing in mouse spermatocytes. *PLoS Genet.* **8**, e1002701.

Bupp, J. M., Martin, A. E., Stensrud, E. S. and Jaspersen, S. L. (2007). Telomere anchoring at the nuclear periphery requires the budding yeast Sad1-UNC-84 domain protein Mps3. *J. Cell Biol.* **179**, 845-854.

Cervantes, M. D., Xi, X., Vermaak, D., Yao, M. C. and Malik, H. S. (2006). The CNA1 histone of the ciliate Tetrahymena thermophila is essential for chromosome segregation in the germline micronucleus. *Mol. Biol. Cell* **17**, 485-497.

Collins, K. and Gorovsky, M. A. (2005). Tetrahymena thermophila. *Curr. Biol.* **15**, R317-R318.

Conrad, M. N., Lee, C.-Y., Chao, G., Shinohara, M., Kosaka, H., Shinohara, A., Conchello, J.-A. and Dresser, M. E. (2008). Rapid telomere movement in meiotic prophase is promoted by NDJ1, MPS3, and CSM4 and is modulated by recombination. *Cell* **133**, 1175-1187.

Cui, B. W. and Gorovsky, M. A. (2006). Centromeric histone H3 is essential for vegetative cell division and for DNA elimination during conjugation in Tetrahymena thermophila. *Mol. Cell. Biol.* **26**, 4499-4510.

Da Ines, O., Abe, K., Goubely, C., Gallego, M. E. and White, C. I. (2012). Differing requirements for RAD51 and DMC1 in meiotic pairing of centromeres and chromosome arms in Arabidopsis thaliana. *PLoS Genet.* **8**, e1002636.

