



DISSERTATION

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„Studies of meiotic recombination in the ciliate
Tetrahymena thermophila“

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ABSTRACT (English)

Meiosis is a special type of cell division, essential for the sexual reproduction of eukaryotic species. It is characterized by a distinct set of cellular and molecular events that lead to the formation of genetically variant haploid gametes. A critical step of meiosis is the process of reciprocal DNA exchange between parental chromosomes as a source of genetic diversity. It starts from the induction of programmed DNA double-strand breaks (DSBs) whose repair via homologous recombination leads to DNA exchange and crossover (CO) formation. All the repair steps have to be accurately fulfilled in order to ensure proper chromosome segregation. If not accomplished, it can cause sterility, stillbirth or chromosome abnormalities.

Meiosis as an evolutionarily and biologically fundamental process has been studied in several model organisms, in particular *S. cerevisiae*, *S. pombe*, *C. elegans*, *Drosophila*, *Arabidopsis* and mouse. In the work presented here, I studied meiotic recombination in the phylogenetically distant protist *Tetrahymena thermophila*, which does not feature a synaptonemal complex (SC), the specialized meiotic pairing structure. I investigated the function and evolutionary conservation of recombination proteins such as Mre11, Sae2/Com1, Sgs1 and Mus81 in the context of this ciliate's unusual meiosis.

Mre11 and Sae2/Com1 proteins are crucial for the first step of meiotic recombination, which is the preparation of broken DNA ends for repair. In *S. cerevisiae* and *C. elegans*, Mre11 has an additional function in DSB generation. In *Tetrahymena*, Mre11 or Sae2/Com1 deletion causes sterility. Taking advantage of the ability of *Tetrahymena* to undergo synchronous meiosis in mass cultures, I adapted a pulsed-field gel electrophoresis (PFGE)-based method for DSB detection. Combined biochemical and cytological analysis showed that the lack of viable progeny is accounted for by a defect in DSB processing and repair in the absence of Mre11 or Sae2/Com1, but not a failure in the induction of DSBs in Mre11 depleted cells. The dispensability of Mre11 for meiotic break formation puts *Tetrahymena* together with *S. pombe* and plants to the group of eukaryotes that share the feature of Mre11-independent DSB formation. On the other hand, the obtained results suggest that the required cooperativity of Mre11 and Sae2/Com1 in DSB processing, described in *S. cerevisiae* and *S. pombe* meiosis, might be not the case in *Tetrahymena*. *Tetrahymena* may share this feature with *C. elegans*, which will be addressed in the discussion in this PhD thesis.

Sgs1 and Mus81 are involved in later steps of DSB recombinational repair. The Mus81 nuclease resolves recombination intermediates and it is implicated in meiotic CO formation. However, since there exist other resolving factors, such as the Mlh1-Mlh3 complex, the contribution of Mus81 resolvase to CO generation differs among investigated organisms. In *S. cerevisiae* and *C. elegans*, Mus81 generates a subset of COs, in mouse and *Arabidopsis*, only a minor CO fraction depends on

Mus81, whereas CO formation in *S. pombe*, predominantly, if not exclusively, depends on Mus81. The Sgs1 helicase on the other hand, possesses a well-characterized anti-CO activity, which leads to the disassembly of recombination intermediates and noncrossover (NCO) products formation.

In this PhD study, I investigated the depletion phenotypes of Mus81 and Sgs1 in *Tetrahymena* meiosis. In situ observations revealed that in the absence of Mus81 or Sgs1, chromosomes fail to separate during meiotic divisions. The PFGE analysis of these mutants showed the accumulation of unresolved recombination intermediates. Thus, both Mus81 and Sgs1 are required for the processing of recombination intermediates in *Tetrahymena*. Moreover, I excluded a possible participation of the Mlh1-Mlh3 complex as a resolving factor. Therefore, *Tetrahymena* relies on the predominant (if not only) use of the Mus81 nuclease similar to *S. pombe*, with whom it also shares the lack of an SC. Therefore, these two unrelated eukaryotes, which both lost their SC during evolution, evolved similar ways of executing meiotic recombination. On the other hand, *Tetrahymena* is the first organism where Sgs1 is dispensable for meiosis, and its probable engagement in NCO formation is presented in a model of the meiotic recombination pathway in *Tetrahymena*.

KURZFASSUNG (Deutsch)

Die Meiose, eine spezielle Zellteilung, reduziert den diploiden Chromosomensatz somatischer Zellen und ermöglicht dadurch die sexuelle Reproduktion von Eukaryoten. Spezifische zelluläre und molekulare Vorgänge, die charakteristisch für die meiotische Teilung sind, führen zu genetisch unterschiedlichen, haploiden Gameten. Homologe Rekombination, ein reziproker DNA-Austausch zwischen elterlichen Chromosomen, ist ein entscheidender Prozess während der Meiose und eine Quelle für genetische Diversität. Sie beginnt mit der Entstehung von DNA-Doppelstrangbrüchen (DSBs) und der Reparatur dieser Brüche am homologen Chromosom. Dies führt zum Austausch von DNA und zur Bildung von Crossovers (COs). Für eine fehlerfreie Chromosomenaufteilung muss die Reparatur aller DSBs korrekt erfolgen. Fehler in diesem Prozess können die Ursache von Sterilität, Totgeburten oder chromosomal aberranten Nachkommen sein.

Die Meiose ist ein biologisch und evolutionär grundlegender Prozess und wurde in zahlreichen Modellorganismen, im speziellen *S. cerevisiae*, *S. pombe*, *C. elegans*, *Drosophila*, *Arabidopsis* und der Maus studiert. Im Zuge dieser Arbeit untersuchte ich die meiotische Rekombination im phylogenetisch weit entfernten Protisten *Tetrahymena thermophila*, der während der Meiose keinen Synaptonemalen Komplex (SC), eine Meiose-spezifische Paarungsstruktur, aufbaut. Ich studierte die Funktion und evolutionäre Konservierung der Rekombinationsproteine Mre11, Sae2/Com1, Sgs1 und Mus81 im Kontext der ungewöhnlichen Meiose dieses Ciliaten.

Mre11 und Sae2/Com1 sind für die erste Stufe der meiotischen Rekombination, der Aufbereitung gebrochener DNA-Enden für die Reparatur, verantwortlich. In *S. cerevisiae* und *C. elegans* hat Mre11 eine zusätzliche Funktion in der Erzeugung meiotischer DSBs. Deletion von Mre11 oder Sae2/Com1 führt zu Sterilität in *Tetrahymena*. Ich adaptierte eine Puls-Feld-Gelelektrophorese (PFGE) basierende Methode zur Detektion von DSBs in synchron induzierten Meiosen von *Tetrahymena* Massenkulturen. Kombiniert mit zytologischen Analysen zeigte dieser biochemische Ansatz, dass das Fehlen von lebensfähigen Nachkommen in der Abwesenheit von Mre11 oder Sae2/Com1 durch einen Defekt in der DSB-Verarbeitung und Reparatur, nicht jedoch in der Entstehung von DSBs verursacht wird. Damit gehört *Tetrahymena*, gemeinsam mit *S. pombe* und Pflanzen, in eine Gruppe von Eukaryonten, die Mre11-unabhängige meiotische DSBs bildet. Weiters lassen meine Untersuchungen den Schluss zu, dass, anders als für *S. cerevisiae* und *S. pombe* Meiose beschrieben, Mre11 und Sae2/Com1 unabhängig voneinander funktionieren. Die Ähnlichkeit zwischen *Tetrahymena* und *C. elegans* in dieser Hinsicht wird in der Diskussion dieser Dissertation angesprochen.

Sgs1 und Mus81 sind in späteren Schritten der rekombinatorischen DSB-Reparatur beteiligt. Die Nuklease Mus81 löst Zwischenprodukte der Rekombination auf und ist dadurch an der meiotischen

CO-Bildung beteiligt. Der Beitrag von Mus81 bei der Bildung von COs ist in den verschiedenen Modellorganismen unterschiedlich, da weitere Faktoren, wie z.B. der Mlh1-Mlh3 Komplex, die Rekombinationsintermediate auflösen können. Mus81 erzeugt einen Teil der COs in *S. cerevisiae* und *C. elegans*. In der Maus hängt nur ein geringer Anteil der COs von Mus81 ab, während in *S. pombe* COs überwiegend, wenn nicht ausschließlich, von Mus81 erzeugt werden. Die Sgs1 Helicase hingegen besitzt eine gut charakterisierte Anti-CO-Aktivität, die zur Auflösung von Rekombinationszwischenprodukten und dabei zur Bildung von Noncrossover-Produkten (NCO) führt. In der hier vorgelegten Dissertation untersuchte ich die Auswirkung des Fehlens von Mus81 und Sgs1 auf die Meiose von *Tetrahymena*. *In situ* Beobachtungen zeigten, dass Chromosomen während der Meiose nicht aufgeteilt werden können, wenn Mus81 oder Sgs1 inaktiviert sind. Weiteres belegte die Analyse der Meiosen dieser Mutanten mittels PFGE die Akkumulation von unaufgelösten Rekombinationszwischenprodukten. Diese Beobachtungen erlauben den Schluss, dass in *Tetrahymena* beide Proteine, Mus81 und Sgs1, benötigt werden, um Rekombinationsintermediate zu prozessieren. Weiteres konnte ich einen möglichen Beitrag des Mlh1-Mlh3 Komplexes zur Auflösung von meiotischen Rekombinationszwischenprodukten ausschließen und dadurch zeigen, dass *Tetrahymena*, ähnlich wie *S. pombe* hauptsächlich (wenn nicht ausschließlich) die Nuklease Mus81 zur Erzeugung von COs verwendet. Folglich entwickelten diese beide entfernt verwandten Organismen, welche jeweils den SC verloren, ähnliche Methoden der meiotischen Rekombination. Allerdings ist, anders als bei den üblichen Modellsystemen, Sgs1 für die Meiose von *Tetrahymena* essenziell. Schliesslich wird der wahrscheinliche Beitrag dieses Proteins zur Bildung von NCO in einem Modell des Rekombinationsmechanismus in *Tetrahymena* diskutiert.

INTRODUCTION

1. Meiosis

Sexually reproducing eukaryotes undergo meiosis, a specialized type of cell division, generating haploid gametes from a diploid cell. Meiosis consists of two consecutive divisions which are not separated by DNA replication. This leads to a reduction of the parental genome by half, thus restoring the diploid number of chromosomes after the fusion of two gametes. Importantly, since the fused haploid cells originate from two different individuals, the offspring represent a unique genetic mixture. Another source of genetic diversity is the process of meiotic recombination, which produces a reciprocal exchange of the genetic information between paternal and maternal chromosomes preceding their separation. This DNA shuffling makes every gamete an inimitable blend of the parental genome. Therefore, the process of meiosis is a key force driving evolutionary success of sexually reproducing organisms.

1.1. Meiotic events

In order to produce viable gametes, chromosomes have to be transmitted in a faithful manner during two successive meiotic divisions, meiosis I and meiosis II. Similarly to mitosis, meiosis I and II are each divided into prophase, metaphase, anaphase and telophase. However, in contrast to the mitotic prophase, the meiotic prophase I is prolonged and comprises five cytologically discernible stages, which have no equivalent in the mitotic division. These are leptotene, zygotene, pachytene, diplotene and diakinesis. In the course of these stages, highly complex meiotic processes take place, and their accomplishment is essential for proper chromosome segregation.

Cells entering meiosis are diploid, therefore each chromosome has its paternal and maternal equivalent. These parental pairs are known as homologous chromosomes (homologs) (see Fig. 1). Since prophase I is preceded by pre-meiotic DNA replication, each chromosome consists of two sister chromatids connected by cohesins (van Heemst and Heyting, 2000). In order to fulfill meiotic functions, homologous chromosomes have to pair. The process of homologous pairing takes place during the leptotene/zygotene transition (for review see Gerton and Hawley, 2005; Roeder, 1997). In leptotene, chromosomes start to condense and thin threads of DNA become visible. Throughout the zygotene phase, the degree of homolog interactions increases. This leads to a close apposition of homologous chromosomes along their entire length, which is defined as chromosome alignment. In most organisms, the pairing process is accompanied by the formation of a synaptonemal complex (SC), a meiosis-specific proteinaceous structure built between two homologs (Moses, 1958; for review see Page and Hawley, 2004). This emerging intimate homolog association is known as

chromosome synapsis. Characteristically, in pachytene, chromosome pairs are fully synapsed. In parallel with synapsis, homologous recombination takes place (for review see Kleckner, 2006) (see Fig. 1). Although the exchange of DNA fragments might occur between any of the four chromatids within a synapsed chromosome pair, only nonsister recombination results in crossover (CO) formation (for review see Lao and Hunter, 2010). Since the CO is flanked by cohesed sister chromatids, it constitutes a physical linkage between the homologs. This connection, manifested cytologically as chiasmata, is crucial for accurate homolog separation at anaphase I (see below). Thus, CO formation not only gives rise to recombinant chromosomes, but also itself guarantees their faithful segregation in meiosis. Following CO establishment, cells exit the pachytene stage, the SC is disassembled and homologous chromosomes stay connected only at the CO sites. Throughout diplotene and diakinesis, chromosomes condense to form distinct bivalents in metaphase I (see Zickler and Kleckner, 1999). Attributable to the presence of chiasmata, the correct bipolar orientation of bivalents is achieved and homologs segregate to the opposite poles in anaphase I. Importantly, during the first meiotic division, cohesins are removed from the chromosome arms, but are maintained between centromeres (see Fig. 1). The centromere cohesion protection prevents sister separation in meiosis I and is important for their proper segregation in meiosis II (for review see Watanabe, 2004). During the second meiotic division, which is similar to mitosis, sister chromatids separate, and eventually, four haploid daughter cells are formed.

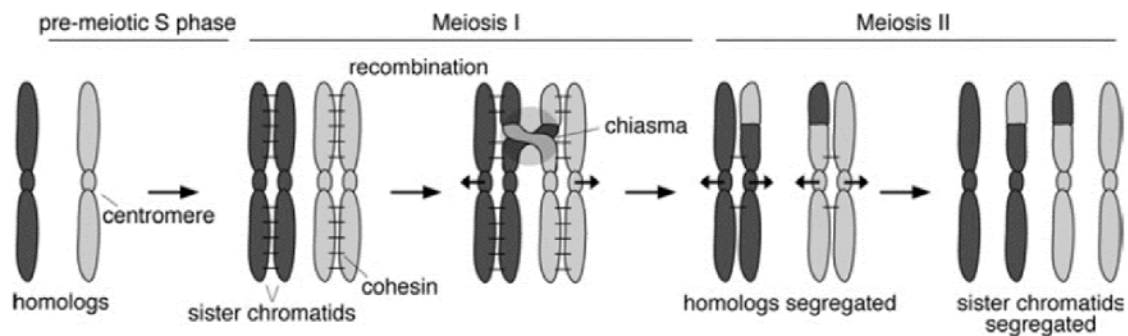


Fig. 1 Chromosome segregation in meiosis (Whitby, 2005). See main text for details.

2. Meiotic recombination

Homologous recombination (HR) is an obligatory process by which COs are produced in meiosis. At the molecular level, it is initiated by the induction of programmed DNA double-strand breaks (DSBs) by the Spo11 endonuclease (see Fig. 2) (Keeney et al., 1997). DNA at the DSB site is resected in the 5' to 3' direction, revealing single-stranded 3' overhangs. The free 3' end is a substrate for recombinases, Rad51 and Dmc1, which promote strand exchange. Consequently, the Rad51/Dmc1

nucleoprotein filament invades a homologous DNA duplex (single-end invasion, SEI) to form a displacement loop (D-loop). Next, the invading end is extended by DNA synthesis using the homologous sequence as a template. In parallel, the second 3' end anneals to the D-loop (second-end capture) and is also extended by DNA synthesis (see Krogh and Symington, 2004). According to the canonical HR model, the subsequent ligation of nicked DNA strands generates a double Holliday junction (dHJ)-like intermediate (see Bell and Byers, 1983; Szostak et al., 1983). This four-way cross-stranded joint molecule (JM) is resolved by endonucleases. The released DNA molecules are resealed, which results in a formation of either a CO or a non-crossover (NCO), depending on the cleavage orientation of the two HJs (see Fig. 2 and for review see Whitby, 2005). Interestingly, CO-biased resolution of dHJs has been described in meiosis (Sourirajan and Lichten, 2008). This is in contrast to the mitotic recombinational repair of spontaneous DSBs, where the preferred outcome is NCO (for review see Andersen and Sekelsky, 2010).

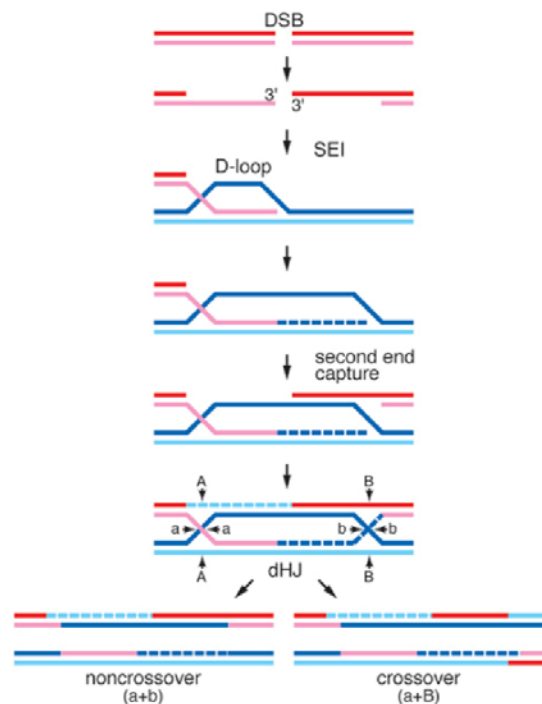


Fig. 2 The canonical DSB repair model by Szostak et al. 1983 (after Whitby, 2005).
See main text for details.

2.1. The function of the Mre11 complex in DSB formation

In addition to Spo11 catalyzing DSB formation, several other factors essential for meiotic break initiation have been identified in budding yeast. These are Ski8, Mre11, Rad50, Xrs2, Rec102, Rec104, Mer2, Rec114, and Mei4 (for review see Keeney, 2001). Of these, Mre11, Rad50 and Xrs2 belong to the *RAD52* epistasis group, which is central for DNA repair via HR (for review see Krogh and Symington, 2004). These three proteins form a highly conserved complex, Mre11-Rad50-Xrs2/Nbs1,

where Nbs1 is the yeast Xrs2 ortholog in other eukaryotes. The Mre11 complex performs multiple functions, both in mitosis and meiosis. It is involved in DNA damage sensing and response, regulation of DSB repair and HR promotion (for review see Borde, 2007; Stracker and Petrini, 2011; Williams et al., 2007). In the budding yeast meiosis, as mentioned above, the Mre11 complex is required for DSB formation, and deletion of any of the subunits blocks meiotic recombination. However, it is not yet known why the Mre11 complex is important for meiotic break formation. It was found that the Mre11 protein associates preferentially to the chromatin of DSB regions. This transient binding depends on the presence of all the other factors required for DSB formation, except of Rad50. In turn, Mre11 is necessary for Rad50 binding to chromatin. Moreover, the localization of Mre11 to chromatin occurs independently of DSB induction. Therefore, Nicolas and colleagues concluded that Mre11 is part of a larger pre-DSB forming complex (Borde et al., 2004).

Isolation of the *rad50S* and *mre11S* alleles (for Separation of function), characterized by specific point mutations, revealed that these proteins are also essential for meiotic recombination downstream of DSB formation (Alani et al., 1990; Nairz and Klein, 1997). In these mutants, DSBs are formed but remain unprocessed. A similar phenotype was observed in the *sae2/com1Δ* mutant. (McKee and Kleckner, 1997; Prinz et al., 1997). Further research showed that Sae2/Com1 and the Mre11 complex display a functional relationship in DSB end processing, both in meiosis and mitosis (for details see next chapter and for review see Krogh and Symington, 2004; Stracker and Petrini, 2011). Collectively, the Mre11 complex has two separate functions in the budding yeast meiosis. Interestingly, the requirement of the Mre11 complex for both meiotic break formation and repair was described besides *S. cerevisiae* only in *C. elegans* (Alpi et al., 2003; Chin and Villeneuve, 2001). In contrast, in *Arabidopsis* and *S. pombe*, DSB induction occurs independently of the Mre11 presence (Puizina et al., 2004; Young et al., 2004). However, its involvement in the 5' end processing is evolutionarily conserved (also see discussion, chapter 1; and for review see Borde, 2007).

2.2. The role of Mre11 and Sae2/Com1 in DSB processing

Spo11-induced DSBs must be appropriately processed in order to generate the 3' overhangs required for the single-end invasion step. This involves Spo11 removal from the break site and 5' strand resection (see Krogh and Symington, 2004). The nature of this activity was broadly studied in the budding and fission yeast meioses. It was found that Spo11, covalently bound to the 5' termini, is removed by an endonucleolytic cut of the DNA at some distance from the DSB site, producing short Spo11-oligonucleotide complexes (Milman et al., 2009; Neale et al., 2005; Rothenberg et al., 2009). This reaction requires Mre11 and Sae2/Com1 nucleases (Hartsuiker et al., 2009; Milman et al., 2009; Moreau et al., 1999; Rothenberg et al., 2009). The precise mechanism of the cleavage by the Mre11 endo/exonuclease has been revealed in budding yeast (see Fig. 3 and Garcia et al., 2011). First, the

Mre11 single-stranded (ss) DNA endonuclease activity is required to nick DNA at ~300 bp away from the Spo11-DSB site. Second, its 3'-5' exonuclease activity is used to resect nucleotides towards the DSB end. How the Spo11-oligonucleotide molecules escape full nucleolytic degradation remains elusive. However, it was suggested that they might be protected by retention of the whole complex of proteins that are initially implicated in DSB formation (Garcia et al., 2011). Nevertheless, Mre11 has no 5' to 3' nuclease activity, which is necessary for the formation of long ssDNA tracts. This function in turn is performed by the Exo1 nuclease (Garcia et al., 2011). Thus, generation of invasive 3' overhangs in budding yeast involves a bidirectional combined activity of Mre11 and Exo1 (see Fig. 3).

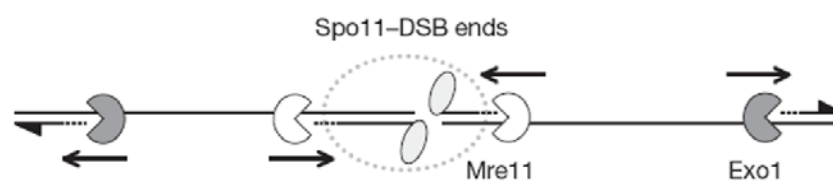


Fig. 3 Model for bidirectional DSB processing in the budding yeast meiosis (Garcia et al., 2011).
See main text for details.

As mentioned above, endonucleolytic Spo11 removal requires the presence of another nuclease, Sae2/Com1. Depletion of this protein, both in *S. cerevisiae* and *S. pombe*, leads to a retention of Spo11 at the broken ends (Hartsuiker et al., 2009; Milman et al., 2009; Neale et al., 2005; Rothenberg et al., 2009). It is not known how Sae2/Com1 contributes to Spo11 removal. However, the similar phenotype of the nuclease dead *mre11* and *sae2/com1Δ* mutants implies a functional interaction between these two proteins.

The Mre11 protein is a member of the Mre11-Rad50-Xrs2/Nbs1 complex (as described in chapter 2.1). The role of Sae2/Com1 and the Mre11 complex in DNA repair has been widely studied in mitosis, where these proteins promote HR by initiating the first step of DSB resection (Mimitou and Symington, 2008; Zhu et al., 2008). It was demonstrated that the fission yeast Sae2/Com1 ortholog, Ctp1, physically interacts via the FHA domain of Nbs1 with the Mre11 complex, and this interaction depends on Ctp1 phosphorylation (see Stracker and Petrini, 2011). A similar phenomenon was reported for the mammalian Sae2/Com1 ortholog, CtIP. In addition, Wu and colleagues showed that the phosphorylation dependent CtIP-Nbs1 interaction is a prerequisite for the initiation of end resection (Wang et al., 2013). Also, in budding yeast meiosis, it was shown that phosphorylation of Sae2/Com1 is required for DSB processing (Manfrini et al., 2010; Terasawa et al., 2008). Importantly, a functional relationship between the Mre11 complex and Sae2/Com1 was demonstrated in vitro. Accordingly, Sae2/Com1 stimulates the Mre11 exonuclease activity, and also itself has an

endonucleolytic activity on ssDNA. In turn, the Sae2/Com1 endonucleolytic property is stimulated in the presence of the Mre11 complex (Lengsfeld et al., 2007).

Altogether, it is likely that Mre11 and Sae2/Com1 cooperate in the initial resection step of mitotic and meiotic DSB ends, similarly. Also removal of Spo11 from DSB ends may require a coordinated action of both nucleases. On the other hand, studies on the meiotic function of Mre11 and Sae2/Com1 in other eukaryotes revealed discrepancies between their requirements for 3' overhang generation (see discussion). Therefore, the clear mechanism of DSB processing in meiosis remains obscure.

2.3. CO formation pathways

Each homologous pair of chromosomes has to receive at least one CO in order to be properly separated in meiosis I (see Youds and Boulton, 2011). This highly obligatory process, termed CO assurance, employs many different proteins involved in the meiotic HR program, which assist or are directly involved in CO formation. The most potent proteins involved in the CO-pathway are the so-called ZMM proteins, which are Zip1, Zip2, Zip3, Msh4, Msh5, Mer3, Spo16 and Spo22 (Borner et al., 2004; Lynn et al., 2007; Shinohara et al., 2008). They were initially identified in budding yeast meiosis as mutants displaying reduced CO number and defects in chromosome synapsis. Of these, Zip1, Zip2, Zip3, Spo16 and Spo22 (Zip4) are required for assembly of the SC, and the Zip1 protein itself is a structural component (for review see de Boer and Heyting, 2006; Page and Hawley, 2004). On the other hand, Mer3, Msh4, and Msh5 are specifically active on recombination intermediates. The Mer3 helicase, following the strand invasion step, extends the DNA heteroduplex in the 3'-5' direction, which stabilizes the intermediate and hence facilitates second DSB end capture (Mazina et al., 2004). Msh4 and Msh5 belong to the mismatch repair protein group and they act together as a meiosis-specific complex, known as MutSy (see Lynn et al., 2007). The Msh4-Msh5 heterodimer is believed to preserve unstable recombination intermediates such as SEIs, by forming a clamp around two homologous DNA duplexes. This mechanism stabilizes newly generated JMs (Snowden et al., 2004). Therefore, Mer3 and the Msh4-Msh5 complex play a fundamental role in converting nascent JMs into mature dHJs. This final recombination intermediate has to be resolved in order to produce a CO. A prominent role in CO formation is attributed to another mismatch repair protein complex, Mlh1-Mlh3 (also known as MutLy). This complex seems to work specifically on ZMM-stabilized JMs to exclusively yield COs (De Muyt et al., 2012; Zakharyevich et al., 2012). Although Mlh3 possesses a conserved nuclease motif, direct evidence of Mlh1-Mlh3's ability to resolve HJs in vitro, has not been demonstrated (Zakharyevich et al., 2012). It was shown that the putative MutLy endonuclease complex functions together with Exo1 in promoting COs. Interestingly, the interaction of Exo1 with

Mlh1-Mlh3 is required, but the nuclease activity of Exo1 is dispensable for this step (Zakharyevich et al., 2010). Therefore, Exo1 is involved in two distinct meiotic processes in budding yeast, which are DSB end resection and CO formation, with its nuclease activity reserved for resection. Collectively, ZMM proteins and the MutLγ complex define a major CO pathway in *S. cerevisiae*. Similar to budding yeast, in meiosis of *Arabidopsis* and mammals, the ZMM- MutLγ pathway predominates (for review see Schwartz and Heyer, 2011). The remaining COs are generated via an alternative recombination pathway, which involves Mus81 and Mms4/Eme1 (Mms4 in *S. cerevisiae*) (Berchowitz et al., 2007; de los Santos et al., 2003; Holloway et al., 2008). These two proteins form a resolving complex, with Mus81 responsible for the JM endonucleolytic cleavage (for review see Hollingsworth and Brill, 2004). Whereas the Mus81 pathway generates a minor fraction of CO in budding yeast, *Arabidopsis* and mammals, it determines a major, if not exclusive, CO pathway in the fission yeast meiosis (Boddy et al., 2001; Cromie and Smith, 2008).

Importantly, in the fission yeast meiosis, the majority of the JMs examined by electron microscopy represent a single HJ (sHJ) (Cromie et al., 2006). This is in contrast to the dHJ-JMs observed in budding yeast (Bell and Byers, 1983). Another striking difference between these two recombination pathways is the position of COs generated along the chromosome. While the ZMM- MutLγ-dependent COs are distributed in a regulated manner, where COs suppress other COs in their vicinity (termed CO interference), those formed via the Mus81-Mms4/Eme1 pathway are not interfering (for review see Bishop and Zickler, 2004).

Furthermore, studies of meiotic recombination pathways in *C. cerevisiae* revealed the existence of additional meiotic resolvases. These were the Slx1-Slx4 complex and Yen1/GEN1. However, they seem to be active only on JMs which are missed by the Mus81-Mms4 complex. Thus, they play a very modest role in wild-type meiosis (De Muyt et al., 2012; Matos et al., 2011; Zakharyevich et al., 2012). Unlike the proteins of the ZMM- MutLγ pathway, Mus81-Mms4/Eme1, Slx1-Slx4 and Yen1/GEN1 are not meiosis-specific, and they play a significant role in mitotic DNA damage repair, as well (for review see Chapman et al., 2012; Svendsen and Harper, 2010).

Contrary to the situation in budding yeast, Slx1 and Slx4 contribute considerably to CO formation in *C. elegans* meiosis. It has been reported recently that the worm ortholog of Slx4, HIM-18, works in two parallel CO-pathways. In detail, it functions either together with MUS-81 and SLX-1 or with XPF-1, the ortholog of budding yeast Rad1 (Agostinho et al., 2013; O'Neil et al., 2013; Saito et al., 2013). In addition, an unidentified JM-resolving complex must still exist, since the two described pathways do not deliver all COs. This unusual way of CO generation does not seem to be absolutely exotic, because in *Drosophila*, the vast majority of COs is formed with assistance of another resolving complex, which consists of a partially similar complement of proteins. These are MEI-9, ERCC1 and MUS12, which represent the *S. cerevisiae* Rad1, Rad10 and Slx4 homologs, respectively (Radford et

al., 2005; Yildiz et al., 2004; Yildiz et al., 2002). Importantly, the Rad1-Rad10 and Rad1-Rad10-Slx4 complexes have well-established roles in several mitotic DNA repair processes. Moreover, the mammalian SLX4 protein interacts with ERCC4(XPF)-ERCC1, SLX1 and MUS81-EME1 (for review see Svendsen and Harper, 2010). Altogether, CO formation may depend on the utilization of different mitotic repair nucleases, like Rad1, Slx1 or Mus81, and their specific function could rely on interactions with other proteins.

2.4. The role of helicases in NCO formation

Promotion of the CO outcome in terms of CO assurance is essential for the completion of the first meiotic division, and several recombination pathways have been found to effectively produce COs (as described in the chapter above). On the other hand, the excess of DSBs over the final CO number is a common meiotic feature in all investigated eukaryotes. Thus, a subset of DSBs formed at the onset of meiosis is distinctively repaired via a mechanism which generates NCO products (for review see Martinez-Perez and Colaiacovo, 2009).

The NCO outcome can be achieved via several processes, which can take place at different steps of recombinational repair. After DSB formation, a very early recombination intermediate which can be readily transformed to a NCO, is the D-loop. This intermediate is typically susceptible to disassembly by helicases, which can unwind the invading strand and reject it from the homologous DNA duplex. If the end was extended by DNA synthesis prior to dissociation, it can anneal to the second end of the original DSB, and subsequent DNA synthesis and ligation seals the break. This mechanism of NCO generation is termed synthesis-dependent strand annealing (SDSA), and it plays a prominent role in DSB repair in mitosis, where NCOs are the preferred recombination outcome (for review see Andersen and Sekelsky, 2010; Krejci et al., 2012). Currently available data indicate that the SDSA pathway might also promote the NCO outcome in meiosis. In this regard, several helicases were described to limit the CO number. These were/are, Fml1 in *S. pombe*, FANCM (the Fml1 ortholog) in *Arabidopsis*, RTEL-1 in *C. elegans* and Sgs1 (a homolog of vertebrate BLM) in *S. cerevisiae* (Crismani et al., 2012; De Muyt et al., 2012; Lorenz et al., 2012; Youds et al., 2010).

Another mechanism that specifically drives NCO formation is dissolution. It involves an alternative processing of the dHJ intermediate, directed by a Sgs1-Top3-Rmi1 complex. The Sgs1 helicase promotes branch migration of the two neighboring HJs to form a hemicatenane, which is then disentangled by the Top3 topoisomerase, and the Rmi1 protein stimulates this action (Cejka et al., 2010b). In this way, a coordinated action of the dissolution complex leads to dHJ disentanglement without CO formation. Importantly, the dissolution-driven NCO formation by Sgs1-Top3-Rmi1 was thoroughly demonstrated in vitro (Cejka et al., 2010b; Wu and Hickson, 2003). Also, collective in vivo data indicate that this process plays a role in suppressing mitotic COs (Mankouri et al., 2011;

Mankouri and Hickson, 2007). In meiosis, the existence of this mechanism as a NCO pathway awaits further research. However, in budding yeast, genetic studies implicate that some of the NCOs may arise from dHJ dissolution (Martini et al., 2011). Moreover, Hunter and colleagues suggested that dHJs undergo dissolution in the absence of all potential resolvases (Zakharyevich et al., 2012). Yet, in wild-type cells, it is believed that Sgs1 channels SEI intermediates, rather than dHJs, into the SDSA-NCO pathway (De Muyt et al., 2012; Zakharyevich et al., 2012). Nonetheless, regardless of the substrate that it may disrupt, Sgs1 acts as an anti-CO factor. Importantly, this activity seems to be antagonized by ZMM proteins, which are thought to stabilize early recombination intermediates (Jessop et al., 2006; Rockmill et al., 2003). Surprisingly, an anticipated pro-CO role of Sgs1 has been recently described in budding yeast meiosis (Zakharyevich et al., 2012). The mechanism of how Sgs1 might promote the CO outcome is not clear. The present model of CO formation in budding yeast assumes that in the presence of Sgs1, COs are primarily formed via the ZMM-MutL γ pathway, whereas the Mus81 complex resolves aberrant JMs or intermediates that escaped the ZMM-dependent stabilization. Conversely, in the absence of Sgs1, the Mus81 pathway becomes predominant (De Muyt et al., 2012; Jessop and Lichten, 2008; Oh et al., 2007; Oh et al., 2008; Zakharyevich et al., 2012). Therefore, the pro-CO role of Sgs1 might be indirect and associated with its function as the regulator of the recombination pathway choice.

3. Synaptonemal complex function in homologous pairing and recombination

CO-biased JM resolution represents one of the mechanism promoting COs in meiosis (described in chapter 2.3). In fact, the HR outcome can be controlled at several points. First of all, the CO precursor has to be formed between two non-sister chromatids of the homologous pair, since only interhomolog COs establish chiasmata. Therefore, in most eukaryotes, DSB recombinational repair is strictly coordinated with homologous chromosome pairing and synapsis during meiotic prophase. An integral element of the chromosome pairing process is SC formation (see Kleckner, 2006; Page and Hawley, 2004; Roeder, 1997; Walker and Hawley, 2000).

The SC is a tripartite proteinaceous structure assembled between paired homologs. It consists of two parallel lateral elements (LEs) connected via transverse filaments (TFs) of the central element (CE) (Page and Hawley, 2004; Zickler, 2006). SC formation is preceded by the development of axial elements (AEs), which are built along two sister chromatids early in prophase I. When the AEs become connected by the TFs, they are referred to as the LEs. It was shown that cohesins are required for AE assembly. Importantly, cohesins become a part of the AE and they are essential for loading of the LE components and SC formation. The Rec8 protein, a widely conserved meiosis-specific subunit of the cohesion complex, plays a prominent role in this process (see Page and

Hawley, 2004). Although the tripartite SC structure is evolutionarily conserved, the LE and TF proteins are divergent. The LE proteins identified in different eukaryotes are respectively: SCP2 and SCP3 in mammals, Red1 and Hop1 in *S. cerevisiae* and HIM-3, HTP-1, HTP-2 and HTP-3 in *C. elegans*. With regard to the TF components, several proteins have been described. These were SCP1 in mammals, Zip1 in *S. cerevisiae*, SYP-1, SYP-2 and SYP-3 in *C. elegans*, ZYP1 in *Arabidopsis* and C(3)G in *Drosophila* (de Boer and Heyting, 2006; Garcia-Muse and Boulton, 2007; Osman et al., 2006; Page and Hawley, 2004; Smolikov et al., 2007b).

In most organisms (except worms and flies), homologous pairing and synapsis is coupled to DSB formation and repair (for review see Gerton and Hawley, 2005; Page and Hawley, 2004; Roeder, 1997; Zickler, 2006). The DSB-dependent homology search occurs in leptotene and leads to a close alignment of the AEs. It is believed that some of the early homologous interactions (presumably SEIs) are stabilized to form so-called axial associations. These stable intimate connections between the LEs represent the sites where synapsis initiates. Importantly, ZMM proteins specifically localize to these sites, thus they are also referred to as the synapsis initiation complex proteins. Since ZMM proteins are well-characterized pro-CO factors, it is believed that CO designation takes place very early in meiosis, before synapsis is complete (Bishop and Zickler, 2004; Borner et al., 2004). Full SC assembly is achieved in the pachytene stage. Although it seems that the extension of the SC only follows the initiation of meiotic recombination, full synapsis turns out to be essential for CO formation and the completion of meiosis. One possible explanation is that the transition of SEI intermediates to HJs occurs in the context of the full SC (Borner et al., 2004).

On the contrary, in *C. elegans* and *Drosophila* females, homologous chromosomes pair and synapse independently of meiotic break formation (see Gerton and Hawley, 2005). In flies, pairing is promoted by centromere clustering (Takeo et al., 2011), whereas in worms it is mediated by specialized chromosome regions known as pairing centers (see Garcia-Muse and Boulton, 2007).

In contrast to the eukaryotes mentioned above, there exist organisms which do not form an SC. These are *S. pombe* (Loidl, 2006), *Asperigillus nidulans* (Egel-Mitani et al., 1982) and *Tetrahymena thermophila* (Loidl and Scherthan, 2004; Wolfe et al., 1976). Interestingly, in the fission yeast prophase I, various linear structures, termed linear elements (LinEs), are microscopically observed. Although LinEs never organize a continuous stretches, their formation is essential for meiotic recombination and complete homologous pairing (for review see Loidl, 2006). The composition of LinEs is reminiscent of AEs in budding yeast. The major component of the LinEs is Rec10, which shows partial sequence similarity to Red1. Another protein, Hop1 (orthologous to *S. cerevisiae* Hop1) also localizes to the LinEs. Thus, LinEs are thought to be a rudimentary, though fully functional, form of AEs. (Lorenz et al., 2004).

In summary, homologous pairing is essential for promoting interhomolog exchange, regardless of the factors that may drive this process. However, bringing homologs in close proximity does not eliminate the possibility of a preferential use of the sister chromatid as a repair template, which normally occurs in mitosis. Nevertheless, meiotic recombination takes place with a higher probability between homologous chromosomes. Several factors appear to be involved in template choice in meiosis (for review see Lao and Hunter, 2010). In budding yeast, the AE proteins, Red1 and Hop1, and the axis-associated meiosis-specific kinase Mek1, specifically suppress intersister recombination. This phenomenon, called a barrier to sister chromatid recombination (BSCR), is specific to meiotic DSB repair (Niu et al., 2005; Schwacha and Kleckner, 1997; Thompson and Stahl, 1999). A similar function was proposed for axis-associated proteins in other eukaryotes, such as HIM-3 in *C. elegans*, ASY1 in Arabidopsis and HORMAD1 and -2 in mice. These proteins, like Hop1, represent HORMA domain-containing proteins (see Garcia-Muse and Boulton, 2007; Sanchez-Moran et al., 2007; Wojtasz et al., 2009).

Another factor, fundamental for template choice and interhomolog bias, is the meiosis-specific Dmc1 recombinase. Dmc1 assembles on the 3' single-stranded end and directs DSB repair toward the homolog (for review see Keeney and Neale, 2006). Interestingly, Hop1, Red1 and Mek1 promote Dmc1-dependent repair by suppressing the recombinase activity of the mitotic Rad51 (see Sheridan and Bishop, 2006). Yet, it has been shown recently that Rad51 contributes to interhomolog bias as a Dmc1 accessory factor (Cloud et al., 2012). At the same time, Dmc1-mediated strand invasion is required for homologous pairing and synapsis (Bishop et al., 1992; Rockmill et al., 1995). Therefore, there exists a strong interrelation between DSB repair, pairing, synapsis and the CO fate of a DSB. In flies and worms, where pairing and synapsis is DSB-independent, the strand invasion step relies solely on the Rad51 recombinase (see Gerton and Hawley, 2005). This suggests that Dmc1 is required both for homology recognition and interhomolog CO, whereas Rad51-mediated strand invasion is sufficient for interhomolog CO once the homology recognition step has taken place.

4. Inhibition of the NHEJ repair mechanism in meiosis

An additional aspect of successful meiosis is the inhibition of a nonhomologous end-joining (NHEJ) pathway. NHEJ is a common DSB repair mechanism in mitosis, alternative to HR (for review see Chapman et al., 2012). It relies on the direct rejoining of broken ends by the Lig4 ligase, and it is mediated by the Ku70/Ku80 complex, which binds to DSB ends and blocks their resection. In contrast to HR, this repair mechanism is known to be error prone. Moreover, during the mitotic cell cycle, NHEJ is favored in G1, whereas the HR pathway dominates after DNA replication in S/G2 phase, when DSBs can be securely repaired based on the undamaged sister chromatid. Logically, it makes sense to suppress NHEJ in meiosis. First of all, only the HR pathway delivers COs, and second, a random

ligation of DNA ends may lead to a fusion of nonhomologous chromosomes. Also, even if the correct DSB ends are sealed, this pathway often results in loss or gain of base pairs, thus causing mutations (see Chapman et al., 2012). Nevertheless, the NHEJ suppression mechanism in meiosis is poorly understood. In mouse, it is believed that Ku proteins are downregulated on the transcriptional or translational level (Goedecke et al., 1999). On the other hand, in *C. elegans* meiosis, it has been found recently that MRE-11 and COM-1 play a role in the meiotic NHEJ prevention (Lemmens et al., 2013; Yin and Smolikove, 2013; also see discussion, chapter 1). In addition, it was shown that the NHEJ pathway can be utilized in worm mutants defective in HR or cohesion and synapsis (Martin et al., 2005; Smolikov et al., 2007a). Also in *Drosophila*, a few proteins involved in the NHEJ block were identified (Joyce et al., 2012). In general, the NHEJ process is not desirable in meiosis and is widely avoided.

5. *Tetrahymena thermophila* as a model organism for studying meiosis

Tetrahymena thermophila is a unicellular ciliated protist. Like most of the ciliates, it characteristically possesses two distinct nuclei, a polyploid somatic macronucleus (MAC) and a diploid germline micronucleus (MIC) (see Karrer, 2012). The MAC is transcriptionally active, and it regulates the life cycle of *Tetrahymena*, whereas the MIC genome is not translated. However, only the MIC's genetic information is passed to the progeny during sexual reproduction, and only the MIC undergoes meiosis. The sexual stage of *Tetrahymena*'s life cycle represents an unusual biological process (see Fig. 4 and for review see Orias et al., 2011). When two cells of opposite mating types are exposed to starvation conditions, they conjugate and their MICs synchronously enter the meiotic program. Consequently, four haploid products are formed by the end of meiosis. Of these, three are degraded, and the one selected undergoes mitosis to form two gametic pronuclei. This stage is followed by the reciprocal exchange of one of the pronuclei between the conjugants. The following cross fertilization produces a diploid nucleus, which next undergoes two rounds of postzygotic mitosis. During this step of the sexual stage, the conjugating cells enter the programmed macronuclear differentiation that results in a formation of new MACs and MICs, while the parental MACs are destroyed. When the sexual reproduction process is complete, the conjugants split and continue vegetative growth by binary fission.

During the new MAC development two sorts of DNA elimination have been described (see Collins and Gorovsky, 2005; Orias et al., 2011). First, an RNAi-based mechanism mediates removal of around 6000 of the so-called internal eliminated sequences (IESs). This generates DNA fragments, which are then appropriately ligated. The IES elimination accounts for the difference between the MAC and MIC genome size, which is ~104 Mb versus ~120 Mb, respectively. The second process involves cleavage of the so-called chromosome breakage sequences (Cbs). In contrast IEs elimination,

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the DNA ends at the Cbs sites are not rejoined but are subjected to telomere addition. The newly-formed minichromosomes are then endoreplicated. Consequently, two nuclei display different chromosome number and structure. While the diploid MIC contains 5 pairs of metacentric chromosomes, the polyploid MAC consists of approximately 180 minichromosomes, each in ~45 copies. Because the MAC does not divide mitotically, but distributes its genome via amitosis, it is postulated that chromosome fragmentation and polyploidization might bypass a potential loss of chromosomes during their random distribution. Interestingly, there exists an uncharacterized control mechanism which ensures that the right chromosome copy number is restored in descendant cells.

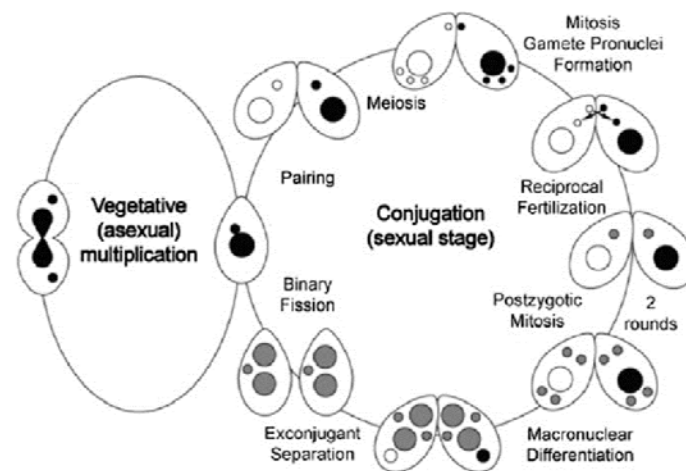


Fig. 4 *Tetrahymena* life cycle (Orias et al., 2011). See main text for details.

As mentioned above only the MIC undergoes the process of meiosis, which in *Tetrahymena* progresses in a unique manner (see Fig. 5). The non-dividing MIC is situated in a pocket of the MAC, but at the time when two cells conjugate, it leaves the MAC and starts to elongate. This leads to the formation of a morphologically distinct structure, termed the crescent. Next, the enormously elongated MIC exits the crescent stage and shortens again, which is accompanied by chromatin condensation. Eventually, 5 bivalents are visible at metaphase I (Loidl and Scherthan, 2004).

The extreme changes of the MIC shape correspond to specific meiotic events that take place during prophase I, namely, meiotic homologous pairing and recombination. The process of HR initiation is conserved in *Tetrahymena* and starts with meiotic break formation by Spo11 (Mochizuki et al., 2008). The ATR-dependent signaling of induced DSBs is responsible for full MIC elongation (Loidl and Mochizuki, 2009). Characteristically, within the crescent, chromosomes are arranged in a way such that all telomeres are on one end and all centromeres are on the opposite end (Loidl et al., 2012). This polarized chromosome arrangement is essential for interhomolog CO formation. If crescent formation is disturbed, for instance by adding the microtubule poison nocodazole, homologous chromosomes stay unpaired, and despite completed DSB repair, bivalents are not

formed (Kaczanowski et al., 1985; Loidl et al., 2012). On the other hand, MIC elongation is not sufficient to produce interhomolog COs, and has to be accompanied by simultaneous interhomolog recombinational repair. This in turn, similarly to the other studied eukaryotes, is promoted by the meiosis-specific Dmc1 recombinase (Howard-Till et al., 2011). Overall, successful completion of meiosis requires both crescent formation and interhomolog-directed DSB repair.

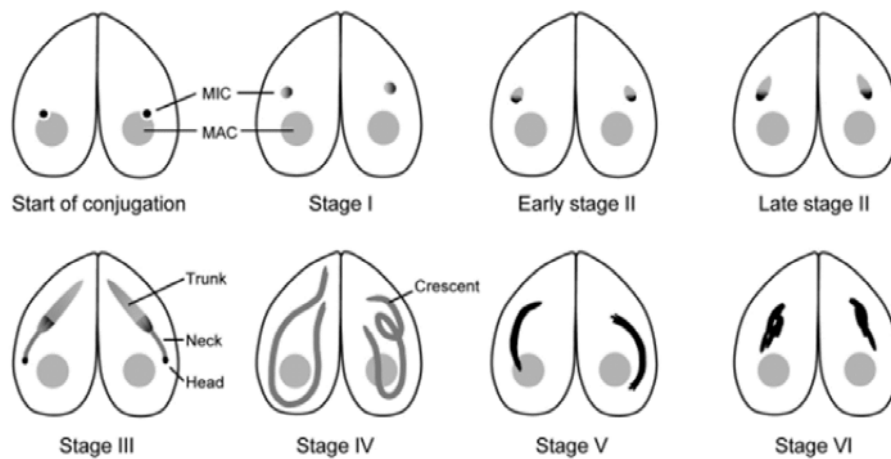


Fig. 5 The morphology change of the MIC throughout meiotic prophase (Loidl and Scherthan, 2004). See main text for details.