Davidson, L. and LaFountain, J. R., Jr (1975). Mitosis and early meiosis in Tetrahymena pyriformis and the evolution of mitosis in the phylum Ciliophora. *Biosystems* **7**, 326-336.
Davis, L. and Smith, G. R. (2006). The meiotic bouquet promotes homolog interactions and restricts ectopic recombination in Schizosaccharomyces pombe. *Genetics* **174**, 167-177.
Ding, D. Q., Haraguchi, T. and Hiraoka, Y. (2010). From meiosis to postmeiotic events: alignment and recognition of homologous chromosomes in meiosis. *FEBS J.* **277**, 565-570.
Falk, J. E., Chan, A. C. H., Hoffmann, E. and Hochwagen, A. (2010). A Mec1- and PP4-dependent checkpoint couples centromere pairing to meiotic recombination. *Dev. Cell* **19**, 599-611.
Gaertig, J. and Fleury, A. (1992). Spatio-temporal reorganization of intracytoplasmic microtubules is associated with nuclear selection and differentiation during the developmental process in the ciliate Tetrahymena thermophila. *Protoplasma* **167**, 74-87.
Harper, L., Golubovskaya, I. and Cande, W. Z. (2004). A bouquet of chromosomes. *J. Cell Sci.* **117**, 4025-4032.
Hiraoka, Y. and Dernburg, A. F. (2009). The SUN rises on meiotic chromosome dynamics. *Dev. Cell* **17**, 598-605.
Howard-Till, R. A. and Yao, M. C. (2006). Induction of gene silencing by hairpin RNA expression in Tetrahymena thermophila reveals a second small RNA pathway. *Mol. Cell. Biol.* **26**, 8731-8742.
Howard-Till, R. A., Lukaszewicz, A. and Loidl, J. (2011). The recombinases Rad51 and Dmc1 play distinct roles in DNA break repair and recombination partner choice in the meiosis of Tetrahymena. *PLoS Genet.* **7**, e1001359.
Kaczanowski, A., Gaertig, J. and Kubiak, J. (1985). Effect of the antitubulin drug nocodazole on meiosis and postmeiotic development in Tetrahymena thermophila. Induction of achiasmatic meiosis. *Exp. Cell Res.* **158**, 244-256.
Karrer, K. M. (2000). Tetrahymena genetics: two nuclei are better than one. In *Tetrahymena thermophila* (ed. D. J. Asai and J. D. Forney), pp. 127-186. San Diego, CA: Academic Press.
Koestler, T., von Haeseler, A. and Ebersberger, I. (2010). FACT: functional annotation transfer between proteins with similar feature architectures. *BMC Bioinformatics* **11**, 417.
Koszul, R. and Kleckner, N. (2009). Dynamic chromosome movements during meiosis: a way to eliminate unwanted connections? *Trends Cell Biol.* **19**, 716-724.
Lee, C.-Y., Conrad, M. N. and Dresser, M. E. (2012). Meiotic chromosome pairing is promoted by telomere-led chromosome movements independent of bouquet formation. *PLoS Genet.* **8**, e1002730.
Linger, B. R. and Price, C. M. (2009). Conservation of telomere protein complexes: shuffling through evolution. *Crit. Rev. Biochem. Mol. Biol.* **44**, 434-446.
Loidl, J. and Mochizuki, K. (2009). Tetrahymena meiotic nuclear reorganization is induced by a checkpoint kinase-dependent response to DNA damage. *Mol. Biol. Cell* **20**, 2428-2437.
Loidl, J. and Scherthan, H. (2004). Organization and pairing of meiotic chromosomes in the ciliate Tetrahymena thermophila. *J. Cell Sci.* **117**, 5791-5801.
Lukaszewicz, A., Howard-Till, R. A., Novatchkova, M., Mochizuki, K. and Loidl, J. (2010). MRE11 and COM1/SAE2 are required for double-strand break repair and efficient chromosome pairing during meiosis of the protist Tetrahymena. *Chromosoma* **119**, 505-518.
McDonald, B. B. (1966). The exchange of RNA and protein during conjugation in Tetrahymena. *J. Protozool.* **13**, 277-285.
Mochizuki, K., Novatchkova, M. and Loidl, J. (2008). DNA double-strand breaks, but not crossovers, are required for the reorganization of meiotic nuclei in Tetrahymena. *J. Cell Sci.* **121**, 2148-2158.
Moore, G. and Shaw, P. (2009). Improving the chances of finding the right partner. *Curr. Opin. Genet. Dev.* **19**, 99-104.
Morimoto, A., Shibuya, H., Zhu, X., Kim, J., Ishiguro, K., Han, M. and Watanabe, Y. (2012). A conserved KASH domain protein associates with telomeres, SUN1, and dynactin during mammalian meiosis. *J. Cell Biol.* **198**, 165-172.
Obeso, D. and Dawson, D. S. (2010). Temporal characterization of homology-independent centromere coupling in meiotic prophase. *PLoS ONE* **5**, e10336.
Orias, E., Hamilton, E. P. and Orias, J. D. (2000). Tetrahymena as a laboratory organism: useful strains, cell culture, and cell line maintenance. In *Tetrahymena thermophila* (ed. D. J. Asai and J. D. Forney), pp. 189-211. San Diego, CA: Academic Press.
Penfold, C. A., Brown, P. E., Lawrence, N. D. and Goldman, A. S. H. (2012). Modeling meiotic chromosomes indicates a size dependent contribution of telomere clustering and chromosome rigidity to homologue juxtaposition. *PLOS Comput. Biol.* **8**, e1002496.
Phillips, C. M., Meng, X., Zhang, L., Chretien, J. H., Urnov, F. D. and Dernburg, A. F. (2009). Identification of chromosome sequence motifs that mediate meiotic pairing and synapsis in C. elegans. *Nat. Cell Biol.* **11**, 934-942.
Qiao, H. Y., Chen, J. K., Reynolds, A., Höög, C., Paddy, M. and Hunter, N. (2012). Interplay between synaptonemal complex, homologous recombination, and centromeres during mammalian meiosis. *PLoS Genet.* **8**, e1002790.
Rao, H. B. D. P., Shinohara, M. and Shinohara, A. (2011). Mps3 SUN domain is important for chromosome motion and juxtaposition of homologous chromosomes during meiosis. *Genes Cells* **16**, 1081-1096.
Ray, C., Jr (1956). Meiosis and nuclear behaviour in Tetrahymena pyriformis. *J. Protozool.* **3**, 88-96.
Santaguida, S. and Musacchio, A. (2009). The life and miracles of kinetochores. *EMBO J.* **28**, 2511-2531.
Scherthan, H. (2001). A bouquet makes ends meet. *Nat. Rev. Mol. Cell Biol.* **2**, 621-627.
Scherthan, H. (2007). Telomere attachment and clustering during meiosis. *Cell. Mol. Life Sci.* **64**, 117-124.
Schmitt, J., Benavente, R., Hodzic, D., Höög, C., Stewart, C. L. and Alsheimer, M. (2007). Transmembrane protein Sun2 is involved in tethering mammalian meiotic telomeres to the nuclear envelope. *Proc. Natl. Acad. Sci. USA* **104**, 7426-7431.
Stewart, M. N. and Dawson, D. S. (2008). Changing partners: moving from non-homologous to homologous centromere pairing in meiosis. *Trends Genet.* **24**, 564-573.
Subramanian, V. V. and Hochwagen, A. (2011). Centromere clustering: where synapsis begins. *Curr. Biol.* **21**, R920-R922.
Sugai, T. and Hiwatashi, K. (1974). Cytologic and autoradiographic studies of the micronucleus at meiotic prophase in Tetrahymena pyriformis. *J. Protozool.* **21**, 542-548.
Takeo, S., Lake, C. M., Morais-de-Sá, E., Sunkel, C. E. and Hawley, R. S. (2011). Synaptonemal complex-dependent centromeric clustering and the initiation of synapsis in Drosophila oocytes. *Curr. Biol.* **21**, 1845-1851.
Tanneti, N. S., Landy, K., Joyce, E. F. and McKim, K. S. (2011). A pathway for synapsis initiation during zygotene in Drosophila oocytes. *Curr. Biol.* **21**, 1852-1857.
Terasawa, M., Ogawa, H., Tsukamoto, Y., Shinohara, M., Shirahige, K., Kleckner, N. and Ogawa, T. (2007). Meiotic recombination-related DNA synthesis and its implications for cross-over and non-cross-over recombinant formation. *Proc. Natl. Acad. Sci. USA* **104**, 5965-5970.
Tsai, J.-H. and McKee, B. D. (2011). Homologous pairing and the role of pairing centers in meiosis. *J. Cell Sci.* **124**, 1955-1963.
Tsubouchi, T. and Roeder, G. S. (2005). A synaptonemal complex protein promotes homology-independent centromere coupling. *Science* **308**, 870-873.
Tsubouchi, T., Macqueen, A. J. and Roeder, G. S. (2008). Initiation of meiotic chromosome synapsis at centromeres in budding yeast. *Genes Dev.* **22**, 3217-3226.
Wimber, D. E. and Prensky, W. (1963). Autoradiography with meiotic chromosomes of the male newt (Triturus viridescens) using H^3-thymidine. *Genetics* **48**, 1731-1738.
Wolfe, J., Hunter, B. and Adair, W. S. (1976). A cytological study of micronuclear elongation during conjugation in Tetrahymena. *Chromosoma* **55**, 289-308.
Yamamoto, A. and Hiraoka, Y. (2001). How do meiotic chromosomes meet their homologous partners?: lessons from fission yeast. *Bioessays* **23**, 526-533.