As mentioned in chapter 3, *Tetrahymena* does not possess an SC or SC-related structures. Moreover, no homologs of the SC components have been identified (Loidl and Scherthan, 2004). This implies that a barrier to sister chromatid recombination imposed by the AE/LE proteins (for details and references see introduction, chapter 3), does not exist in *Tetrahymena*. Only the Rec8 cohesin, which in other eukaryotes forms a structural basis for the other AE components (see Page and Hawley, 2004), is present and essential for meiotic DSB repair in *Tetrahymena* (Howard-Till et al., 2013). However, it is not clear if Rec8 has a direct function in break repair or if the defect is caused by a failure in defining chromosome axes. Summarizing, the remarkable reorganization of the meiotic nucleus is most likely an adaptation to the SC-less meiosis.

Importantly, *Tetrahymena* is phylogenetically distant to any other model eukaryote representing the kingdom of animals (mouse, *C. elegans* and *Drosophila*), plants (*Arabidopsis*) or fungi (*S. cerevisiae* and *S. pombe*) (Baldauf, 2003). In addition, this ciliate is an excellent model system, due to its ability to undergo inducible synchronous meioses in mass cultures. Therefore, studying unusual meiosis in this evolutionarily remote model organism, allows the investigation of conserved and non-conserved features of meiotic processes.

Here, I specifically study the molecular aspects of recombination in *Tetrahymena*'s unusual SC-less meiosis.

RESULTS

1. MRE11 and COM1/SAE2 are required for double-strand break repair and efficient chromosome pairing during meiosis of the protist *Tetrahymena*

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Rachel A. Howard-Till: part of Fig. 6, part of Fig. 7, part of Fig. S7

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MRE11* and *COM1/SAE2* are required for double-strand break repair and efficient chromosome pairing during meiosis of the protist *Tetrahymena

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Abstract Programmed DNA double-strand breaks (DSBs) are generated during meiosis to initiate homologous recombination. Various aspects of DSB formation, signaling, and repair are accomplished or governed by Mre11, a component of the MRN/MRX complex, partially in cooperation with Com1/Sae2/CtIP. We used *Tetrahymena* to study evolutionarily conserved and changed functions of Mre11 and Com1. There is a difference between organisms with respect to the dependency of meiotic DSB formation on Mre11. By cytology and an electrophoresis-based assay for DSBs, we found that in *Tetrahymena* Mre11p is not required for the formation and ATR-dependent signaling of DSBs. Its dispensability is also reflected by wild-type-like DSB-dependent reorganization of the meiotic nucleus and by the phosphorylation of H2A.X in *mre11Δ* mutant.

However, *mre11Δ* and *com1Δ* mutants are unable to repair DSBs, and chromosome pairing is reduced. It is concluded that, while *MRE11* has no universal role in DNA damage signaling, its requirement for DSB repair is conserved between evolutionarily distant organisms. Moreover, reduced chromosome pairing in repair-deficient mutants reveals the existence of two complementing pairing processes, one by the rough parallel arrangement of chromosomes imposed by the tubular shape of the meiotic nucleus and the other by repair-dependent precise sequence matching.

Introduction

Meiosis is the nuclear division that compensates for chromosome doubling during fertilization by separating the parental pairs of chromosomes, so that gametes will be supplied with a single copy of each chromosome. To sort out the pairs to be separated, they must be first identified as the corresponding (homologous) chromosomes. This happens during a process in which closely juxtaposed chromosomes are matched along their lengths (chromosome pairing) and exchange pieces (crossing over). The latter enforces their physical connection and provides the additional bonus of increased intermingling of parental portions in the gamete's genome, thus enhancing the progenies' genetic diversity (for review see Zickler and Kleckner 1999). While various strategies seem to exist among eukaryotes to facilitate the mutual recognition and pairing of homologues (Loidl 1990; Barzel and Kupiec 2008), in the ciliate *Tetrahymena* this process is promoted by the tight parallel alignment of chromosomes in the enormously elongated tubular meiotic nuclei (Loidl and Scherthan 2004).

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Programmed DNA double-strand breaks (DSBs) induced by the transesterase Spo11p are a conserved feature of meiosis (Keeney 2007). In most organisms, DSBs have a dual function in initiating both meiotic recombination and intimate homologous pairing (Peoples-Holst and Burgess 2005). Numerous factors contribute to the generation and/or the (recombinogenic) repair of meiotic DSBs. Among those, Mre11 is a versatile protein with different DSB-related functions in meiosis. Mre11 functions as part of the MRN (in the budding yeast: MRX) protein complex together with Rad50 and Nbs1/Xrs2 (Rupnik et al. 2008). In some organisms, Mre11 is required for the generation of DSBs by Spo11p. It also acts as a sensor of DSBs and helps to activate the DNA damage sensor kinases, ATM, and ATR (for review see Borde 2007; Rupnik et al. 2008). Finally, with the help of Sae2/Com1, it plays a function in removing covalently attached Spo11p from the 5' DNA ends flanking DSBs to allow further strand resection and invasion of homologous strands by the resulting 3' overhangs (see Raynard et al. 2008).

In the ciliate *Tetrahymena*, meiotic nuclei undergo dramatic elongation during the stages corresponding to zygotene and pachytene, which is accompanied by a bouquet-like arrangement of chromosomes. This reorganization is dependent on meiotic DSBs, and it is mediated by an ATR-like kinase (Loidl and Mochizuki 2009). Therefore, we were interested in finding out whether it requires the activity of Mre11p. We identified *Tetrahymena* Mre11p and Com1p and studied their roles in meiosis, specifically in the generation and repair of DSBs, and asked if Mre11p has an additional function in signaling meiotic DSBs. To this end we produced deletion strains for the two genes and studied the progression of meiosis and the course of DSBs in the mutants, using cytology and an electrophoresis-based method for DSB detection. Our observations demonstrate the requirement of Mre11p for DSB repair but its dispensability for DSB induction and signaling in a eukaryote that is evolutionarily distant to the common animal, plant, and fungal meiotic model systems.

Materials and methods

Strain growth and induction of meiosis

Cells were cultured at 30°C according to standard methods (reviewed by 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 cells of different mating types (B2086 and CU428) at equal densities ($\sim 2 \times 10^5$ cells/ml).

For the induction of DSBs in *spo11Δ* cells, bleomycin at indicated concentrations was added to conjugating cells 120 min after mixing. DNA samples from this culture were prepared 3.5 h after mixing.

Viability testing

The viability of sexual progeny was tested according to Mochizuki et al. (2008). In short, conjugating cells were isolated and transferred to single droplets of growth medium 6–10 h after mixing and kept for 2 days at 30°C. Drops in which cells had grown were transferred to multiwell plates with medium containing 100 µg/ml paromomycin and 1 µg/ml CdCl₂. Since only mitotically propagated cells but not sexual progeny express the *neo4* paromomycin resistance gene, only colonies that were dead after 2 days of paromomycin treatment were scored as viable meiotic progeny.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen) from wild-type (B2086 and CU428) strains, and residual genomic DNA was eliminated using the Turbo DNase Kit (Ambion). cDNA was synthesized from 5 µg of total RNA using a RevertAid H Minus First Strand cDNA Synthesis Kit (Fermentas) with a random hexamer as a primer. cDNA from 12.5 ng total RNA was amplified by PCR (94°C for 20 s, 55°C for 30 s, 72°C for 1 min). The following primers were used to amplify cDNAs of the indicated genes. *MRE11*: 5'-ACG ACC CTA TTA GAG GGA ACG ACT C-3' and 5'-AGC TAC TGT TGT ATT GCC TTT CTG G-3'; *COM1*: 5'-TCT GAA TTA GAA ACA CAC AGC TAG TG-3' and 5'-TCA ATT AGA ATC ATC TTC TGA CTA GG-3'; *RPL21* (control): 5'-AAG TTG GTT ATC AAC TGT TGC GTT-3' and 5'-GGG TCT TTC AAG GAC GAC GTA-3'.

Somatic gene disruption

Wild-type strains B2086 and CU428 were used for macronuclear gene disruption. For the design of knockout constructs, see Supplement S1. The *MRE11* disruption construct was made as follows: A genomic region flanking the 5' end of the *MRE11* gene was amplified by PCR using total genomic DNA of CU428 as a template and primers MRE11KO5FW and MRE11KO5RV. Likewise, a region 3' of the *MRE11* gene was amplified by PCR with primers MRE11KO3FW and MRE11KO3RV. The 5' and 3' flanking sequences were then connected to a *neo4* cassette, which confers paromomycin resistance (Mochizuki et al. 2008) and amplified by overlapping PCR using MRE11-KO5FW and MRE11KO3RV. The PCR product was used for transformation.

For the *COM1* disruption construct, a genomic region flanking the 5' end of the *COM1* gene was amplified by PCR with primers COM1KO5FW and COM1KO5RV. A 3' region was amplified with primers COM1KO3FW and COM1KO3RV. The two flanking sequences and the *neo4* cassette were connected and amplified by overlapping PCR using COM1KO5FW and COM1KO3RV, and the PCR product was used for transformation (see [Supplement S1a](#)). All primer sequences are listed in [Supplement S1b](#).

The *MRE11* and *COM1* disruption constructs were introduced into the corresponding macronuclear loci of B2086 and CU428 cells by biolistic transformation (Cassidy-Hanley et al. 1997). The transformants were exposed to increasing concentrations of paromomycin to gradually select for cells in which all macronuclear copies of the wild-type allele were replaced by the deletion construct due to phenotypic assortment (Cassidy-Hanley et al. 1997). Successful deletion was confirmed by Southern hybridization with probes that distinguished between wild-type and disruption sequences. They were generated by PCR using primer pairs MRE11-KO5FW, MRE11KO5RV and COM1KO5FW, COM1KO5RV ([Supplement S1c](#)).

Preparation of chromosome-sized DNA in agarose plugs and pulsed-field gel electrophoresis

Chromosome-sized DNA was prepared in agarose plugs following Schwartz and Cantor (1984) and Gerring et al. (1991) (see also Loidl and Nairz 1997). For a similar procedure see Cassidy-Hanley et al. (2005). In short, 4×10^6 cells (~20 ml of cell culture) were centrifuged for 3 min at $1000 \times g$. The pellet was resuspended in 50 μ l ice-cold 0.05 M Na₂EDTA, 0.01 M Tris–Cl (pH 7.5). The suspension was transferred to a reaction tube and mixed with 250 μ l of 1% low-melt agarose at 42°C. After the agarose had solidified on ice, 400 μ l of LET buffer (0.5 M Na₂EDTA, 0.01 M Tris–Cl, pH 7.5) were added. For time-course experiments, the tubes were kept on ice until all samples were collected. The LET was replaced with 400 μ l of 1% N-lauroyl sarcosine (pH 9.5) in LET supplemented with 2 mg/ml proteinase K, and the samples were incubated overnight at 50°C. The agarose plugs were transferred to 24-well microtiter plates and washed three times for 30 min in 1 M Tris–Cl (pH 7.5). The buffer was replaced with 50 μ g/ml RNaseA in 1 M Tris–Cl (pH 7.5) and the plugs were incubated for 1 h at 37°C. Finally, the plugs were washed four times for 1 h in 0.05 M Na₂EDTA, 0.01 M Tris–Cl, and stored at 4°C in the same buffer.

Slices of agarose plugs ($4 \times 4 \times 1$ mm) were inserted into the wells of an agarose gel. Great care was taken that slices were of equal size. In all cases, meiotic DNA samples

were taken from cultures in which more than 85% of the cells engaged in conjugation (and thus entered meiosis) to ensure that signal strengths after Southern hybridization reflected relative DSB frequencies rather than the absolute amounts of broken MIC DNA.

Pulsed-field gel electrophoresis (PFGE) was performed using a contour-clamped homogeneous electric field apparatus. Two different running conditions were applied: In one set of experiments, the run was performed in 1% agarose with $0.5 \times$ TBE buffer at 200 V, 6°C for 14 h with 60-s pulses, 10 h with 90-s pulses, and 1 h with 120-s pulses. This procedure caused DNA fragments within a certain size range to enter the gel but run as a distinct band. Better separation but weaker signals were achieved in 0.8% agarose gel with $1 \times$ TBE buffer at 130 V for 24 h with 300-s pulses followed by 90 V for 48 h with 1,000-s pulses. The gel was stained for 30 min in 0.5 μ g/ml ethidium bromide in $0.5 \times$ TBE and destained for 30 min in water.

Southern blotting and hybridization

DNA was transferred from pulsed-field gels onto Hybond N+ membrane (GE Healthcare) by alkaline transfer. The pMBR2 vector (NCBI accession number AF451863) carrying an 8.5-kb fragment of the conserved internal region of Tlr elements (Wuitschick et al. 2002) was provided by Kathleen Karrer. The Tlr sequence was excised from the vector, gel-isolated, and radioactively labeled by random priming using ³²P-dATP and hybridized to micro-nuclear DNA on the membrane.

Cytological preparation and microscopy

For subsequent staining with 4',6'-diamidino-2-phenylindole (DAPI), 5 ml aliquots of conjugating cell suspension were transferred to a centrifuge tube and 250 μ l 10% Triton X-100 and 500 μ l 37% formaldehyde were added. After 30 min, the fixed cell suspension was centrifuged for 2 min at $1,000 \times g$, and the pellet was resuspended in 500 μ l 4% paraformaldehyde + 3.4% sucrose solution. A drop of this mixture was spread onto a slide and dried in air. After 2×5 min-washes with $1 \times$ PBS and a 5-min-wash with $1 \times$ PBS + 0.05% Triton X-100, the preparations were mounted in Vectashield supplemented with 0.05 μ g/ml DAPI.

For Rad51p/Dmclp immunostaining, cells were spread by adding 500 μ l of a mixture (450 μ l 10% Triton + 50 μ l 37% formaldehyde) to 5 ml of cell culture. After 25–30 min on ice, another 450 μ l of 37% formaldehyde were added. After 5 min, the cell pellet was collected by centrifugation and resuspended in 500 μ l of 4% paraformaldehyde + 3.5% sucrose. The mixture (80 μ l) was spread on a slide and dried in a chemical hood. Slides were incubated with monoclonal mouse anti-Rad51p antibody (1:50, Clone

51RAD01, NeoMarkers, Fremont, CA, USA) and Cy3-conjugated anti-mouse antibody (Mochizuki et al. 2008). We found this antibody to react with both of the related recombination proteins Rad51p and Dmc1p.

For immunostaining of γ -H2A.X, cells were fixed with partial Schaudin's fixative (saturated HgCl₂, ethanol 2:1) and incubated with mouse anti- γ -H2A.X antibody (Clone 2F3, BioLegend, San Diego, CA, USA; 1:200 and Cy3-conjugated anti-mouse antibody (Mochizuki et al. 2008).

For FISH, cells were fixed with Carnoy's fixative (methanol, chloroform, acetic acid 6:3:2). A compound FISH probe was produced by amplifying a 22.1-kb intercalary chromosomal locus by PCR (Loidl and Mochizuki 2009). The purified PCR products were labeled with Cy3 by nick translation. The probe and chromosomal DNA were denatured by hot formamide and hybridized for 36 h at 37°C.

z stacks of pictures were taken under a fluorescence microscope equipped with the appropriate filters. Picture stacks were recorded using MetaView software (Universal Imaging, Downingtown, PA, USA), deconvolved using AutoDeblur (AutoQuant Imaging, Watervliet, NY, USA) and projected with ImageJ (Wayne Rasband, N.I.H.; <http://rsb.info.nih.gov/ij/>) software. Different color channels were recorded separately, false-colored, and merged.

The "Analyze particle" tool of ImageJ was used to quantify Rad51p/Dmc1p immunofluorescence foci in wild-type, *mre11Δ* and *com1Δ* MICs. Images of stained nuclei were adjusted to the same level of background signal intensity, which was assumed as constant for all strains. Subsequently, half tone images were transformed to binary images, and a threshold was set to remove the nonnuclear background. Overall MIC signal intensities were compared by measuring the total area of particles (in pixels), which integrates brightness, size, and number of foci. Twelve MICs were evaluated and averaged for each strain.

Results

Tetrahymena possesses *MRE11* and *SAE2/COM1* homologues which are expressed in meiotic cells and are essential for meiosis

Tetrahymena ORF TTHERM_00721450 encodes an 884-amino acid protein and is a putative orthologue of Mre11 proteins as established by using the InParanoid program for fully automatic orthology assignment (O'Brien et al. 2005) as well as Reciprocal Best BLAST analyses with metazoan, fungal, and plant Mre11 representatives. Since this similarity was supported by phenotypic analysis (see below), we designate TTHERM_00721450 as Mre11p in the following. The N-terminal region of *Tetrahymena* Mre11p shows

highest sequence conservation and contains hits to two known protein domains—*Calcineurin-like phosphoesterase* (PF00149 24–257aa) followed by *Mre11 DNA-binding presumed domain* (PF04152 263–484aa). Even more striking is the conservation of known Mre11 functional motifs in *Tetrahymena* Mre11p, e.g., the phosphodiesterase motifs that form the nuclease active site of Mre11 (Supplement S2).

Mre11 orthologues are widely present in eukaryotes where they play diverse roles during replication and in the repair of mitotic and meiotic DSBs (see Borde and Cobb 2009; Rupnik et al. 2008). We found the *Tetrahymena* *MRE11* gene to be transcribed in exponentially growing, starving, and conjugating cells (Fig. 1a) and also microarray analysis indicated a low level of transcription in nonmeiotic cells (Miao et al. 2009). Despite the gene's ubiquitous expression, vegetative cultures of *mre11Δ* cells did not show an acute growing defect. Thus, unlike in vertebrates where deletion of *MRE11* was found to be lethal (Haber 1998), this gene is not essential for *Tetrahymena*.

Since in the organisms studied so far, Mre11 together with Rad50 and Nbs1/Xrs2 forms the MRN (a.k.a. MRX) protein complex (Rupnik et al. 2008), we wondered whether the corresponding ensemble exists in *Tetrahymena*. There exists a good Rad50 homologue (encoded by ORF TTHERM_00773790) in *Tetrahymena* (Mochizuki et al. 2008), and its expression profile is very similar to the one of *MRE11* (Miao et al. 2009). Therefore, it is likely that Mre11p and Rad50p act in a complex related to MRN although an Xrs2 or Nbs1 homologue has not yet been detected.

Tetrahymena possesses a member of a family of distant orthologues represented by budding yeast Sae2/Com1, mammalian CtIP, fission yeast Ctp1 (see Takeda et al. 2007), *Arabidopsis* *AtCom1(ATGR1)* (Uanschou et al. 2007), and *Caenorhabditis elegans* COM-1 (Penkner et al. 2007). It is a 657-amino acid protein encoded by ORF TTHERM_00460480. In the organisms where these proteins have been characterized so far, they were found to collaborate with Mre11 in the 5' to 3' resection of DSBs. While CtIP functions in vegetative cells, mutation of the genes encoding the budding yeast, *Arabidopsis* and *C. elegans* orthologues elicited primarily meiotic defects and only mild mitotic phenotypes. Ctp1 may be important both for mitosis and meiosis (Farah et al. 2009). Following its budding yeast name referring to the mutant's inability for *completion of meiosis* (Prinz et al. 1997), which also applies to the *Tetrahymena* deletion mutant (see below), we call the gene and the protein *COM1* and Com1p, respectively. In *Tetrahymena*, *COM1* expression was prevalent in proliferating and in conjugating cells (Fig. 1a and Miao et al. 2009).

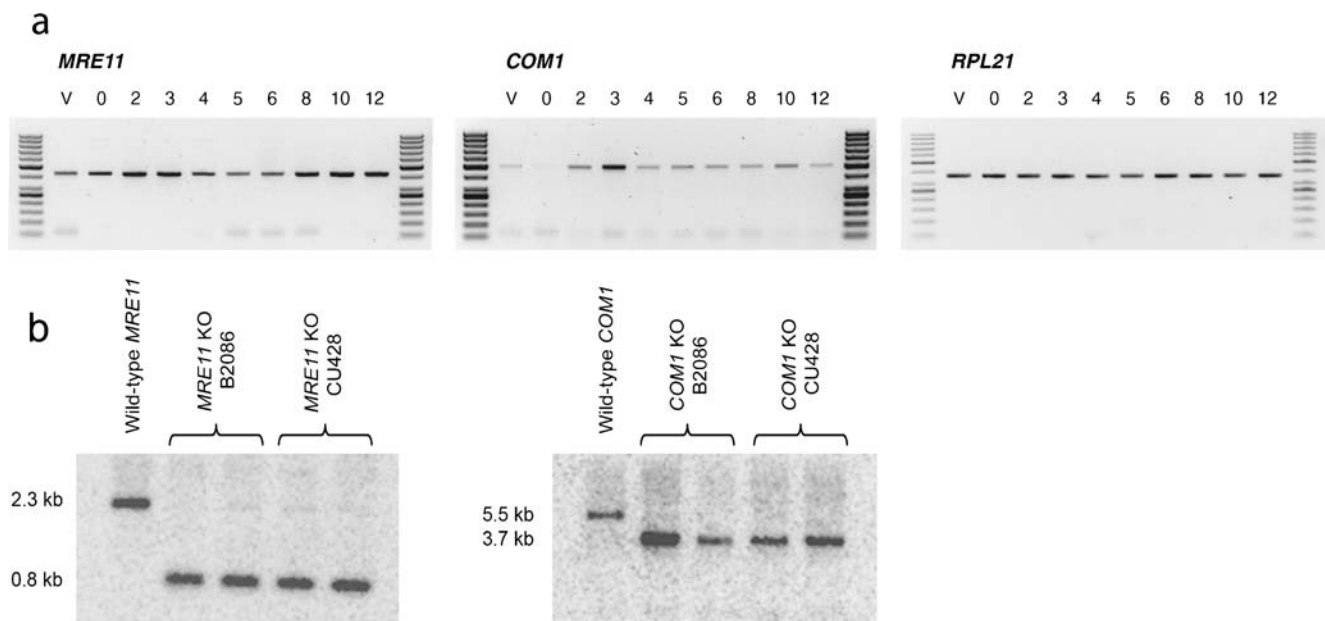


Fig. 1 *MRE11* and *COM1* expression and knockout. **a** Expression of *MRE11*, *COM1*, and *RPL21* in vegetative cells (V) and in cultures at the indicated time (in h) after induction of meiosis as visualized by RT-PCR. *MRE11* shows ubiquitous expression whereas *COM1* is transcribed in proliferating and meiotic cells but not or weakly

expressed in starving cells right before meiosis starts ($t=0$ h). *RPL21* is an ubiquitously expressed control gene. **b** Southern hybridization with probes reflecting the different sizes of the wild-type *MRE11* and *COM1* loci and the corresponding gene replacement constructs confirmed the macronuclear knockout of these genes

We produced somatic deletion strains for *MRE11* and *COM1* by replacing the respective macronuclear loci with antibiotic-resistance cassettes and confirmed their knockout by Southern blot analysis (Fig. 1b). Consistent with a conserved meiotic function, *Tetrahymena mre11Δ* and *com1Δ* cells were infertile. Whereas, 67.7% ($n=192$) of wild-type conjugants (B2086×C428) produced viable sexual progeny, various crossings of different independent knockout clones ($n=128$ conjugants for each of them) produced no viable progeny in either mutant (Supplement S3).

MRE11 is not required for ATR activation and meiotic MIC elongation

Tetrahymena cells possess two nuclei, the endopolyploid somatic macronucleus (MAC) and the germ line micronucleus (MIC). When two cells of complementary mating types conjugate, meiosis simultaneously takes place in their MICs (Fig. 2a). During meiosis, MICs stretch to about 50 times their original diameter (Ray 1956; Wolfe et al. 1976) and within these elongated MICs, homologous pairing and recombination take place (Loidl and Scherthan 2004; Mochizuki et al. 2008). After this elongation stage, MICs shorten again and undergo two meiotic divisions. Based on the degree of MIC elongation, meiotic prophase has been subdivided into six stages (Sugai and Hiwatashi 1974). Stage IV, maximally elongated MICs, may correspond to

pachytene when pairing is complete, stage V to diplotene when intimate pairing along the entire length of chromosomes is resolved, and stage VI to diakinesis–metaphase I when the five bivalents achieve maximal condensation (Fig. 2a).

We have previously demonstrated that the DSB-inducing activity of Spo11p triggers MIC elongation, and that *Tetrahymena*'s only ATM/ATR homologue (*ATR1*) is required for this process (Mochizuki et al. 2008; Loidl and Mochizuki 2009). Thus, we proposed a DNA damage signaling pathway involving *ATR1* to promote homologous chromosome alignment via MIC elongation. Since in a variety of organisms and in vitro systems the MRN complex (and implicitly, Mre11) is required for the activation of ATM and ATR (see Harrison and Haber 2006; Borde 2007; Rupnik et al. 2008), we tested if Mre11p is needed for MIC elongation. This was not the case. In strains lacking *MRE11*, we found that MIC elongation proceeded as in the wild type (Fig. 2b).

Another indicator of ATM/ATR recruitment and activation is the phosphorylation of histone variant H2A.X (H2AX) surrounding DSBs (see Williams et al. 2007; Borde and Cobb 2009). Also in *Tetrahymena*, H2A.X phosphorylation depends on the presence of *ATR1* since in *atr1Δ* meiosis we did not detect γ -H2A.X by immunostaining (Fig. 2d and Loidl and Mochizuki 2009). However, in the absence of Mre11p, γ -H2A.X labeling of meiotic nuclei was as intense as in the wild type (Fig. 2d). Likewise, H2A.

X phosphorylation in response to bleomycin-induced DSBs in vegetatively growing cells was not dependent on Mre11p (Supplement S4). Thus, in *Tetrahymena* two checkpoint kinase-dependent processes, namely MIC elongation and H2A.X phosphorylation, take place normally in the absence of Mre11p. It is therefore likely that in *Tetrahymena*, the activation of ATR kinase does not depend on Mre11p.

In the absence of Mre11p and Com1p, metaphase I nuclei arrest with disintegrated chromosomes

MIC elongation and H2A.X phosphorylation in *mre11Δ* mutant meiosis (see above) suggest that the mutant is proficient in forming DSBs. As an additional indicator of DSB formation, we monitored recombination-dependent foci with an antibody reacting with Rad51p and Dmc1p. Recombination proteins Rad51 and Dmc1 form helical filaments on ssDNA in vitro (see San Filippo et al. 2008) and can be cytologically visualized as foci which accompany meiotic DSBs (Alpi et al. 2003). Because in *Tetrahymena*, Rad51p is expressed and localizes to meiotic MICs even in the absence of DSBs (Mochizuki et al. 2008), we applied a detergent spreading method by which chromatin-associated proteins are retained, but unbound nuclear proteins are washed out (see “Materials and methods” section). Accordingly, foci were present in the wild type but not in a *spo11Δ* control (Fig. 2e). In the *mre11Δ* mutant, Rad51p/Dmc1p foci were reduced as compared to the wild type (Fig. 2e). This reduction was quantified by measuring the area of MICs covered by foci above a threshold intensity (see “Materials and methods” section). While in the wild type, this area (in pixels) was $4,641 \pm 915$, it was $1,858 \pm 524$ in the *mre11Δ* mutant (and $1,880 \pm 313$ in the *com1Δ* mutant). (12 MICs were evaluated for each strain). This corresponds to a reduction in signal intensity to 40.0% (and 40.5%) of that of the wild type. The presence of diminished foci confirms that DSBs are formed, but there may be reduced loading of recombination proteins at DSBs.

Consistent with the generation of DSBs, all meiotic stages up to diplotene appeared cytologically normal in the *mre11Δ* mutant (Fig. 2b), and their temporal appearances and durations were similar to wild-type (Fig. 3). However, whereas in the wild-type condensed bivalents appeared in metaphase I (stage VI), mutant nuclei at the corresponding stage displayed a dotted chromatin distribution (Fig. 2a, b). Moreover, whereas in the wild type metaphase I was immediately followed by a first and second meiotic division, aberrant metaphase I nuclei accumulated in the *mre11Δ* mutant (Fig. 3b). This disintegration and arrest phenotype suggests that DSBs are not repaired.

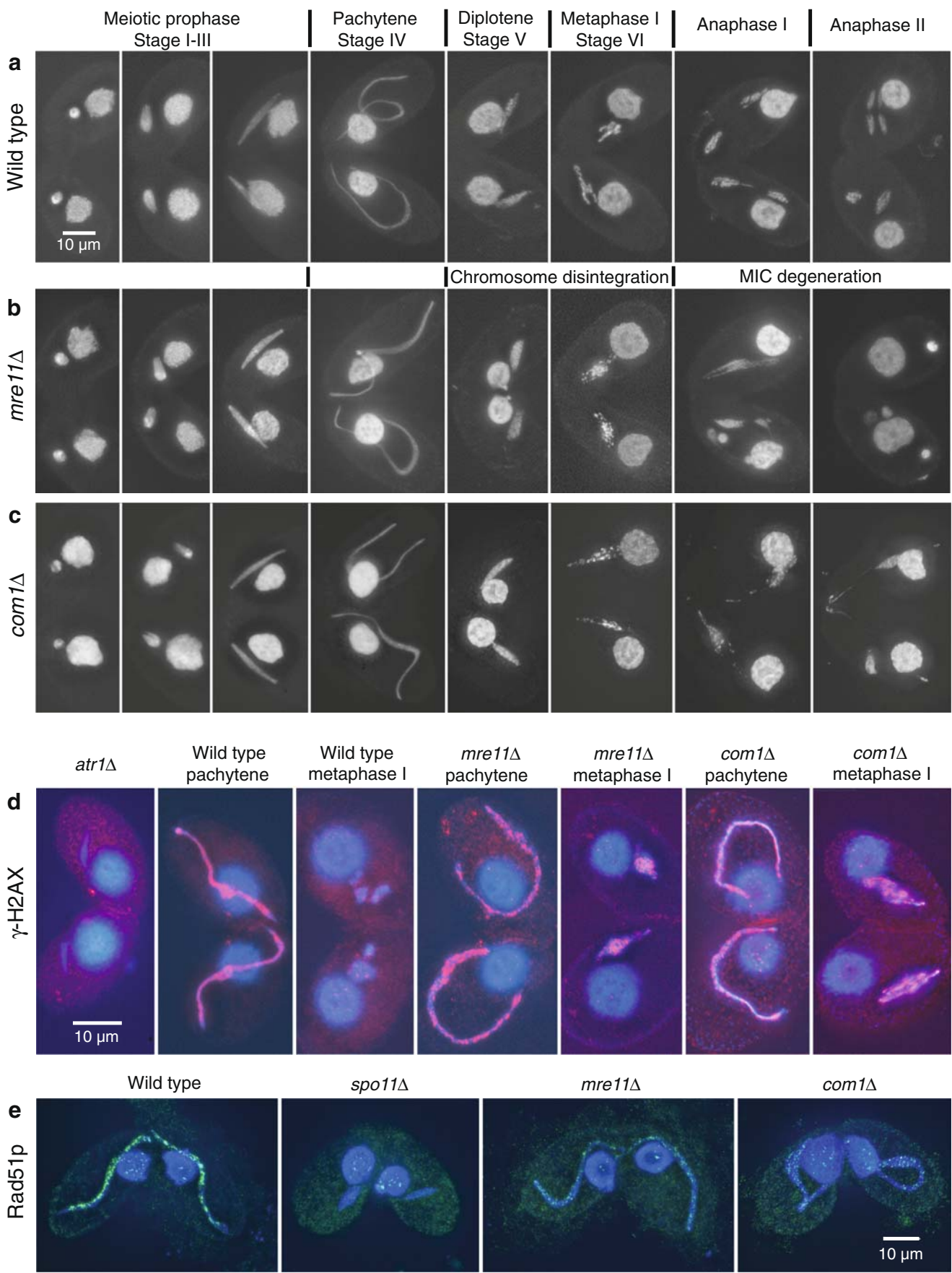
Because Mre11 was found to require the collaboration of Sae2/Com1 for meiotic DSB repair in the organisms tested

Fig. 2 Meiosis progression in the wild-type and mutants. **a** In the wild type, the MICs of conjugating cells elongate during meiotic prophase and achieve their maximal length at stage IV, which corresponds to pachytene. Thereupon, MICs shorten and condensed bivalents are visible at metaphase I. This stage is followed by a first and second meiotic division resulting in four meiotic products per cell. Meiosis in the **b** *mre11Δ* and **c** *com1Δ* mutants is cytologically normal during prophase but chromatin appears fragmented in both mutants in the stage corresponding to metaphase I. Most cells arrest at that stage (see Fig. 3b, c); only in a small minority, aberrant divisions and meiotic products are found. **a–c** DAPI staining. **d** Immunostaining of phosphorylated histone H2A.X (γ -H2A.X; red). In an *atr1Δ* control, H2A.X is not phosphorylated (see Loidl and Mochizuki 2009). (Shown are cells with DNA-poor MICs arrested at meiotic prophase). In the wild type, γ -H2A.X labeling (red) is intense in pachytene MICs but is virtually gone by metaphase I. In the *mre11Δ* and *com1Δ* mutants, strong labeling of γ -H2A.X is observed in pachytene and persists in aberrant metaphase I MICs. **e** Immunostaining of Rad51p/Dmc1p (green) of spread cells. There is massive formation of Rad51p/Dmc1p foci in meiotic MICs of the wild type, while they are completely absent from a *spo11Δ* control where DSBs are not formed. In the *mre11Δ* and *com1Δ* mutants, micronuclear Rad51p/Dmc1p foci are diminished

in this respect (see Raynard et al. 2008; Longhese et al. 2009), we wanted to know if cells lacking Com1p exhibit a similar phenotype. Indeed, similar to the *mre11Δ* mutant, the *com1Δ* mutant underwent wild-type MIC elongation (Fig. 2c), expressed γ -H2A.X (Fig. 2d), and formed diminished Rad51p/Dmc1p foci (Fig. 2e) in meiotic MICs. Notably, like the *mre11Δ* mutant, the *com1Δ* mutant displayed chromosome disintegration and arrest at metaphase I (Figs. 2c and 3c), which is consistent with the idea that the cooperation of the two genes in DSB repair is conserved in *Tetrahymena*.

A molecular assay for DSBs in *Tetrahymena*

To examine the course of DSB formation and to verify persistent DSBs as being the cause for metaphase I chromosome disintegration in the mutants, we developed a direct assay for meiotic DSBs in *Tetrahymena*. It is based on the electrophoretic separation of DSB-generated DNA fragments. In the absence of known recombination hotspots in *Tetrahymena*, we adopted a method by which DSBs can be detected as a smear during the separation of chromosome-sized DNA molecules by PFGE (Game et al. 1989; Loidl et al. 1998). In *Tetrahymena*, the region of the gel that is expected to display a DSB-generated smear is densely covered with an excess of MAC-derived chromosome fragments (Cassidy-Hanley et al. 2005 and Supplement S5). Therefore, we probed the gel with a MIC-specific DNA sequence. The Tlr sequence family consists of approximately 30 sequences with an ~22-kb conserved internal region. It is eliminated from the developing MAC and hence restricted to the MIC (Wuitschick et al. 2002). We used an ~8.5-kb fragment of this sequence as Southern probe.



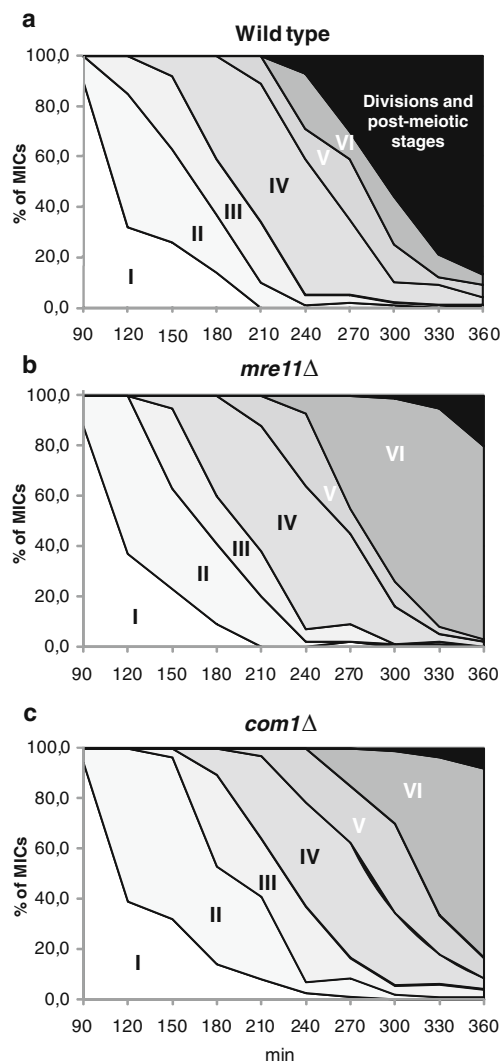


Fig. 3 Time course of meiotic stages. Staging was performed by morphological criteria according to Sugai and Hiwatashi (1974); I–IV prophase up to pachytene, V diplotene, VI first metaphase. The temporal appearance and duration of prophase stages are similar in the wild type (a) and in the *mre11Δ* mutants up to diplotene (b), with a possible slight delay in the *com1Δ* mutant (c). In the wild type, metaphase I is short and immediately followed by the two meiotic divisions and the appearance of four meiotic products, whereas the mutants arrest at an aberrant metaphase I stage (see Fig. 2a–c) with only a few cells attempting divisions at very late time points

When we extracted chromosome-sized DNA from a nonmeiotic wild-type culture, we found that MIC chromosomes (which are of an estimated 50 Mb each) did not enter the gel. However, micronuclear DNA of meiotic wild-type cells, where DSBs are expected to occur, was separated and produced a smear (Fig. 4a). This suggested that meiotic DSBs break MIC chromosomes into pieces, most of which were of a size around an estimated 1.5 Mb. As expected, micronuclear DNA from a *spo11Δ* meiotic culture did not produce a smear. As an additional control for the correspondence of the Southern signal with fragmented

DNA, we ran DNA from a meiotic *spo11Δ* culture treated with 50 μ g/ml bleomycin. This known inducer of DSBs indeed caused the appearance of a signal resembling the one in wild-type meiosis (Fig. 4a).

Because macronuclear DNA that does not undergo meiotic DSBs is present in >20-fold excess in *Tetrahymena* cells, large amounts of DNA had to be loaded onto the gel in order to visualize DSB-dependent micronuclear DNA fragments. Yet the smears were often quite weak. Therefore, we changed PFGE running conditions to increase signal intensities by concentrating labeled DNA into a smaller area. Upon shorter runs of the gel in combination with shorter pulse times, DNA fragments of different lengths migrated as a distinct band (Fig. 4b). This is due to the phenomenon of limit mobility, which is the uniform mobility of fragments above a certain size (Southern and Elder 1995). Using this procedure, no band was observed for wild-type nonmeiotic DNA and *spo11Δ* meiotic DNA, but a band appeared in wild-type meiotic DNA and bleomycin-treated *spo11Δ* meiotic DNA (Fig. 4b). Besides being faster, this protocol provided the advantage that the signal in the form of a distinct band could be more readily detected than a smeared signal of the same amount of DNA. Therefore, we applied the short procedure throughout this study.

mre11Δ and *com1Δ* mutant cells arrest in meiosis with unrepaired DSBs

Rad51 foci (Alpi et al. 2003) or γ -H2A.X foci or patches (Mahadevaiah et al. 2001; Song et al. 2007) in meiotic nuclei accompany meiotic DSBs but neither is a reliable indicator of DSB formation (see “Discussion” section). In the *mre11Δ* and *com1Δ* mutants, meiotic MICs displayed (diminished) Rad51p/Dmclp (Fig. 2e) as well as γ -H2A.X foci (Fig. 2d). Unlike in the wild type (Song et al. 2007; Mochizuki et al. 2008), the latter did not disappear by metaphase I (Fig. 2d), which might indicate that DSBs remain unrepaired.

The above PFGE-DSB assay overcomes the uncertainties intrinsic to these cytological indicators, and we used it to study DSB formation in wild-type and mutant meioses. For the wild type, it confirmed the appearance of DSBs as early as ~2.5 h after induction of meiosis (Fig. 5) which is in good agreement with the appearance of γ -H2A.X and strand exchange proteins Rad51p and/or Dmclp in elongating (stage II) MICs (Song et al. 2007; Mochizuki et al. 2008) around this time. The gradual loss of DNA fragments after their maximal abundance between 3 and 5 h post-meiosis induction corresponds with the disappearance of γ -H2A.X before metaphase I (Fig. 2d) and the formation of intact bivalents (Fig. 5). (For a high-temporal-resolution gel, see Supplement S6.) In agreement with the conserved

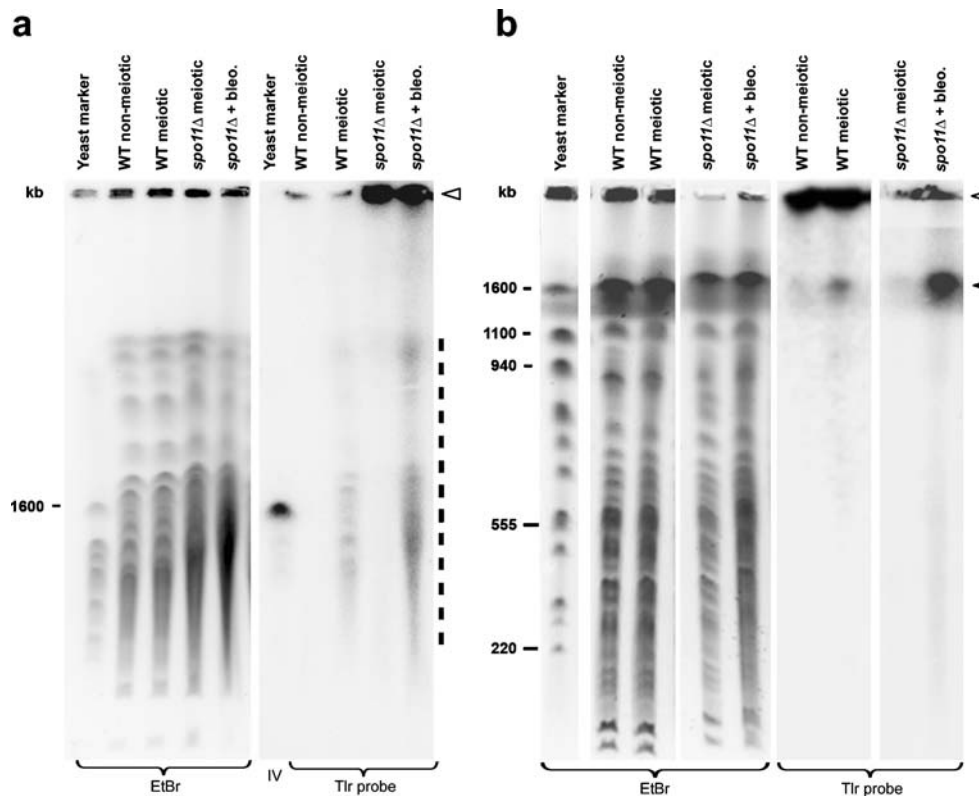


Fig. 4 Detection of DSBs by DNA electrophoresis. **a** The small macronuclear chromosomes are separated by PFGE and form a ladder after ethidium bromide (*EtBr*) staining. Southern hybridization with a MIC-specific probe (*Tlr1*) does not label nonmeiotic wild-type (*WT*) DNA because the intact ~50-Mb-sized MIC chromosomes are too large to enter the gel. However, MIC DNA of meiotic cells enters the gel and produces a smear in the region indicated by the dashed line. In the *spo11Δ* mutant, no meiotic DSBs are formed, and fragmented DNA is not detected. However, DSBs induced in the mutant DNA by bleomycin (50 μ g/ml) produce a smear. The budding yeast size marker was probed with DNA from chromosome *IV* (1,600 kb). **b** During

shorter PFGE runs under different conditions, DSB-dependent fragments do not separate, but migrate as a single band (solid arrowhead) due to a phenomenon known as limit mobility. This improves detectability of the fragments because the Southern signal is concentrated in a smaller area. The position of the loading wells is marked by open arrowheads in **a** and **b**. Different Southern signal intensities in the loading wells do not reflect different amounts of DNA loaded or labeled, but are due to the loss of agar plugs during handling of the gels. Instead, similar *EtBr* staining intensities indicate that comparable amounts of DNA were loaded for each of the different samples

role of Spo11 in inducing meiotic DSBs (Keeney 2007), bands were absent in the *spo11Δ* mutant except for weak signals which probably reflect preparation-induced DNA breakage, and chromosomes remained univalent (Fig. 5).

In stark contrast, DNA fragments persisted in the *mre11Δ* and *com1Δ* mutants to 6–7 h post-meiosis induction (Fig. 5) at which time abnormal metaphase I MICs arrested with disintegrated chromosomes (Figs. 2b, c and 5). This is a clear evidence that the observed cytological defect is due to unrepaired DSBs. At the same time, this arrest suggests the existence of a meiotic DNA damage or recombination checkpoint in *Tetrahymena*.

Homologous pairing is reduced in *mre11Δ* and *com1Δ*

We have shown previously that the confinement of chromosomes within the tubular pachytene MIC promotes the juxtaposition of homologous loci but is not sufficient

for precise pairing (Loidl and Mochizuki 2009). To study whether repair of DSBs is required for the interaction and precise pairing between homologous loci, we tested pairing in the *mre11Δ* and the *com1Δ* mutants by FISH (Fig. 6a). Homologous pairing in pachytene nuclei was determined for an ~22-kb locus in the middle of a chromosome (Loidl and Mochizuki 2009). Two loci were scored as paired if the corresponding FISH signals merged into a single dot. We found that pairing was significantly reduced in both mutants (Fig. 6b). Thus, in *Tetrahymena* like in most other organisms, precise and/or stable pairing may require the matching of homologous DNA sequences during the interaction of DSB sites with homologous chromosomal loci (Peoples-Holst and Burgess 2005; Zickler 2006).

In addition, we noticed that MICs of the *com1Δ* mutant frequently contained three or four FISH signals (Supplement S7). The respective frequencies were 21.8% and 4.4% ($n=206$ pachytene nuclei counted). In comparison, only 2.4%

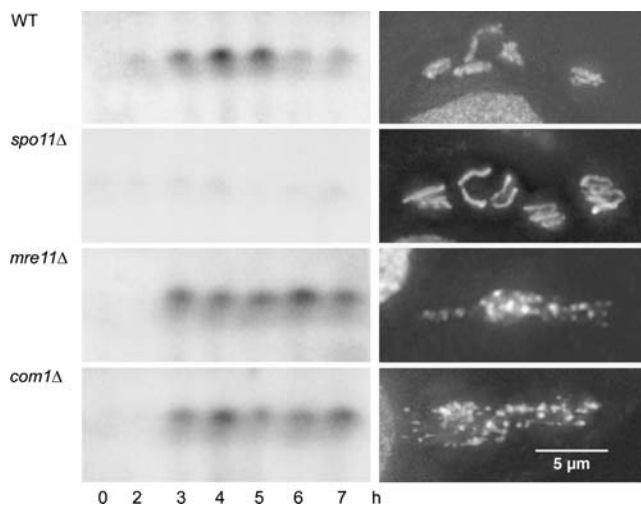


Fig. 5 The course of meiotic DSB formation and repair. *Left*: DSBs are detected as DNA fragmentation in DNA electrophoresis. *Right*: Chromosomes at diakinesis–metaphase I visualized by in situ DAPI staining. In the wild type, DSB-dependent bands appear from $t=3$ h to $t=5$ h after induction of meiosis. Recombinational repair of DSBs results in crossovers and five bivalents are formed at diakinesis–metaphase I. In the *spo11Δ* mutant, DSBs are not formed and chromosomes remain univalent. In the *mre11Δ* and *com1Δ* mutants, DSBs-dependent chromosome fragments appear like in the wild-type and are still prevalent at $t=7$ h after induction of meiosis. This is reflected by fragmented chromatin at diakinesis–metaphase I. DNA samples were taken from cultures in which cells entered meiosis synchronously and with high efficiency to ensure that signal strengths reflected relative DSB frequencies between different time points and strains

and zero of wild-type MICs ($n=208$) displayed three or four signals. (In the *mre11Δ* mutant, this phenomenon was less prevalent with 6.2% and 1.4% MICs displaying three or four FISH signals; $n=210$). While the frequent presence of more than two FISH signals suggests impaired sister chromatid

cohesion in the *com1Δ* mutant, we do not at present have evidence to support this notion.

Discussion

Direct evidence for meiotic DSBs in *Tetrahymena*

Meiotic DSBs were first observed in Southern blots as a meiosis-specific subfragment of a restriction fragment containing a meiotic recombination hotspot of *Saccharomyces cerevisiae* (Sun et al. 1989; Cao et al. 1990), and similar direct observation of meiotic DSBs was made in *Schizosaccharomyces pombe* (see Cromie and Smith 2007). In addition, the ubiquitous occurrence of *SPO11* homologues (Malik et al. 2007) provides support to the universality of Spo11-dependent meiotic DSBs. However, the possibility remains that some recombination events derive from other pathways (Farah et al. 2005) or that alternatives are employed by evolutionarily distant eukaryotes. In experimental systems where the direct detection of meiotic DSBs is not feasible, only various indirect evidence of their occurrence was produced. It was based on the induction of recombination by artificially induced DSBs (Dernburg et al. 1998) and on the cytological detection of chromosome fragmentation in meiotic mutants (Pasierbek et al. 2001; Puizina et al. 2004). Also, the cytological detection of Rad51 foci (Alpi et al. 2003) or γ -H2A.X foci or patches (Mahadevaiah et al. 2001; Song et al. 2007) provides indirect evidence for the presence of DSBs and is used for the characterization of meiotic mutants. However, these methods are not always reliable. First, the formation of Rad51 (or Dmc1) foci depends on DSB resectioning. Also, H2A.X phosphorylation is not an unmistakable sign

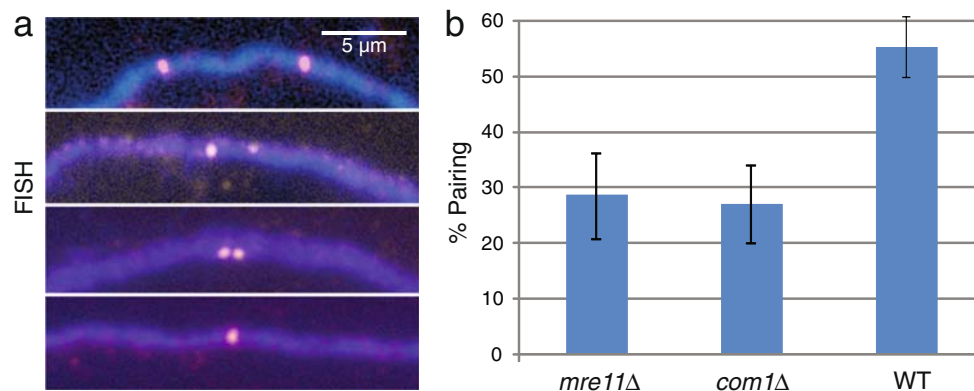


Fig. 6 Homologous pairing in the wild type and mutants. **a** Evaluation of homologous pairing in elongated (pachytene) MICs by FISH to a chromosomal locus (pink). Two separated FISH signals (upper three panels) indicate unpaired loci whereas a single FISH signal (bottom) indicates precise pairing. **b** In both the *mre11Δ* and

com1Δ mutants precise pairing is significantly reduced. Values are a mean of seven countings by three persons of three biological repeats per genotype, with 50 pachytene MICs evaluated in each counting. Error bars denote standard deviations

of DSBs, as several cases were described where it was induced by DNA lesions other than DSBs (Marti et al. 2006; Hanasoge and Ljungman 2007; Ismail and Hendzel 2008; Smart et al. 2008).

Here, we made use of the inducible and highly synchronous meiosis of *Tetrahymena* to mass-isolate meiotic DNA for PFGE-based detection of DSBs, and we confirmed that meiotic recombination in the protist *Tetrahymena* follows the canonical Spo11- and DSB-dependent process. In combination with its suitability for cytological, genetic, and molecular analyses of meiotic processes (Turkewitz et al. 2002), the availability of a direct assay for meiotic DSBs makes *Tetrahymena* an ideal model system for meiosis.

Mre11p is dispensable as a sensor of meiotic DSBs

Numerous studies in yeasts and vertebrates have revealed that DSBs are sensed by the phosphatidylinositol 3-kinase-related checkpoint kinases ATM and ATR. Whereas ATM primarily recognizes blunt-end DSBs, ATR requires single-stranded tails (e.g., those resulting from ATM-dependent processing of blunt-end DSBs; see, Rhind 2009). At least in mitotic cells, ssDNA tails are first engaged by the ssDNA-binding protein replication protein A (RPA), which, with the help of ATRIP recruits ATR. RPA is then replaced by Rad51 (plus Dmcl in meiosis) which allows it to initiate recombinational repair by the strand exchange reaction (see Raynard et al. 2008; Harrison and Haber 2006). Since activation of ATM at blunt-end DSBs depends on the MRN complex (see Williams et al. 2007; Borde and Cobb 2009), the activation of ATR in meiosis might be expected to require the involvement of the MRN complex even at two steps, namely for the activation of ATM and later for removing covalently bound Spo11 and initiating 5' strand resectioning in cooperation with Sae2/Com1 (Klein 2008; Raynard et al. 2008; Garcia-Muse and Boulton 2005).

The situation in *Tetrahymena* turned out different, however. So far *ATR1* (TTHERM_01008650) was identified as the only gene encoding an ATM/ATR-related kinase (with a closer sequence similarity to the ATR/Mec1 family; Yakisich et al. 2006; Loidl and Mochizuki 2009). Moreover, here we found that Mre11p is not part of the ATR-dependent signal cascade from DSBs to meiotic MIC elongation and mitotic and meiotic H2A.X phosphorylation (Figs. 2 and 7). Thus it seems that *Tetrahymena* Mre11p plays a minor role, if any, in DNA damage signaling.

The nature of the DNA lesion (blunt or staggered ends) that triggers the ATR response in *Tetrahymena* meiosis remains unclear, but two scenarios are conceivable: (1) The *Tetrahymena* ATM/ATR homologue, although more closely resembling ATR, can behave like ATM, making the phosphorylation cascade leading to MIC elongation and

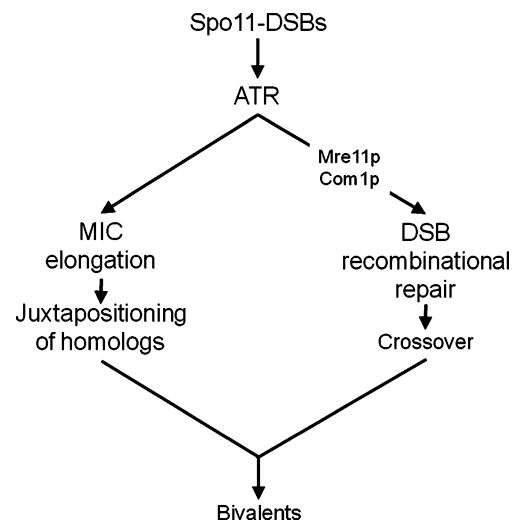


Fig. 7 Meiotic activities of ATR, Mre11p, and Com1p in coordinating homologous alignment and crossover in *Tetrahymena*. Meiotic DSBs (and probably also non-DSB DNA lesions) are sensed by ATR (Loidl and Mochizuki 2009). Mre11p is not required for DSB induction (unlike in *Caenorhabditis elegans* and budding yeast) nor the recruitment and activation of ATR unlike in the cases reported in the literature (see text). ATR triggers MIC elongation which in turn promotes the juxtapositioning of homologous chromosomes. In addition, the failure of Rad51p and/or Dmclp to localize to DSBs in the absence of ATR (data not shown), suggests a role for it in the meiotic DSB repair pathway of *Tetrahymena*. Mre11p and Com1p are involved in DSB repair leading to crossing over and concomitant precise pairing. They are likely to act downstream of ATR because numerous DSB repair proteins, including Com1/Sae2, are known as ATM/ATR targets (see Longhese et al. 2009). The juxtapositioning of homologous chromosomes is necessary to promote homologous recombination over ectopic recombination or sister chromatid recombination, and thus the MIC elongation and DSB repair pathways collaborate in the formation of bivalents

H2A.X phosphorylation independent of ssDNA overhangs. (2) There occurs (at least limited) MRN/Mre11p (and Com1p)-independent ssDNA exposure, sufficient to trigger ATR. The observation of residual Rad51p/Dmclp foci formation by *mre11Δ* and *com1Δ* mutants (Fig. 2e) supports the latter possibility because these recombination proteins probably require ssDNA tracts for loading.

Tetrahymena MRE11 and *COM1* are required for meiotic DSB repair

Mre11 and Sae2/Com1 and their respective orthologues have been shown in various organisms to be required for the production of fertile gametes. The roles of Mre11 during meiosis are many and vary between organisms with respect to its requirement for DSB formation. In *C. elegans* and budding yeast, Mre11 and its partners in the MRN/MRX complex are required for the induction of DSBs by Spo11, whereas this is not the case in *S. pombe*, *Coprinus cinereus*, *Arabidopsis thaliana*, and mammals (Gerecke and Zolan 2000; Bleuyard et al. 2004; Puizina et al. 2004;

Young et al. 2004; Keeney 2007; Cherry et al. 2007). Similarly, in *Tetrahymena*, Mre11p is dispensable for DSB formation. Moreover, we could not find evidence for a function of Mre11p in DSB signaling (see above). We rather observed similar cytological and electrophoresis phenotypes indicative of chromosome fragmentation for both the *mre11* Δ and *com1* Δ mutants, suggesting a cooperative role of the two proteins in the repair of meiotic DSBs (Fig. 7).

It is believed that Sae2/Com1, in cooperation with Mre11 and its partners, prepares DSB ends for degradation by trimming them. Next, the ends are resected, either by Sgs1 helicase plus a nuclease such as Dna2 or by the Exo1 nuclease, to give 3' ssDNA tails (for review, see Klein 2008). In meiosis, the processing of DNA ends flanking DSBs can take place only if they are liberated from covalently bound Spo11 (for review see Williams et al. 2007). It has been directly demonstrated for budding yeast and fission yeast that Mre11(Rad32) and Sae2/Com1(Ctp1) are involved in the removal of covalently bound Spo11, which then allows exonucleolytic strand resectioning (Neale et al. 2005; Hartsuiker et al. 2009). Observations in *Arabidopsis* and *C. elegans* confirmed the evolutionary conservation of this mechanism (Uanschou et al. 2007; Penkner et al. 2007). Here, we extend the range of organisms with conserved meiotic roles of Mre11 and Sae2/Com1 to the kingdom of protists by demonstrating that in the ciliate *Tetrahymena*, meiotic chromosomes accumulate unrepaired DSBs in the absence of these two proteins.

The fact that MICs with unrepaired DSBs arrested prior to the first meiotic division (Fig. 3) might suggest the existence of a meiotic checkpoint monitoring chromosome integrity. However, meiosis could also arrest because the massive chromosome fragmentation (Fig. 5) prevents association of most of the DNA with the spindle, and hence, the assembly of telophase I nuclei. Notably, no meiotic checkpoint activity was found in mutant meioses during which chromosomes were lost and DSBs possibly remained unrepaired due the absence of γ -H2A.X. This led to the conclusion that a recombination (pachytene) checkpoint might be weak or nonexistent in *Tetrahymena* (Song et al. 2007).

Tetrahymena MRE11 and *COM1* are required for precise chromosome pairing

There exist DSB-independent and dependent pathways to meiotic chromosome pairing. Dipterans, *C. elegans*, and a few others completely rely on the former (see Zickler 2006). However, most organisms seem to employ a mixed strategy by achieving rough homologous alignment or random homologous contacts by DSB-independent mech-

anisms such as telomere clustering and horsetail movement followed by DSB-dependent precise matching (Zickler 2006; Barzel and Kupiec 2008). Only if the distance between homologues falls below one that can be bridged by DSB-dependent homology recognition will precise matching occur. Mutants in various factors involved in homologous strand invasion by ssDNA (such as Hop2 and Dmc1) display reduced or abolished pairing, suggesting that it is this step that confers precise meiotic pairing (Peoples-Holst and Burgess 2005). Accordingly, in the absence of 5' to 3' resection of DSBs in an *Arabidopsis com1* mutant, homologue meiotic pairing is abolished (Uanschou et al. 2007).

Tetrahymena employs a two-step pairing process for rough and precise/stable pairing which both are dependent on DSBs (Fig. 7). It has been proposed that a rough alignment of homologous chromosomes is brought about by their parallel and centromere–telomere polarized arrangement within the tubular MIC (Kaczanowski et al. 1985; Loidl and Scherthan 2004; Mochizuki et al. 2008). Here, we show that full pairing does not occur in the absence of Mre11p and Com1p where DSBs are not repaired. This suggests that precise pairing may require the physical linkage of homologues by exchanging DNA strands in the course of DSB repair. The rough alignment of chromosomes ensures that DSB repair takes place by the recombination of allelic rather than ectopic sites and promotes the involvement of homologous chromosomes rather than sister chromatids in recombination. Thus, the DSB-dependent MIC elongation- and repair-branches collaborate in homologous recombination and bivalent formation (Fig. 7).

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Supplementary figure legends

Fig. S1. Macronuclear gene knockout constructs. a. Design of knockout constructs including position of the Southern probe. b. Primer sequences for knockouts and generation of the Southern probes.

Fig. S2. Mre11 protein sequence alignment and functional features. A multiple sequence alignment (MSA) was constructed for Mre11 representatives from archaea, ciliata, plantae, fungi and metazoa using MAFFT (Katoh et al. 2002). (A) Detectable PFAM domain hits (PF00149 and PF04152) and experimentally derived protein features such as DNA-binding domains studied in yeast Mre11 (Usui et al. 1998), GR-motifs in human Mre11 (Déry et al. 2008), DNA-recognition loops (RL 1-6) and nuclease motifs (I-V) defined for *Pyrococcus furiosus* Mre11 (Williams et al. 2008) were mapped to the MSA, and the thereby derived domain architecture is presented. Highest conservation between the selected Mre11 proteins is found around the nuclease motifs (I-V) as illustrated by a conservation quality plot (B) and the presented MSA details (C). The positions of human ATLD mutations N117S and W210C are indicated by arrows below the MSA. Circles denote the residues known to be involved in Mn²⁺ coordination and ester hydrolysis in *Pyrococcus furiosus* Mre11.

Organism abbreviations and sequence identifiers: Tet- *Tetrahymena thermophila* XP_001031877, Pat- *Paramecium tetraurelia* XP_001457861, Art- *Arabidopsis thaliana* NP_200237, Pot- *Populus trichocarpa* jgi269050, Aea- *Aedes aegypti* XP_001654690, Drm- *Drosophila melanogaster* NP_523547, Mum- *Mus musculus* NP_061206, Hos- *Homo sapiens* NP_005582, Sac- *Saccharomyces cerevisiae* NP_013951, Asn- *Aspergillus niger* XP_001392848, Pyf- *Pyrococcus furiosus* Q8U1N9

Fig. S4. γ -H2A.X foci in the MICs of vegetatively growing wild-type (a) and *mre11* Δ (b) cells upon 1h treatment with 10 μ g/ml bleomycin. (For the DSB-inducing activity of the concentration used, see Figure 4.) An untreated wild type control is shown in (c). Both in the treated wild type and *mre11* Δ mutant, MICs displayed γ -H2A.X labelling in 100% of cells (n=200). Staining of MACs was less pronounced and not quantified. H2A.X phosphorylation due to DSB induction in the mutant indicates that Mre11p is not required for DSB sensing in vegetative cells.

Fig. S5. PFGE timecourse of conjugating wild-type cells from t=0 h (mixing of cells = induction of meiosis) to t=10 h after meiotic induction. EtBr staining. PFGE separates the macronuclear chromosomes (a.k.a. autonomously replicating pieces) (Orias 1998). Due to the large amount of DNA loaded in this experiment, the gel displayed fewer than the ~225 macronuclear chromosomes (autonomously replicating pieces) which are normally resolved by PFGE (Orias 1998; Eisen et al. 2006). Micronuclear chromosomes do not enter the gel because of their size (~50 Mb). Unlike in

budding yeast where a DSB-dependent smear appears in meiotic samples (Loidl et al. 1998), fragmentation of micronuclear meiotic chromosomes (expected from about 3 h after induction of meiosis) is not visible in EtBr-stained pulsed-field gels of *Tetrahymena*. Because the MAC (which does not undergo meiosis) is ~45-ploid, it contains a >20 times excess of DNA which conceals the DNA from the diploid MIC. Therefore, meiotic DSBs can be visualized only by Southern hybridization with a MIC-specific DNA probe (see Figure 4). However, beginning at 6 h after induction of meiosis, a smear indicating fragmentation of macronuclear chromosomes starts to form, and 8 h after induction of meiosis, intact macronuclear chromosomes are notably reduced. This loss is due to DNA degradation accompanying the elimination of the old MAC, which is part of *Tetrahymena*'s developmental programme (see (Martindale et al. 1982; Karrer 2000). Only later, a new MAC which is reconstituted from a zygotic MIC, will amplify its DNA. Budding yeast strain SK1 was used as the size marker (Loidl et al. 1998).

Fig. S6. High-temporal-resolution of DSB dynamics in the wild type. DSBs are most abundant from t=3h45' to t=4h15' after induction of meiosis. Independent time courses produced similar results. Intensities of a ca. 940 kb band with ethidium bromide staining well below saturation (upper panel) were measured to determine the relative amount of loaded DNA (ADL) for the different time points. For this, the Gel Analysis Tool of ImageJ (Wayne Rasband, N.I.H.; <http://rsb.info.nih.gov/ij/>) was used. The intensities of hybridized bands (BI) shown in the lower panel were corrected for the amount of DNA. The corrected relative intensities of hybridization signals are plotted at the bottom with the weakest band arbitrarily set to one unit.

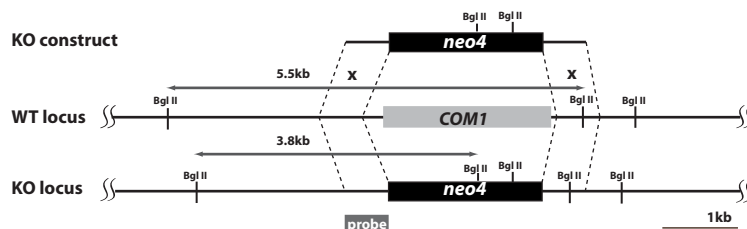
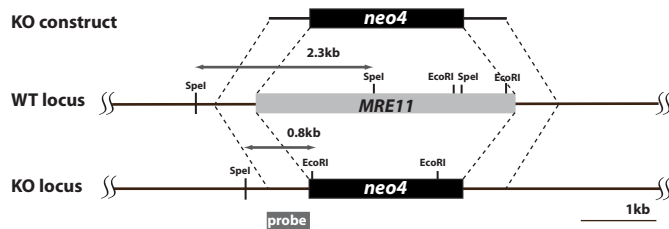
Fig. S7. Examples of exceptional MICs with more than two FISH signals in the *com1Δ* mutant. While attenuated sister chromatid cohesion or over-replication could possibly explain this observation, these anomalies are difficult to reconcile with the supposed defect of the *com1Δ* mutant in DSB repair (see text).

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Figure S1

Construction of gene knockouts and list of primers used

a**b**

MRE11KO5FW 5'-CTA ACC CGT ATT TCC CGC-3' *
 MRE11KO5RV 5'-GTC TAT CGA ATT CCT GCA GCC CTC ATC TTA TTG ATA GTT TGC TTG-3' *
 MRE11KO3FW 5'-CTG GAA AAA TGC AGC CCG CTT TCA TTG ATT GAT TAA GAG-3'
 MRE11KO3RV 5'-GCT GCT ATC TTG TTC CTT TC-3'
 COM1KO5FW 5'-GAG TTT TGC TTT AGA AAA ATC-3' **
 COM1KO5RV 5'-GTC TAT CGA ATT CCT GCA GCC CGA GAG ATT CTT GGT ATA TTT G-3' **
 COM1KO3FW 5'-CTG GAA AAA TGC AGC CCC TTA ATG AGA TTT CAA AAT TG-3'
 COM1KO3RV 5'-AAT AAT CAG TAT TCT GCT AC-3'

The underlined sequences in MRE11KO5RV and COM1KO5RV are complementary to a 5'-flanking region of the neo4 cassette and MRE11KO3FW and COM1KO3FW to a 3'-flanking region. The primer pairs denoted * and ** were used to generate Southern probes that distinguish between wild-type and disrupted loci for the confirmation of successful knockout of MRE11 and COM1, respectively.

Figure S2

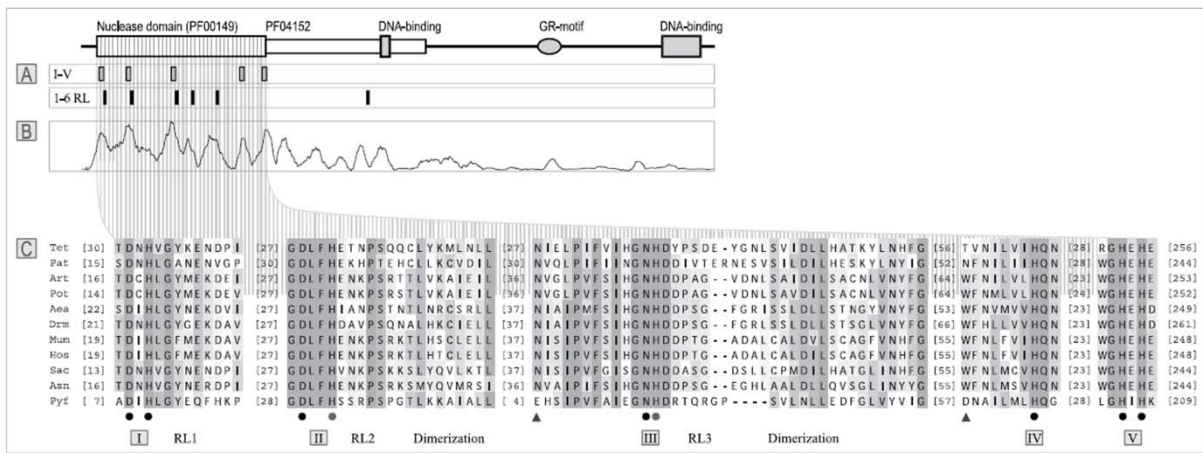


Table S3. Crosses of different *mre11*Δ and *com1*Δ knockout lines all produce dead sexual progeny

Conjugating partners	% Viable progeny	No. of conjugants
Wild type B2086 × C428	67.7	192
B2086 <i>mre11</i> Δ 8(4) × C428 <i>mre11</i> Δ 23(6)	0	128
B2086 <i>mre11</i> Δ 5(6) × C428 <i>mre11</i> Δ 9(3)	0	128
B2086 <i>mre11</i> Δ 8(4) × C428 <i>mre11</i> Δ 9(3)	0	128
B2086 <i>mre11</i> Δ 5(6) × C428 <i>mre11</i> Δ 23(6)	0	128
B2086 <i>com1</i> Δ 8(3) × C428 <i>com1</i> Δ 23(4)	0	128
B2086 <i>com1</i> Δ 1(1) × C428 <i>com1</i> Δ 8(2)	0	128
B2086 <i>com1</i> Δ 8(3) × C428 <i>com1</i> Δ 8(2)	0	128
B2086 <i>com1</i> Δ 1(1) × C428 <i>com1</i> Δ 23(4)	0	128

Figure S4

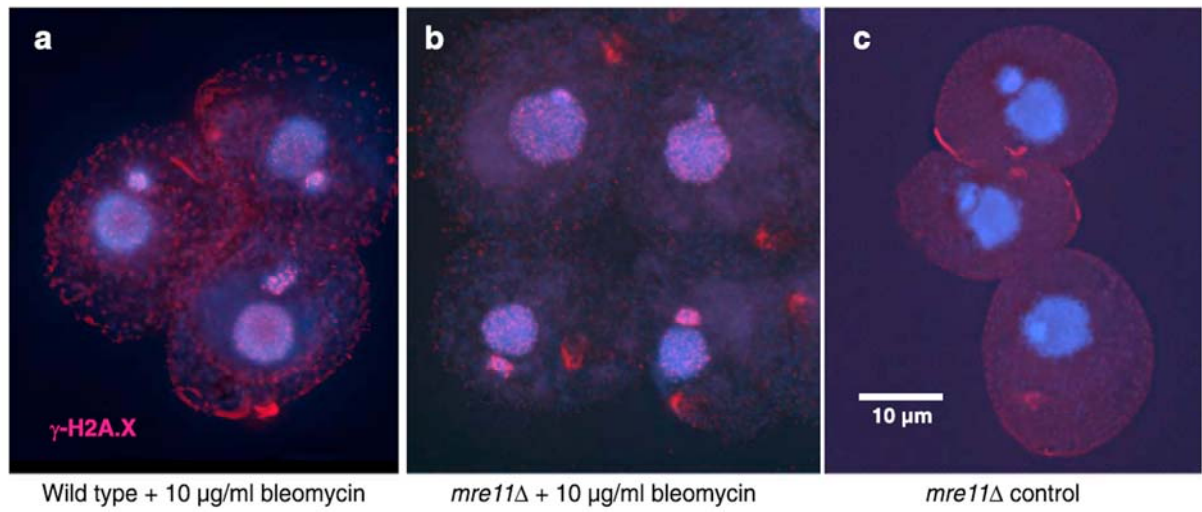


Figure S5

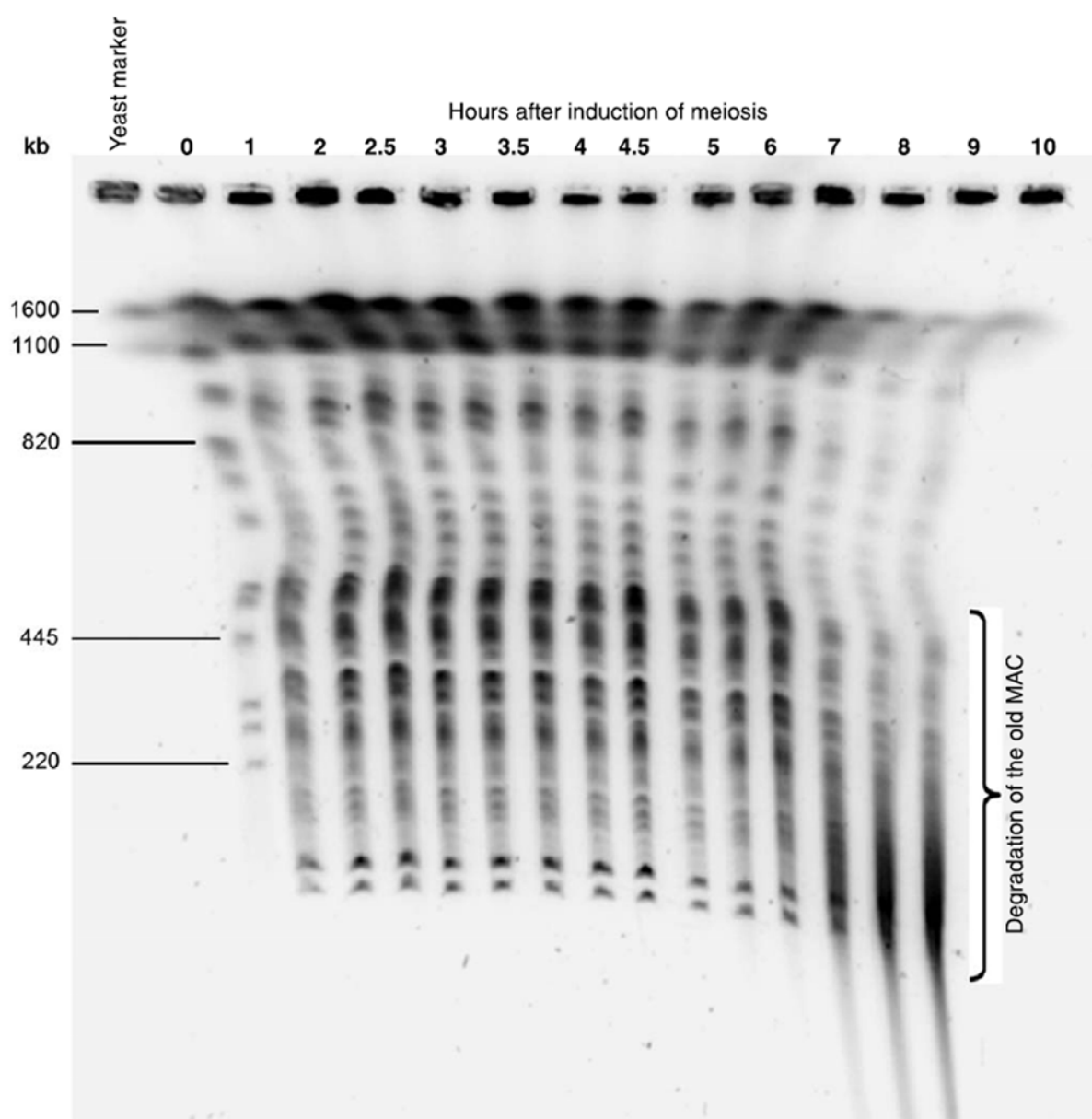


Figure S6

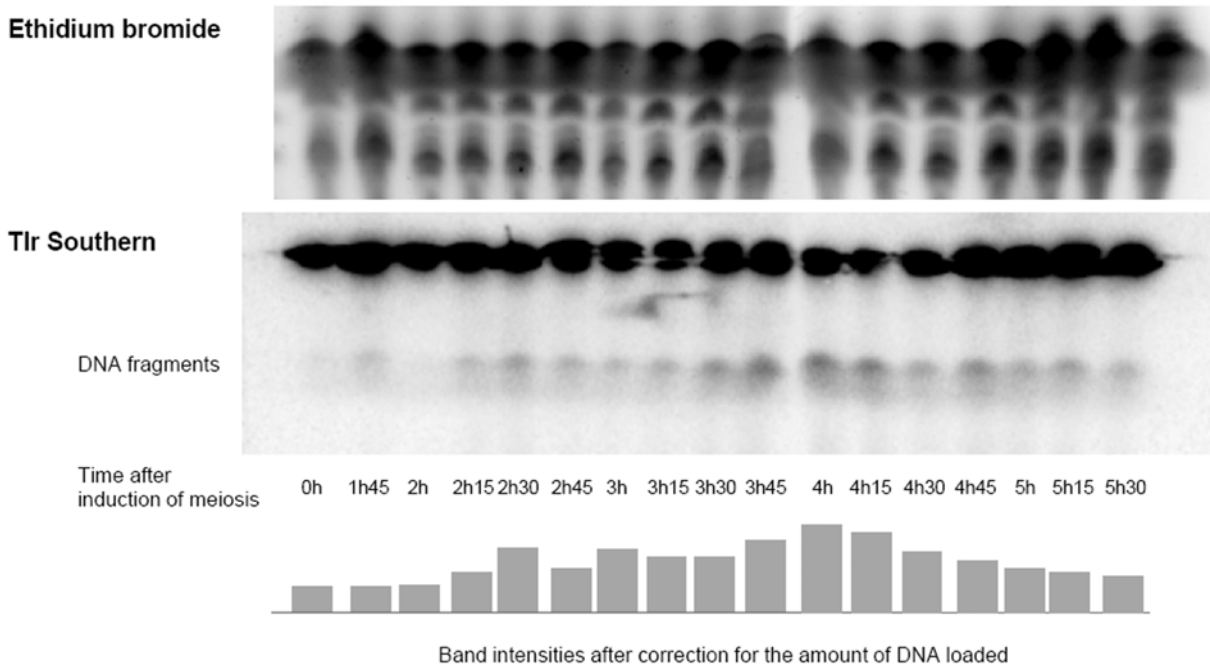
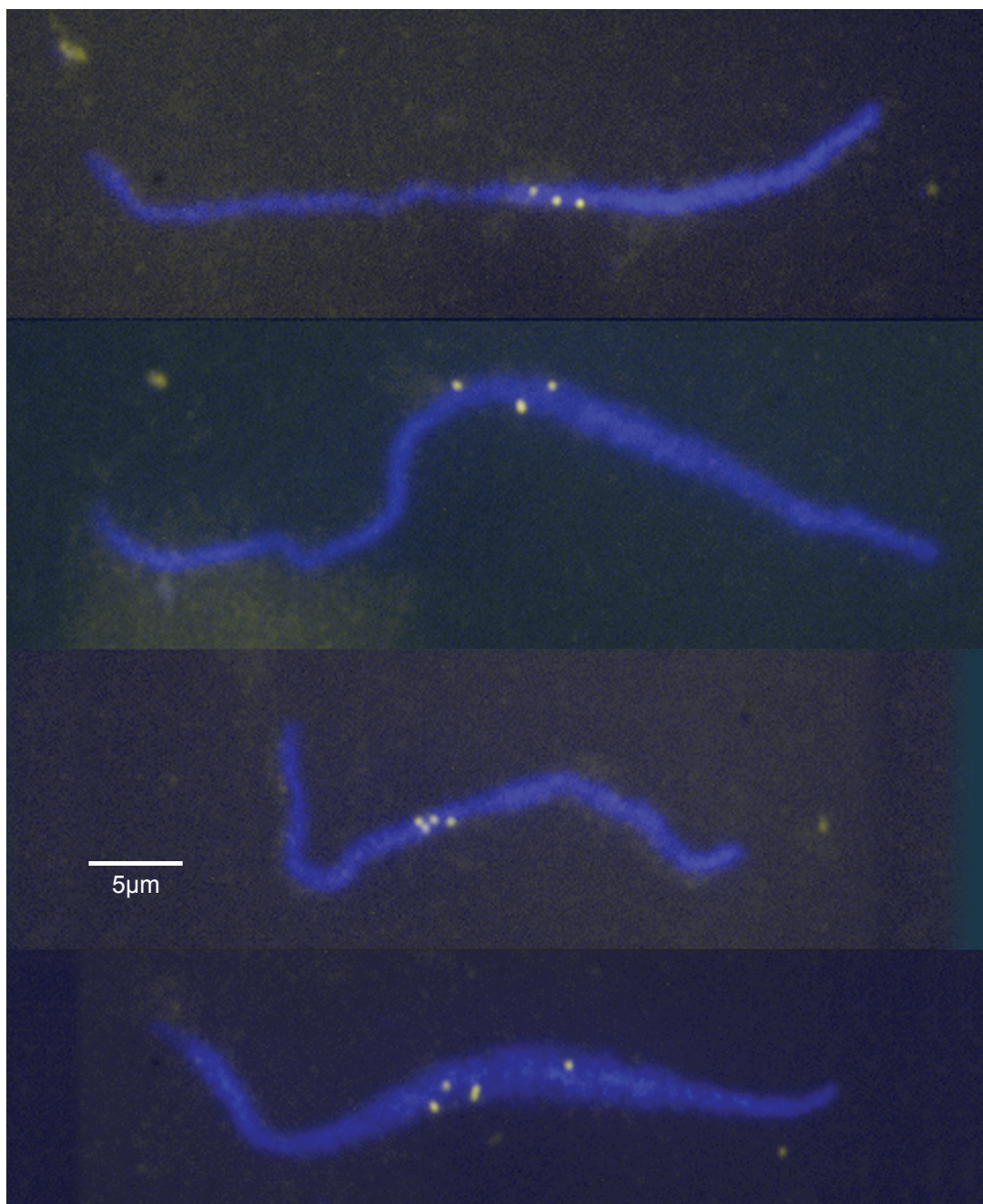


Figure S7



2. Mus81 nuclease and Sgs1 helicase are essential for meiotic recombination in a protist lacking a synaptonemal complex

Author contributions:

Agnieszka Lukaszewicz: Fig. 2a, part of Fig. 2b, part of Fig. 2c, part of Fig. 2d, Fig. 3a, Fig. 3b, Fig. 3c, Fig. 3d, Fig. 3e, part of Fig. 3f, Fig. 4, part of Fig. 5, Table S1, Table S2, Fig. S2, Fig. S3, part of Fig. S4, Fig. S5, Fig. S6, part of Fig. S7

Rachel A. Howard-Till: part of Fig. 2b, part of Fig. 2d, part of Fig. 3f

Josef Loidl: Fig. 1, part of Fig. 2b, part of Fig. 2c, part of Fig. 2d, part of Fig. 3f, part of Fig. 5, Fig. S1, part of Fig. S4, part of Fig. S7, Fig. S8

Mus81 nuclease and Sgs1 helicase are essential for meiotic recombination in a protist lacking a synaptonemal complex

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ABSTRACT

Mus81 resolvase and **Sgs1** helicase have well-established roles in mitotic DNA repair. Moreover, **Mus81** is part of a minor crossover (CO) pathway in the meiosis of budding yeast, plants and vertebrates. The major pathway depends on meiosis-specific synaptonemal complex (SC) formation, ZMM proteins and the MutL γ complex for CO-directed resolution of joint molecule (JM)-recombination intermediates. **Sgs1** has also been implicated in this pathway, although it may mainly promote the non-CO outcome of meiotic repair. We show in *Tetrahymena*, that homologous chromosomes fail to separate and JMs accumulate in the absence of **Mus81** or **Sgs1**, whereas deletion of the MutL γ -component **Mlh1** does not affect meiotic divisions. Thus, our results are consistent with **Mus81** being part of an essential, if not the predominant, CO pathway in *Tetrahymena*. **Sgs1** may exert functions similar to those in other eukaryotes. However, we propose an additional role in supporting homologous CO formation by promoting homologous over intersister interactions. *Tetrahymena* shares the predominance of the **Mus81** CO pathway with the fission yeast. We propose that in these two organisms, which independently lost the SC during evolution, the basal set of mitotic repair proteins is sufficient for executing meiotic recombination.

INTRODUCTION

Meiosis is the division by which germ progenitor cells reduce the somatic diploid chromosome set to the gametic haploid set. The chromosomes of the haploid set are mosaics assembled from corresponding parts of homologous parental chromosomes. The exchange of chromosome parts occurs by crossing over (CO). It

contributes to the recombination of parental genes in the gametes and the genetic diversity of the offspring. At the same time, CO is instrumental in connecting homologous chromosomes by chiasmata, which are required for the correct bipolar orientation of bivalents during the first meiotic division. If CO is compromised, chromosomally unbalanced gametes may be formed.

CO is induced by programmed DNA double-strand breaks (DSBs). At a CO site, one of the four chromatids of a chromosome pair experiences a DSB made by a dedicated endonuclease, Spo11 (1). The DSB is widened to a gap, and DNA flanking the DSB is resected in the 5'–3' direction, exposing single-stranded 3' overhangs. These single-stranded DNA ends associate with strand exchange proteins Rad51 and Dmc1, and one end invades a DNA double strand, which results in a three-way DNA structure, the so-called displacement loop (D-loop). If strands within the D-loop are complementary, they form a heteroduplex, and the invading strand extends by DNA synthesis (2). Most heteroduplexes seem to be short-lived and become unwound by helicases. Other D-loops capture the second DSB end and expand into a stable joint molecule (JM). The standard model of CO, elaborated in budding yeast, invokes a JM consisting of two Holliday junctions (HJs) (3). To disengage, JMs must be resolved by endonucleases. Depending on the cleavage orientation of the two HJs, the ligation of nicked strands may result in a reciprocal exchange of two DNA molecules, corresponding to a CO, or alternatively, in a nonreciprocal exchange, a noncrossover (NCO) (4,5).

Based on their function in meiotic CO formation and their ability to cleave JMs *in vitro*, several potential eukaryotic HJ resolvases were identified. These were the Mlh1–Mlh3 (also known as MutL γ) complex, the Rad1–Rad10 complex, the Mus81–Mms4/Eme1 complex, the Slx1–Slx4 complex and Yen1/GEN1 (6–8). Of these, the MutL γ complex (with Mlh3 containing the nuclease motif) produces (in collaboration with Exo1) the majority of COs in budding yeast (9). The MutL γ complex seems to work on JMs that are generated in the

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context of a synaptonemal complex (SC) and the so-called ZMM proteins (Zip1,2,3, Msh4,5, Mer3, Spo16 and Spo22) (10,11). Paradoxically, Sgs1 (the BLM helicase ortholog), which has long been known for its mitotic anti-CO activity, functions as a CO promoting factor as well (9,12–14). There also exists a minor pathway in the budding yeast that is independent of ZMM proteins and MutL γ , and involves the Mus81–Mms4 nuclease complex (and, to a lesser degree, Yen1).

The fission yeast is believed to feature a nondouble HJ form of JM, either a single HJ (15) or a nonreciprocal (i.e. nicked HJ) CO precursor (16–18). JM resolution largely depends on the Mus81–Emel (the Mms4 ortholog) complex (16,19–21). *Arabidopsis* and mammals feature both a ZMM–MutL γ -dependent pathway and a Mus81-dependent pathway, with the former being predominant (22–24). *Caenorhabditis elegans* and *Drosophila* rely on different resolvase complexes (25,26). Because of the considerable diversity even within such a small selection of organisms, it is of interest to know whether other organisms use similar sets of resolvases or have come up with different solutions. Revealing the variability of CO pathways among different eukaryotes may help to understand the evolutionary flexibility of the meiotic process, and ultimately, the nature of primordial meiosis. To address these questions, we studied meiotic DSB processing in an evolutionarily distant model system, the ciliated protist *Tetrahymena thermophila*.

Tetrahymena is a unicellular organism with two functionally distinct nuclei. One is the polyploid somatic macronucleus, which is transcriptionally active and is propagated only during the vegetative life cycle. The other is the transcriptionally silent micronucleus, which functions as the germ line. Only the micronucleus undergoes meiosis and is passed on to the offspring during sexual reproduction [(27) and [Supplementary Figure S1](#)].

Pairs of mating *Tetrahymena* cells undergo synchronous meioses (28), and the progression of meiosis can be easily followed and staged ([Figure 1](#)). Early steps in meiotic recombination follow the canonical pathway with Spo11 inducing DSBs, and strand exchange requiring Rad51 and Dmc1 (29,30). A remarkable feature of *Tetrahymena* meiosis is the extreme elongation of nuclei during prophase. Nuclear elongation is triggered by DSB formation (31), and it begins ~2 h after meiosis induction. Within an elongated nucleus, chromosomes are arranged in a stretched bouquet-like manner, with centromeres and telomeres attached to opposite ends. This ultimate bouquet arrangement is believed to promote the juxtapositioning of homologous regions and, thereby, homologous pairing and CO (32,33). Following this unusual pairing stage, nuclei shorten and DSBs become repaired (33). Condensed bivalents become discernible at the diplotene/diakinesis stage, which is followed by closed first and second meiotic divisions ([Figure 1](#)). Like the fission yeast (34), *Tetrahymena* also lacks an SC [(31) and literature citation therein], and here we propose that they adapted to this condition by using similar modes of CO generation.

MATERIALS AND METHODS

Cell growth and induction of meiosis

Tetrahymena thermophila wild-type (WT) strains B2086 and Cu428, being of complementing mating types, served as controls and as the source material for the construction of knockdown and knockout strains. Strains were obtained from the *Tetrahymena* Stock Center at Cornell University; <http://tetrahymena.vet.cornell.edu/>) The *spo11* Δ and *dmc1* Δ knockout lines were described previously (30,31).

Cells were cultured at 30°C according to standard methods [reviewed by (35)] and they were made competent for sexual reproduction by starvation in 10 mM Tris–Cl (pH 7.4) for at least 16 h. Meiosis was induced by mixing starved cultures of cells of complementing mating types at equal densities ($\sim 2 \times 10^5$ cells/ml).

Gene knockdown by RNA interference

To create the *mus81*, *sgs1* and *mms4* RNA interference (RNAi) constructs, an ~500 bp fragment of the corresponding open reading frame (ORF) was amplified from genomic DNA using compound polymerase chain reaction (PCR) primers (for primer sequences, see [Supplementary Table S1](#)) to add appropriate restriction sites for cloning into the RNAi hairpin (hp) vector pREC8hpCYH (36) [For a map of the vector pREC8hpCYH, see [Supplementary Figure S6](#) accompanying (36)]. This vector contains the Cd²⁺ inducible *MTT1* metallothionein promoter and the homologous sequence to the native *RPL29* gene, which provides cycloheximide resistance. The PCR products were used to replace the *REC8* fragments in the RNAi hp vector. The finished hp construct was introduced into starved B2086 and Cu428 cells by biolistic transformation (37,38). Transformants were selected initially in media containing 10 μ g/ml cycloheximide, and then were transferred to increasingly higher concentrations, up to 30 μ g/ml. Expression of hp double-stranded RNA was induced by the addition of CdCl₂ to transformed cells (36). To deplete proteins efficiently in meiosis, RNAi was induced in exponentially growing cells before meiosis. To this end, cultures were grown to high density, diluted to half, grown in medium with 0.25 μ g/ml CdCl₂ for 3 h (~1 mitotic cycle) and starved in the presence of 0.075 μ g/ml CdCl₂. Before mixing, CdCl₂ was washed out of the starvation medium. RNAi efficiency was confirmed by reverse transcription-PCR (RT-PCR) ([Supplementary Figure S2](#)).

To create *mus81* *spo11* Δ , *mus81* *dmc1* Δ and *sgs1* *dmc1* Δ strains, the *spo11* and *dmc1* knockout strains were transformed with either *MUS81* or *SGS1* RNAi constructs as described above. *spo11* RNAi was reported previously (36).

Macronuclear gene knockout

For the construction of *mlh1* and THERM_00670390 knockout strains, ~500 bp fragments of genomic *Tetrahymena* DNA upstream and downstream of the respective ORF were amplified using the primers listed in [Supplementary Table S1](#). These fragments were then

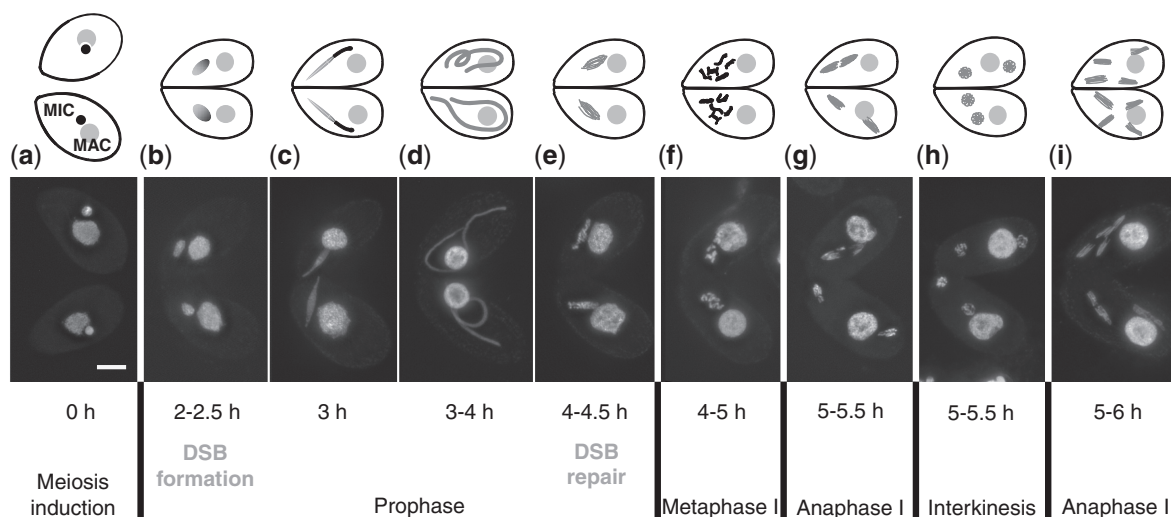


Figure 1. Meiotic stages in the WT (DAPI staining and schematic representation with nuclei not to scale). Each panel displays two mating cells in which meiosis proceed synchronously. Each cell contains a macronucleus (MAC) and a micronucleus (MIC). Only the latter undergoes meiosis, and it becomes extremely elongated during meiotic prophase. Lower case letters a–i indicate the stages referred to in other figures. The approximate times after meiosis induction are given, at which the different stages are most abundant in our laboratory, according to (31) and (29). Stages of DSB formation and repair are indicated according to (31) and (33). Bar: 10 μ m.

joined to each end of the *neo4* selection cassette using overlapping PCR (39). The knockout construct was introduced into B2086 and CU428 cells by biolistic transformation as described previously (37). The *NEO4* resistance gene is expressed under the Cd^{2+} -inducible *MTT1* metallothionein promoter (40). Transformants were selected in media containing 0.1–1 $\mu\text{g}/\text{ml}$ CdCl_2 and increasingly higher concentrations of paromomycin (from 120 μg to 10 mg/ml) until the WT chromosomes were completely replaced by the knockout chromosomes in the somatic nucleus (Supplementary Figure S2). Complete knockout was tested by PCR using primers flanking the ORFs (Supplementary Table S1).

Protein tagging

Sgs1-HA and Mlh1-GFP strains were created by tagging the respective genes at the C-terminus by the macronuclear knock-in method. Two short (~500 bp) DNA fragments were amplified by PCR, one homologous to the 3'-end of the ORF (without the stop codon) and one to the flanking sequence downstream of the gene (primers listed in Supplementary Table S1). These fragments were joined by overlapping PCR with the tagging cassettes of the pHA-Neo4 and pEGFP-Neo4 vectors (gifts of Kazufumi Mochizuki). The constructs were introduced into the macronuclei of B2086 and CU428 cells by biolistic transformation. Transformants were selected using the same strategy as for the knockouts (see above). Somatic transformation introduces transgenes to the vegetative nucleus, which is ~45-ploid. The more WT copies are replaced by tagged copies, the stronger the protein labeling is. The level of replacement was tested using primers listed in Supplementary Table S1.

Standard molecular techniques

cDNA for RT-PCR analysis was prepared as described in (29), and gene expression profiles were obtained by

RT-PCR using the primers listed in Supplementary Table S1.

Cytological methods

Depending on further use, cells were prepared by one of the following methods [for details see (31,32)]: (A) 250 μl 10% Triton X-100 and 500 μl 37% formaldehyde were added to 5 ml of cell suspension. After 30 min, the fixed cell suspension 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. (B) 5 ml of cell suspension were fixed with 20 μl of partial Schaudinn's fixative (saturated HgCl_2 , ethanol 2:1), centrifuged and resuspended in methanol. This suspension was dropped onto slides and air-dried. (C) Cells were pelleted, resuspended in Carnoy's fixative (methanol, chloroform, acetic acid 6:3:2), again pelleted and resuspended in 70% ethanol. This suspension was dropped onto slides and air-dried.

A polyclonal antibody against peptide FNIFSNDDPNLDLQ from the C-terminus of Mms4 was raised in rabbit and affinity purified by a commercial provider. Its specificity was confirmed by the lack of staining of *mms4i* meiotic nuclei (data not shown). For immunostaining, slides prepared after procedure (A) were washed 2 $\times$ 5 min with 1 $\times$ phosphate buffered saline and 5 min with 1 $\times$ phosphate buffered saline + 0.05% Triton X-100. The following primary antibodies were used: anti-Mms4 (1:100), anti-GFP (1:100) and anti-HA (1:100). After washing with buffer as before, appropriate fluorescence-labeled secondary antibodies were applied. Slides prepared after (B) were used for immunostaining with anti Cna1 antibody (1:200; a generous gift from Harmit Malik). For fluorescence *in situ* hybridization (FISH) on slides prepared according to (C), a compound FISH probe was produced by amplifying a 22.1 kb intercalary chromosomal locus by

PCR (41). The purified PCR products were labeled with Cy3 by nick translation. The probe and chromosomal DNA were denatured by hot formamide and hybridized for 36 h at 37°C.

All preparations were mounted in Vectashield anti-fading agent (Vector Laboratories) supplemented with 0.05 µg/ml 4',6'-diamidino-2-phenylindole (DAPI). A fluorescence microscope equipped with the appropriate filters was used for observation and documentation. Z stacks of pictures were recorded using MetaView software (Universal Imaging, Downingtown, PA, USA), deconvolved using AutoDeblur (AutoQuant Imaging, Watervliet, NY, USA) and projected with ImageJ (Wayne Rasband, N.I.H.; <http://rsb.info.nih.gov/ij/>) software.

Preparation, digestion and pulsed-field gel electrophoresis of Mb-sized DNA

Chromosome-sized DNA was prepared in agarose plugs using disposable plug molds (Bio-Rad) following (29). Aliquots of the cultures used for DNA preparation were checked cytologically for efficient induction of meiosis and distribution of meiotic stages. The agarose plugs were washed in 1 M Tris-Cl (pH 7.5), treated with RNaseA and washed and stored in pulsed-field gel electrophoresis (PFGE) TE (0.05 M Na₂EDTA, 0.01 M Tris-Cl) at 4°C (29).

For enzymatic treatment, plugs were incubated for 1 h in 1 mM PMSF in PFGE TE, washed twice with PFGE TE at 4°C for 1 h and three times in TE (0.001 M Na₂EDTA, 0.01 M Tris-Cl, pH 7.5) for 30 min at room temperature. For the *Sma*I digest, plugs were equilibrated in 1× restriction enzyme buffer and treated with 30 U of *Sma*I per plug in restriction digest mixture (300 µl of 1× restriction buffer, 4 mM spermidine, 2 mM DTT, 0.2 mg/ml bovine serum albumin) at 30°C overnight, followed by addition of fresh 30 U of the enzyme and incubation for another 4 h.

The sensitivity of putative branched DNA to *Escherichia coli* JM resolvase RuvC (Abcam) was assayed by digesting the DNA in plugs according to (15) and (42). After *Sma*I digestion, plugs were washed with TE, twice with RuvC buffer [50 mM Tris-acetate, pH 7.5, 10 mM Mg(OAc)₂, 1 mM DTT, 50 µg of bovine serum albumin per ml] for 30 min at room temperature, and once for 30 min with RuvC buffer on ice. The plugs were incubated in 150 µl of buffer with 300 ng RuvC for 4 h at 4°C and then for 1 h at 55°C. The temperature increase promotes migration of JMs to a preferred DNA sequence context for RuvC activity (43). Reactions were stopped by adding 7.5 µl of 0.5 M EDTA, pH 8.0. Plugs with digested DNA were directly used for PFGE or stored in PFGE TE at 4°C.

PFGE was performed using a contour-clamped homogeneous electric field (CHEF) apparatus (Bio Rad Chef-DR III). DNA fragments were separated in a 0.8% agarose gel (pulsed-field certified agarose, Bio-Rad) in 1× TAE at 14°C for 24 h at 3.5 V/cm with a 300 s switch time followed by 48 h at 2.2 V/cm with a 1000 s switch time. After gel staining with ethidium bromide, DNA

was transferred onto nylon membrane by Southern blotting. The majority of DNA in *Tetrahymena* cells is located in the somatic nuclei that do not undergo meiosis, and it obscures bands originating from meiotic DNA. Therefore, meiotic DNA was probed with a radioactively labeled Tlr DNA sequence, which is limited to germ line nuclei (44).

After hybridization and detection, membranes were stripped of the Tlr probe and reprobed with a sequence from a 306 kb macronuclear minichromosome, as a control for equal loading and Southern transfer. Band density of loading controls was measured, and Tlr signal intensities in the indicated areas of the gels in Figure 4B and C were integrated and measured using the 'Analyze' tool of ImageJ (Wayne Rasband, N.I.H.; <http://rsb.info.nih.gov/ij/>) software. Signal intensities were corrected according to the respective loading control intensities. For representation in Figure 4B and C, corrected signal intensities of timepoint t = 0 and -RuvC were arbitrarily assigned a value of 1, and corrected intensities at t = 5, 5 h and +RuvC were expressed as the n-fold.

RESULTS

Candidates for JM resolving factors

Tetrahymena possesses homologs to a number of proteins that have been implicated in JM processing in other organisms. *Tetrahymena* Mus81 and Sgs1, encoded by ORFs TTHERM_00624870 and TTHERM_01030000, respectively, have been reported previously (31). Two *Tetrahymena* proteins, encoded by TTHERM_00127000 and TTHERM_01109940, are close homologs to budding yeast MutL family members, of which Mlh1 and Mlh3 are components of the MutLγ resolvase complex. TTHERM_01109940p is the first candidate in a BLAST search with Mlh3 as bait, but it is the best hit in a reciprocal BLAST search with Pms1. It is also the best hit with Mlh2 as bait. TTHERM_00127000p is the best hit in a reciprocal BLAST search with Mlh1. Altogether, it is likely that TTHERM_00127000p is the ortholog of Mlh1, whereas TTHERM_01109940p could be any of Pms1, Mlh2 or Mlh3, but an unambiguous assignment is not possible (31). (TTHERM_00127000 was given the official name *TMLH1* in the *Tetrahymena* Genome Database, but it will be called *MLH1* in this article.) Other members of the MutL family do not seem to exist in *Tetrahymena* [see also (45)].

TTHERM_00194130p was found as the best Mms4/Eme1-homolog candidate in a reciprocal BLAST search of the *Tetrahymena*, budding yeast and fission yeast proteomes. BLAST searches for protein homologs to budding yeast Yen1 and its human ortholog GEN1 both produced TTHERM_00670390p as the first hit with limited local similarities. Moreover, the comparison of protein domains using the Feature Architecture Comparison Tool [FACT; <http://fact.cibiv.univie.ac.at/index.php>; (46)] revealed that *Tetrahymena* TTHERM_00670390p shares with Yen1 and GEN1 a similar arrangement of XPGN and XPGI regions. This makes TTHERM_00670390p a possible Yen1/GEN1 candidate.

We failed to identify good homologs of Slx1 and Slx4 proteins.

To assess potential roles of the above-mentioned proteins in meiosis, gene expression was studied by reverse transcription-PCR (RT-PCR). As can be seen from Figure 2A, expression of *SGS1* is strongest at ~3 h after induction of meiosis, which coincides with the time when DSBs are generated and Dmc1 foci form on chromatin (30). These temporally coordinated activities are reflected by similar timing of the transcription of *SPO11* and *DMC1* genes. *MUS81*, *MMS4* and *MLH1* display a basal level of expression in vegetatively growing cells but are upregulated throughout meiosis. The patterns of *MUS81* and *MMS4* expression, with maximums at 4–5 h, are similar, which is consistent with the possibility that, like their yeast homologs, they act as a complex. Expression of the *YEN1* homolog, THERM_00670390 peaks at 6 h, which is too late in meiosis to act as a JM resolvase. Thus, the transcription profiles of *MUS81*, *MMS4*, *SGS1* and *MLH1* candidates, which are in good agreement with a microarray analysis of gene expression (47), suggest their potential involvement in meiotic recombination, and we went on to characterize them further.

Mms4, Sgs1 and Mlh1 localize to meiotic nuclei

To study the presence and localization of candidate JM-processing proteins during the course of meiosis, we constructed strains expressing tagged proteins and/or had antibodies raised. We failed to detect Mus81 by immunostaining or protein tagging both *in situ* and in western blot experiments. However, we obtained an antibody against its putative partner Mms4, and we were able to visualize Sgs1 and Mlh1 by tagging with HA and GFP epitopes, respectively.

Little Sgs1-HA was detected in the micronucleus of vegetatively growing cells, but it increased in meiotic nuclei from an early stage onward and was most abundant in elongated prophase nuclei (Figure 2B). By meiotic metaphase, a signal was no longer visible. Mms4 was hardly detectable cytologically in elongated prophase nuclei and was most abundant in nuclei exiting from the elongated state, i.e. after the onset of Sgs1 expression (Figure 2C). This appearance of Mms4 and Sgs1 at different stages reflects the 1–2 h temporal difference in maximal mRNA transcription of the respective genes (Figure 2A). Mlh1-GFP was present in vegetative micronuclei, produced weak signals in meiotic prophase nuclei and appeared to be slightly more abundant only in nuclei that had completed the first and second meiotic divisions (Supplementary Figure S3).

Knockdown of *MUS81* or *SGS1* causes abnormal bivalent formation

Despite their upregulation in meiotic cells, a basal expression level of *MUS81* and *SGS1* during vegetative growth (Figure 2A) suggests their requirement for mitotic propagation. Indeed, *MUS81* appears to be essential because we were not able to generate a full *MUS81* knockout by random gene assortment. To study the function of Mus81 and of Sgs1 in meiosis, we resorted to the

meiosis-specific depletion of the proteins by inducible RNAi. We constructed strains that display a robust reduction of mRNA on induction of double-stranded RNA transcription (Supplementary Figure S2). In the following, we will denote RNAi-knocked-down genes and RNAi mutant cells by the suffix 'i'.

As a first indication of whether *mus81i* or *sgs1i* might cause a meiotic defect, we tested the fertility of these mutants. We found that *mus81i* and *sgs1i* failed to produce any viable sexual progeny (Supplementary Table S2).

Next, we cytologically monitored the progression of *mus81i* and *sgs1i* strains through meiosis. In both mutants, prophase looked normal, and nuclei elongated as in the WT. However, at metaphase, chromosomes did not condense to the same extent as in the WT, and they formed a single mass of chromatin (Figure 2D). To determine whether these chromatin masses consisted of unpaired chromosomes or bivalents, we applied FISH to an intercalary chromosomal locus (41) and scored tightly connected (single signal) versus separate (2 signals) homologous regions (Figure 2D). In both mutants, nuclei with single or closely associated FISH dots prevailed, which confirms that, despite the lack of distinct condensed structures, homologous chromosomes had formed bivalents. We next assessed the closeness of homolog associations by scoring the frequency of nuclei with a single FISH signal and measuring the distances between pairs of FISH signals. In 100 nuclei of the WT, we found 25% with a single FISH signal and the rest with two signals side-by-side. This relaxed association is owing to the fact that diplotene–metaphase I bivalents are not intimately paired along their entire lengths, and only loci in the vicinity of a chiasma are closely aligned. Interestingly, in the *mus81i* mutant, 54 of 100 nuclei displayed fused FISH signals. Also, the average distance (center to center) of FISH foci was 0.47 μ m in the WT and 0.25 μ m in the *mus81i* mutant. This suggests that *mus81i* bivalents are linked at more sites than WT bivalents. No such enforced connection was observed in the *sgs1i* mutant with an average distance of foci of 0.51 μ m ($n = 100$) (Figure 2D).

Homologs do not separate in the absence of Sgs1 and Mus81

We next studied the segregation behaviour of the abnormal *mus81i* and *sgs1i* bivalents and found that they completely failed to undergo anaphase separation. Nuclei were seen to elongate as if they were trying to separate chromosomes, but ultimately they collapsed back into a single nucleus and remained arrested in that stage (Figure 3A and B). Immunostaining with an antibody against the centromeric histone Cna1 (48) revealed that the centromeres were correctly bioriented during attempted anaphase (Figure 3A). However, while the WT formed two haploid late anaphase/telophase ensembles with 5 centromeres, mutants retained nuclei with a diploid set of 10 centromeres. This indicates that the spindle was pulling, but was unable to separate homologous chromosomes.

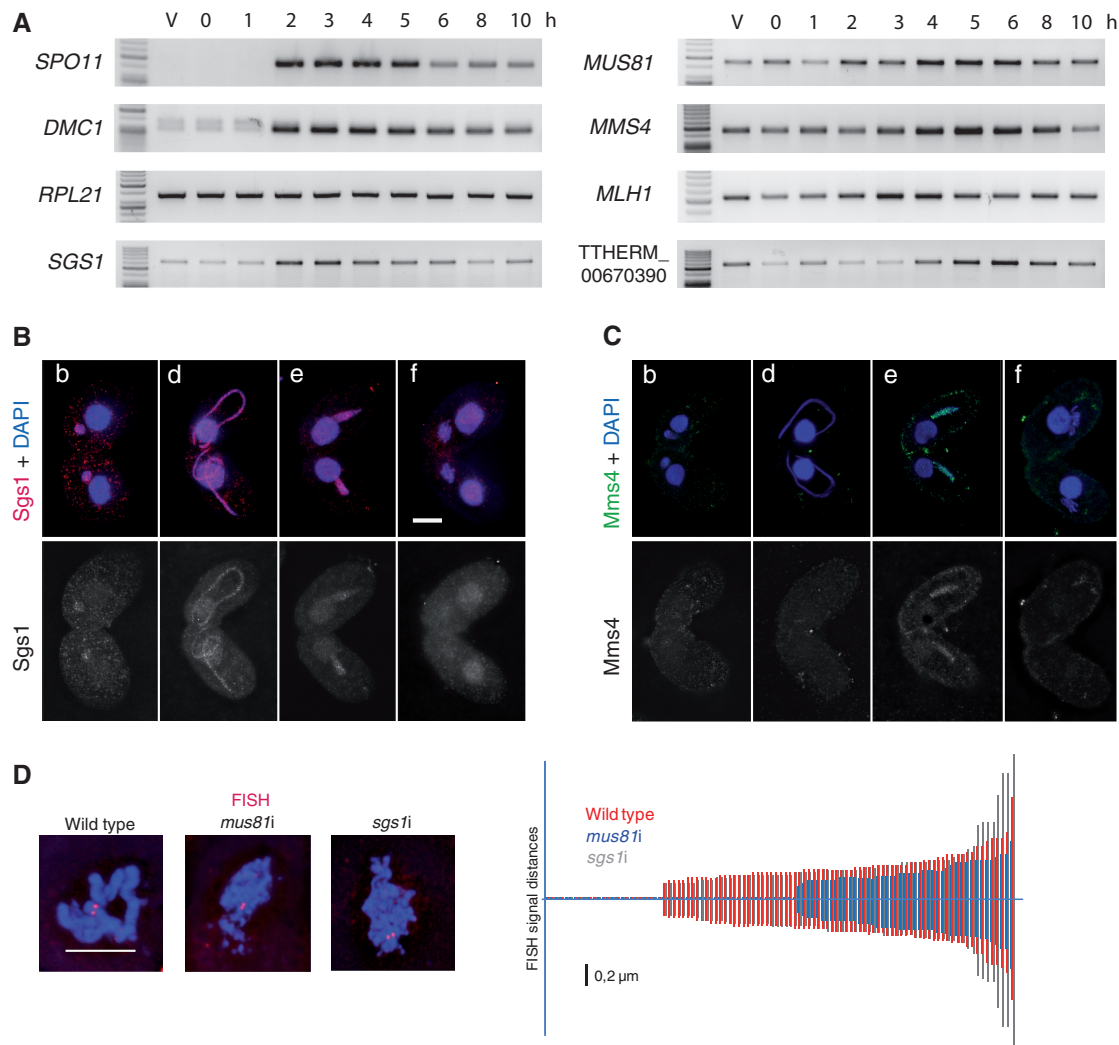


Figure 2. Expression of HJ resolvase candidate genes, Sgs1 and Mms4 localization during meiosis, and aberrant metaphase I in *mus81Δ* and *sgs1Δ*. (A) RT-PCR. Expression patterns of meiosis-specific genes *SPO11* and *DMC1* and the ubiquitously expressed control gene *RPL21* are shown for comparison. V = vegetative cells, 0 = starving cells at the time of meiosis induction, 1–10 = h after meiosis induction. (B) Sgs1-HA (red) localizes to meiotic nuclei throughout prophase. By metaphase I it disappears. (C) Immunostained Mms4 is visible at diakinesis. (D) *mus81Δ* and *sgs1Δ* cells do not show condensed metaphase bivalents, yet association of FISH signal pairs (red) demonstrates that homologous chromosomes are paired in most nuclei. FISH signal distances were measured in 100 nuclei of the WT, *mus81Δ* and *sgs1Δ*, each. Nuclei were plotted along the X-axis according to increasing distances. Single FISH signals, indicating close pairing, were assigned a zero distance on the Y-axis. Bars in **B** and **D**: 10 μm.

Depletion of Mms4 caused an anaphase arrest identical to the one of *mus81Δ* (Supplementary Figure S4). This makes it likely that Mus81 and Mms4 cooperate in *Tetrahymena* as they do in the yeasts, and given the similar behavior of the two mutants, we continued with the further characterization of Mus81 only.

DSB-dependent physical links prevent the separation of homologs in *mus81Δ* and *sgs1Δ*

Because metaphase bivalents of *mus81Δ* and *sgs1Δ* fail to condense properly, it is conceivable that their separation was inhibited by the twisting and entangling of abnormally long chromosomes. Alternatively, segregation could be prevented by inappropriately processed recombination intermediates. To test if the *mus81Δ* and *sgs1Δ* anaphase arrest was due to persistent interchromosomal connections formed during recombination, we created

strains depleted of Spo11. In the absence of Spo11, no DSBs and hence no COs are formed (1). In *Tetrahymena spo11Δ*, micronuclei do not fully elongate, and univalents are formed and segregate randomly in the first meiotic anaphase (31).

spo11Δ strains were transformed with the *MUS81* hp construct to create *spo11Δ mus81Δ* double mutants (see ‘Materials and Methods’ section), and we found that the *mus81Δ* segregation defect was fully rescued by the *spo11Δ* mutation (Figure 3C). Similarly, we simultaneously inactivated *SGS1* and *SPO11* by RNAi. WT strains were transformed with either *SGS1* or *SPO11* hp constructs, selected with cycloheximide, and RNAi was induced by adding cadmium (see ‘Materials and Methods’ section). *spo11* RNAi is mostly transferable and penetrant, i.e. when a *spo11* strain is mated to a WT strain, the Spo11 depletion phenotype is usually elicited in both partners (30). (Here,

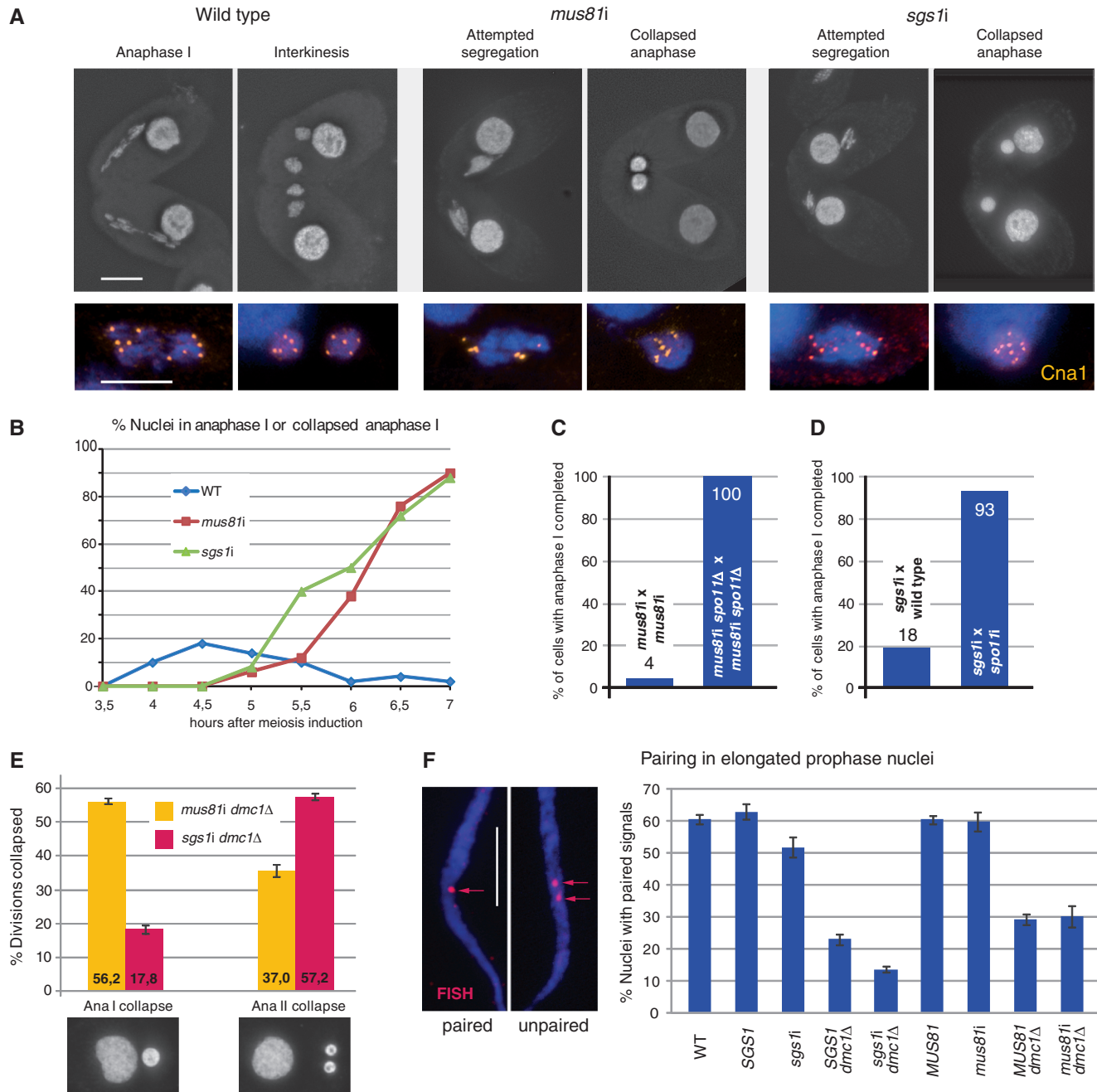


Figure 3. Depletion of Mus81 and Sgs1 causes DSB-dependent meiotic segregation defects. (A) In the WT, homologous chromosomes separate in anaphase I and form two distinct chromatin masses in telophase I–interkinesis. In *mus81i* and *sgs1i* meiosis, chromosomes collapse back into a single diploid nucleus after an attempted anaphase I. Staining of the centromere marker Cna1 (orange) shows the orientation of five centromeres to each of the opposite poles and five centromeres in each daughter nucleus after complete separation of the homologs in the WT. In the mutants, collapsed nuclei contain 10 centromeres. (B) Quantification of the segregation defect. WT meiotic nuclei pass through anaphase I, whereas mutants arrest with collapsed anaphase I nuclei. Hundred cells were evaluated for each genotype and timepoint. (C) Spo11 depletion rescues the *mus81i* segregation defect. *mus81i* strains were depleted of Spo11 by *SPO11* knockout. All *mus81i spo11Δ* cells completed anaphase I 5.5 h after induction of meiosis ($n = 50$ cells for each phenotype). (D) Spo11 depletion rescues the *sgs1i* segregation defect. *sgs1i* cells were depleted of Spo11 by *spo11* RNAi of their mating partner. *spo11* RNAi is transferable, i.e. if one of the mating partners expresses interfering RNA, both cells display the depletion phenotype. *spo11* RNAi efficiency was monitored by the failure of micronuclei to elongate during meiotic prophase (see main text). The *sgs1i* phenotype is limited to the cell expressing siRNA and is attenuated by mating to a non-*sgs1i* partner. Therefore, the frequency of successful anaphases was scored in the partner affected by *spo11 sgs1* double RNAi and compared with *sgs1i* x WT pairs. $n = 100$ cells for each genotype. (E) *dmc1Δ mus81i* and *dmc1Δ sgs1i* double mutants are partially rescued from the anaphase I defect and undergo defective anaphase II. Shown for each genotype are the mean values $\pm$ S.D. from three experiments with 100 nuclei counted in each. An unaccounted-for percentage of cells contained three or four nuclei, where one or both products of a first division may have undergone a second division or where nonseparating bivalents or chromosomes had formed extra nuclei. (F) Pairing in the elongated prophase nucleus. Examples of paired (FISH signals fused) and unpaired (FISH signals separate) homologous loci (red arrows) are shown. *SGS1* and *MUS81* genotypes carry the respective RNAi hp constructs, but RNAi was not induced; hence, they resemble the WT control. In the absence of Sgs1 but not of Mus81, homologous pairing is reduced both in the WT and in the *dmc1Δ* background, in which recombination takes place primarily between sisters (30). Values are means of three repeats with 100 nuclei evaluated, each. Error bars indicate standard deviation. Bars in A and F: 10 μ m.

micronuclear elongation was abolished in 82 of 100 mating pairs, indicating that *spo11* RNAi was efficient.) *sgs1* RNAi is not systemic, and if an *sgs1* cell is mated to WT, the Sgs1 depletion phenotype is limited to the *sgs1* partner (data not shown). Therefore, in *spo11* × *sgs1* pairs, one partner is Spo11[−], the other mostly Spo11[−]/Sgs1[−]. Of 100 *spo11* × *sgs1* pairs, where one partner (*spo11*) had completed anaphase I, there were 93, where the other partner had also completed anaphase I (Figure 3D). Thus, in *sgs1 spo11* double RNAi cells, rescue of anaphase segregation was robust. These results indicate that both in *mus81* and *sgs1*, persistent Spo11-dependent recombination intermediates are responsible for the anaphase collapse.

The separation of sister chromatids is inhibited in *mus81* and in *sgs1* meiosis

While the anaphase I arrest revealed the persistence of recombination-dependent interhomolog links, we wanted to know if intersister links are also formed and remain unresolved. To this end, we created a situation where cells overcame the anaphase I arrest. In *Tetrahymena*, like in most other eukaryotes, the meiosis-specific recombination protein Dmcl is required to direct recombinational repair toward the homolog [(30) and literature citation therein]. Hence, in a *dmc1Δ* mutant, there is little interhomolog CO, and mostly univalents are formed at metaphase I. The random segregation of univalents at anaphase I is followed by the separation of sister chromatids at anaphase II (30). However, anaphase II segregation of sisters will be impaired if unresolved intersister links are present.

We scored meiotic configurations in a *mus81 dmc1Δ* × *mus81 dmc1Δ* crossing 5.5 h after induction of meiosis, i.e. at a time when, in the WT, ~90% of cells have completed the second meiotic division. We found that about half of the cells (56%, *n* = 150) had abolished anaphase I (Figure 3E). Thus, while a residual CO is formed in only 9% of *dmc1Δ* cells (30), unresolved NCO intermediates may contribute to the substantial anaphase I arrest in double mutants. Nevertheless, we found that 37% of cells had completed anaphase I but became arrested at anaphase II.

Next, we quantified anaphase I and II segregation in a *sgs1 dmc1Δ* × *sgs1 dmc1Δ* double mutant crossing and found that only 18% of cells abolished anaphase I, whereas 57% arrested at anaphase II (Figure 3E). The anaphase II defect in both double mutants suggests that links are formed between sisters and that Mus81 and Sgs1 are required for resolving them.

Homologous pairing is slightly reduced in the absence of Sgs1

The above experiment showed that *sgs1* bypasses the anaphase I segregation defect more efficiently than *mus81* in the *dmc1Δ* background. We wondered whether this might be owing to a lower abundance of interhomolog recombination intermediates in the *sgs1 dmc1Δ* mutant. Previous experiments have shown that interhomolog strand exchange promotes pairing in elongated prophase

nuclei (30). Therefore, we determined the pairing frequency of a chromosomal locus by FISH (Figure 3F). We found that *mus81* in the *dmc1Δ* mutant background has not reduced pairing as compared with the uninduced RNAi control. In contrast, *sgs1* in the *dmc1Δ* background reduced pairing by ~50%. This suggests that reduced interhomolog connections may be the reason for the relatively successful anaphase I segregation in the *sgs1 dmc1Δ* double mutant. Next, we evaluated pairing frequency in *mus81* and *sgs1* single mutants. Again, Mus81 depletion did not reduce pairing, whereas in *sgs1* it was reduced by 20% (Figure 3F). This is consistent with the possibility that Sgs1 promotes or stabilizes interhomolog interactions.

Branched DNA molecules accumulate in the absence of Mus81 or Sgs1

To test if the persistent links consist of DNA JMs, we analyzed the mobility of meiotic DNA species in pulsed-field gel electrophoresis. Chromosomes of generative nuclei are too big to enter a pulsed-field gel. They will do so only when they are fragmented by meiotic DSBs (29). Therefore, in the WT, a smear diagnostic of DSBs appeared transiently (3 h after meiosis induction), and disappeared later when DSBs were repaired (5.5 h after meiosis induction) (Figure 4A). (For the timing of DSB appearance and disappearance, see also Supplementary Figure S5.) By contrast, no such smear appeared in the *spo11Δ* control, which does not form DSBs. A smear formed at 3 h and became stronger at 5.5 h in the *mre11Δ* control, which we showed previously to accumulate unrepaired DSBs (29). In meiotic cultures of *mus81* or *sgs1*, the DSB smear appeared and disappeared similarly to the WT (Figure 4A). We speculated that in these cases the loss of the smear was not due to the restoration of intact chromosomes but due to the persistence of branched molecules that would not enter the gel. To test this hypothesis, we treated DNA with *Sma*I, which cleaves the *Tetrahymena* genome into fragments with an average size of 1.5 Mb (M. Novatchkova, personal communication). Equal amounts of *Sma*I-generated fragments entered the gel in WT samples from before and after meiotic DSB formation (0 and 5.5 h after meiosis induction—Figure 4B). In contrast, in the *mus81* and *sgs1* samples, the amount of DNA entering the gel at *t* = 5.5 h was reduced to about half that of *t* = 0 h (Figure 4B). It was shown previously that branched DNA molecules remain trapped in the loading well, whereas linear molecules of the same size enter the gel (49,50). Therefore, it is likely that *mus81* and *sgs1* meiosis arrest with branched DNA.

Persistent branched DNA molecules are RuvC sensitive

To study in more detail the molecular nature of the branched molecules that fail to reenter the gel after the disappearance of DSB-generated fragments, we treated the samples with RuvC. RuvC has been shown to cleave HJs *in vitro* (15,42) and hence to produce linear pieces of DNA, which would migrate into the gel. Indeed, RuvC digestion restored entry of DNA fragments into the gel

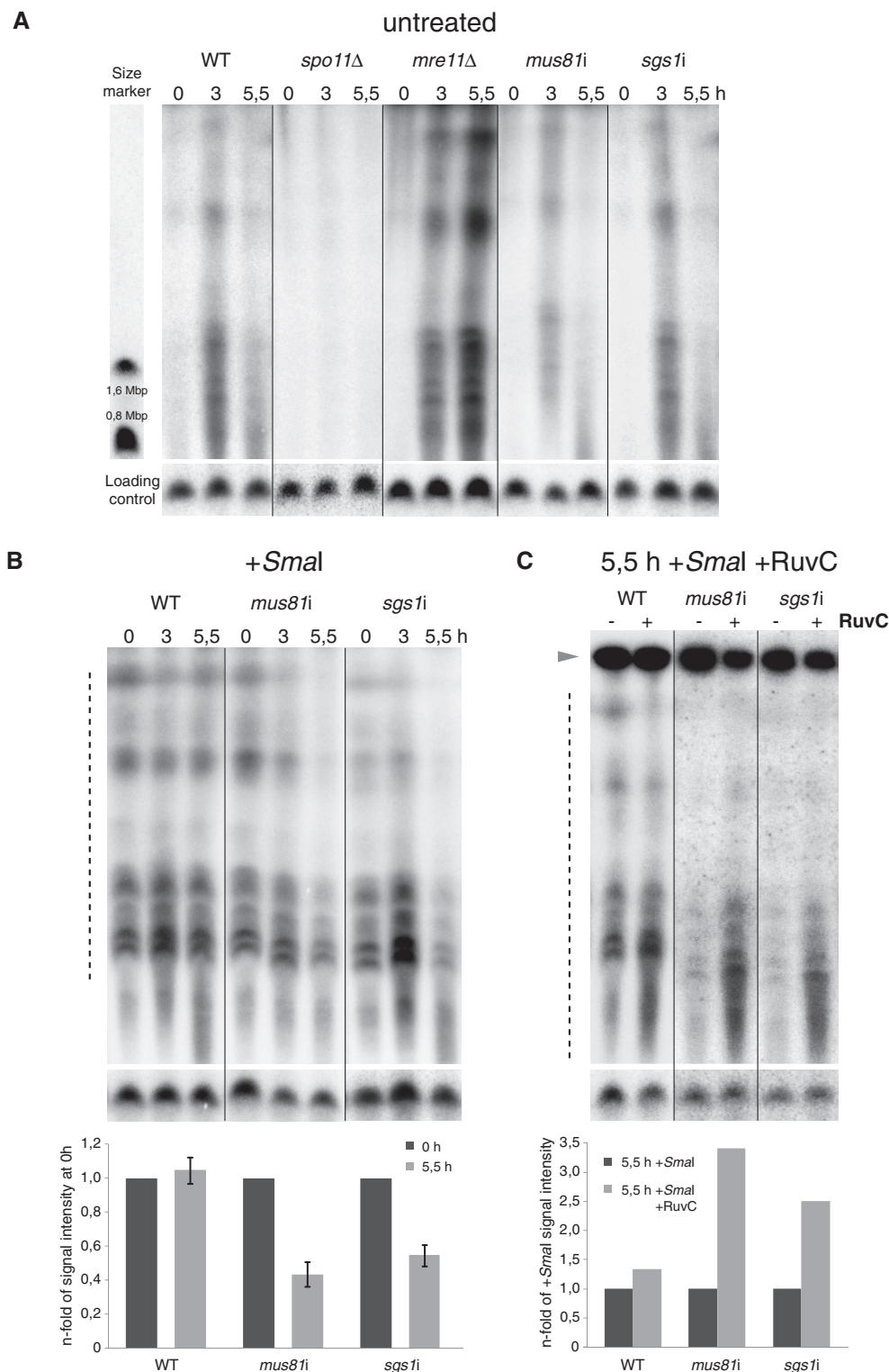


Figure 4. Detection of DSBs and JMs by PFGE. (A) A discontinuous smear in the range of ~400 kb to 3 Mb represents DSB-generated micronuclear chromosome fragments. In the WT, no DSBs are present at the time when meiosis is induced (0h). DSBs are maximally induced 3 h after meiosis induction, and have disappeared 5.5 h after meiosis induction. *spo11Δ*, where DSBs are not formed, and *mre11Δ*, where DSBs are not repaired (29), are shown as controls. In *mus81i* and *sgs1i*, DSB-generated fragments appear transiently, similar to the WT (see also Supplementary Figure S4). WT DNA was prepared from cultures grown in the presence of Cd to guarantee equal conditions to Cd-induced *mus81i* and *sgs1i*. Yeast chromosomes IV (1,6 Mb) and X (0,8 Mb) served as size markers. Discontinuity of the smear is caused by the incomplete separation of fragments of similar size. (B) Upper panel: After *Sma*I digestion, micronuclear chromosomes, which otherwise would be too large to enter the gel, run as a discontinuous smear of fragments at the time of meiosis induction (0h). In the WT, the same *Sma*I fragments appear 5.5 h after meiosis induction, i.e. at a time when DSBs have been repaired (see A and Supplementary Figure S5). In *mus81i* and *sgs1i*, the amount of DNA entering the gel is reduced at 5.5 h after meiosis induction as compared with 0 h, suggesting that unlike in the WT, recombination is not completed.

(continued)

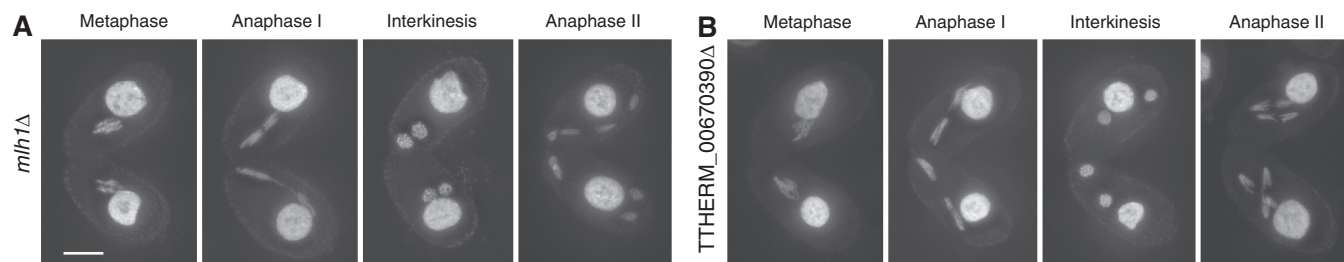


Figure 5. Deletion of *MLH1* and of ORF THERM_00670390, encoding a Yen1 homolog, does not affect meiotic divisions. *mlh1Δ* (A) and THERM_00670390Δ (B) undergo normal first and second anaphases (for comparison, see the WT in Figure 1). Bar: 10 μm.

(Figure 4C). [To exclude the possibility that the handling of samples during RuvC treatment caused DNA fragmentation or JM separation, we did a control mock treatment in the absence of RuvC (Supplementary Figure S6).] This indicates that the retainment of post-DSB DNA in the loading well is caused by the presence of JMs, which are RuvC-sensitive. RuvC cleaves mature HJs as well as three-stranded DNA molecules that are structurally similar to D loops or nicked HJs (51). Therefore, the precise nature of JMs that accumulate in *mus81Δ* and *sgs1Δ* meioses remains unclear.

mlh1 and THERM_00670390 mutant cells do not undergo meiotic catastrophe

The inability of the *mus81Δ* mutant to segregate homologs is not a sufficient indication for the exclusive function of Mus81 in resolving JMs because a small number of unresolved JMs is sufficient to block nuclear division (52). Therefore, it is possible that another activity contributes to the completion of meiotic recombination in *Tetrahymena*. A potential candidate is the MutLγ (Mlh1–Mlh3) complex which, in the budding yeast, plays a prominent role in the same pathway as the ZMM proteins in JM to CO transformation (9,52,53). Another candidate is Yen1, which may resolve JMs that escape from the main pathway (54).

Despite a possible role of Mlh1 and the Yen1 homolog THERM_00670390p for cell viability or vegetative propagation, we were able to produce stable *mlh1* and THERM_00670390 knockout lines (Supplementary Figure S2). When two *mlh1Δ* strains or two THERM_00670390Δ strains were mated, both failed to initiate postmeiotic mitoses of the gametic nuclei (for WT postmeiotic development of the gametes, see Supplementary Figure S1). Importantly, however, the segregation of chromatids at anaphase I and II was uninhibited (Figure 5A and B). This was expected in the case of THERM_00670390Δ, given that the peak expression

of the protein occurs only at $t = 6$ h after meiosis induction (see above). In *mlh1Δ*, on the other hand, anaphase could be largely rescued by the activity of Mus81. However, *mlh1Δ* could have a more subtle meiotic phenotype. If Mus81 was not fully compensating for the lack of MutLγ, Sgs1 might step in and convert persistent JMs to NCOs, which would result in an increase in univalents. Therefore we used FISH to score for univalent formation at metaphase (Supplementary Figure S7). We found 100% bivalent formation both in the mutant and in the WT ($N = 50$ metaphases, each).

Altogether, our results suggest that Mlh1 (and a potential MutLγ complex of which it may be part) and the Yen1-like THERM_00670390p may have roles in nuclear development after fertilization, but play a nonessential role, if any, in the resolution of HJs.

DISCUSSION

Mus81 is an essential resolvase of recombination intermediates

Here, we showed that in the absence of Mus81, DNA JMs (probably HJs) persist in meiosis and homologs do not separate. Moreover, at diplotene/metaphase I, chromosomes are less condensed and homologs are more tightly connected than in the WT. This confirms that an abnormally high number of interhomolog connections are present. In the absence of Mlh1, normal-looking intact bivalents are formed, and both meiotic divisions progress uninhibited. Together, our observations indicate that Mus81 is an essential, if not the only, HJ resolvase in *Tetrahymena*. This raises the question of whether Mus81-dependent COs in *Tetrahymena* are interfering.

COs within a certain distance tend to suppress each other. This is called CO interference, and it is likely related to the mechanism ensuring that each pair of chromosomes obtains at least one CO (CO assurance)

Figure 4. Continued

Lower panel: Quantification of DNA released into the gel at 0 h and 5.5 h. Error bars indicate SD of the mean from three biological repeats. (C) Upper panel: RuvC treatment releases DNA from the loading well in *mus81Δ* and *sgs1Δ* but not in the WT. This is seen by the appearance of a smear in the gel and by the reduced amount of DNA in the loading well (arrowhead). Lower panel: Quantification of DNA released into the gel after – RuvC and +RuvC treatment. In B and C, signal intensities were integrated from the gel regions indicated by dotted lines. Smaller fragments were excluded because they are formed at late timepoints owing to the degradation of postmeiotic nuclei. A signal produced by Southern hybridization to a 306 kb macronuclear chromosome, which served as a loading control, is shown underneath each lane. For the measurement of signal intensities and correction for the amount of DNA loaded, and the calculation of relative band intensities, see the ‘Materials and Methods’ section.

[reviewed in (5,55,56)]. ZMM–MutL γ -dependent COs were found to be interfering, whereas residual COs in the absence of ZMM proteins in budding yeast, *Arabidopsis* and mouse, and COs in fission yeast are noninterfering. Thus, in the organisms where both pathways co-exist, Mus81-dependent COs have been generally assumed to be noninterfering (22–24,57). In the fission yeast, which has only the Mus81 pathway, interference does not exist (58), and in *C.elegans*, where COs are mainly ZMM-dependent, interference is strong (59,60).

In *Tetrahymena*, there are an estimated number of >200 DSBs (30). As in most eukaryotes, only a subset of DSBs may give rise to mature COs. Because of the link between CO interference and assurance, low numbers of COs/chiasmata are considered a sign of interference. Unfortunately, the number of interhomolog COs or chiasmata could not yet be reliably determined in *Tetrahymena*. In the future, we hope to be able to test the link between the mode of CO generation and CO interference.

A possible role of Sgs1 in NCO and a proposed additional function in discouraging intersister recombination

The RecQ family helicases of budding yeast and humans (Sgs1 and BLM) are known as anti-CO factors in mitosis. They act by reversing or destabilizing early strand invasion intermediates (D-loop structures) (61) and by dissolving double HJs in collaboration with DNA topoisomerase III (62) (5,14). In meiosis, destabilization of recombination intermediates by Sgs1 and other helicases is assumed to channel the majority of DSBs into a NCO pathway to avoid the adverse effects of excessive COs on homolog segregation (52,63–65). Another function of Sgs1 is to eliminate multichromatid or other aberrant recombination intermediates, to prevent potentially deleterious COs (66,67). Conversely, a pro-CO activity of Sgs1 (9,52) and its orthologs in fission yeast, worm and *Drosophila* (68–70) has become evident. Also in the budding yeast, Sgs1 was suggested to contribute directly to CO formation by being part of the major ZMM-dependent CO pathway (9). Alternatively, it is possible that its pro-CO role is owing to its recycling the above-mentioned toxic intermediates and feeding them into the CO pathway.

It is conceivable that Sgs1 serves similar functions in *Tetrahymena*. Because, like in most other organisms, there seems to be an excess of recombination intermediates compared with interhomolog COs (see above), Sgs1 could play a role in limiting CO number by redirecting DSB repair to NCO outcomes, such as synthesis-dependent strand annealing (SDSA), or by HJ dissolution, in a subset of intermediates. In the absence of Sgs1, excess persisting JMs could overwhelm Mus81's capacity of JM resolution, hence causing the *sgs1i* phenotype observed here.

However, Sgs1 may have an additional earlier function. In its absence, we noticed reduced homologous pairing and, revealed by the *dmc1* Δ background, decreased interhomolog JM formation. Therefore, we propose that Sgs1 promotes interhomolog over intersister recombination. In the majority of eukaryotes, axial element components of the SC pose a barrier against intersister

exchange [see (71) and literature citation therein]. This may even be true for the truncated axial element-like structures of fission yeast (72). In *Tetrahymena*, homologous chromosomes become aligned within the stretching prophase nucleus. In the absence of axial elements, DSB ends would tend to invade sister molecules, when homologs are not yet aligned. Sgs1 was present in prophase nuclei from the beginning to after the end of the elongation stage (Figure 2B). This corresponds to the entire ≥ 90 -min interval from DSB formation to recombination-related DNA synthesis (Supplementary Figure S8). Its presence before recombination-related DNA synthesis makes a role in SDSA or CO during this period unlikely. Instead, it might destabilize D-loops, the majority of which would be intersister, and recycle DSBs for use in homologous recombination once homologs have become aligned. Consistent with this proposed function of Sgs1, increased intersister double HJ formation in the absence of Sgs1 was observed in budding yeast (66). This suggests that the role of Sgs1 in discouraging intersister recombination might be conserved.

Tetrahymena and fission yeast have independently evolved streamlined meioses

Studies in model systems revealed that there exist different pathways for the transformation of recombination intermediates into COs (8). In most model systems tested so far, the majority of COs is dependent on ZMM proteins, which are also required for SC formation. Furthermore, they need the MutL γ complex for JM resolution, and thus it seems that they are components of a single pathway, which may also include Exo1 and Sgs1 (9,53). Only a subset of COs requires the Mus81–Eme1 (Mms4) complex (8), which seems to be part of an independent pathway. In contrast, in *Schizosaccharomyces pombe*, which lacks the ZMM proteins, and hence an SC, and MutL γ , CO formation depends almost entirely on Mus81–Eme1 (see ‘Introduction’ section). Besides SC-less *Aspergillus* (73), of which little is known, *Tetrahymena* is the only other organism so far where the SC and a full set of ZMM proteins are missing. Moreover, although the MutL γ -component Mlh1 is present, this complex may not play a prominent role in meiotic segregation. These parallels lead us to suggest that *Tetrahymena* and the fission yeast have similarly developed Mus81-dependent meiotic recombination as their primary (or only) CO pathway. However, they differ in the use of Sgs1, which is essential in *Tetrahymena*, but only has a moderate contribution to spore viability in the fission yeast (68,74). While Sgs1 has been considered by some to be part of the ZMM–MutL γ -dependent pathway (9), its importance for *Tetrahymena* CO formation may mainly come from its role in discouraging intersister recombination.

Tetrahymena and the fission yeast possess meiosis-specific factors, such as Spo11 and Dmc1, for allowing programmed DSB formation and homolog-directed recombinational repair. However, unlike the majority of eukaryotes, they depend exclusively on mitotic repair factors for the processing of recombination intermediates. Another parallel, which may also be related to the loss of

an SC, is the homology searching and pairing by peculiar forms of bouquet arrangement (33). It will be interesting in future work to detect further parallels between these two evolutionarily distant organisms and to find out which adaptations led them to their parsimonious way of meiotic recombination.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online, including [75–78].

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Supplementary Data

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Table S1. List of primers

Primers used for RNAi:

mus81i

Mus81PmeFW: CGTTTAAACGATGGGTAGTATTTACAATCCTC
 Mus81XmaRV: GCCCGGGCAGCAGACACCATTTTCCTC
 Mus81ApaFW: CGGGCCCGATGGGTAGTATTTACAATCCTC
 Mus81XhoRV: GCTCGAGCAGCAGACACCATTTTCCTC

sgs1i

Sgs1PmeFW: GTTTAACTTATTGATGAAGCTCACTGTG
 Sgs1XmaRV: CCCGGGCATTTGACATCCAAAGTTATTG
 Sgs1ApaFW: GGGCCCTTATTGATGAAGCTCACTGTG
 Sgs1XhoRV: CTCGAGCATTTGACATCCAAAGTTATTG

mms4i

Eme1PmeFW: GTTTAAACGATGAAGGAGAATAAGGGTTG
 Eme1XmaRV: CCCGGGCCACTGCTTTCTTTTCACTG
 Eme1ApaFW: GGGCCCGATGAAGGAGAATAAGGGTTG
 Eme1XhoRV: CTCGAGCCACTGCTTTCTTTTCACTG

Primers used for gene knockout

mlh1

MLH1KO5FW: AAAGGAGGGGACAATCAATC
 MLH1KO5RV: GTCTATCGAATTCCTGCAGCCCGGACGGATTCAAATACCTGC
 MLH1KO3FW: CTGGAAAAATGCAGCCCCGAAAGATGTTGAGTTATTCATTG
 MLH1KO3RV: GTTTGTTTTCTATTTGGTAATTTGG

THERM_00670390 (*yen1* homolog)

YEN1KO5FW: GGAAGACACCACAAATTACAAT
 YEN1KO5RV: GTCTATCGAATTCCTGCAGCCCATTTTGTAGACTGTTTGCTTTTG
 YEN1KO3FW: CTGGAAAAATGCAGCCCTTTATGTGCCAATTTTGAGTAGC
 YEN1KO3RV: TTTTAACAAAGCCATATTCTAAAGC

Primers used for knockout testing

mlh11KO

MLH1KOFW: CAGAACATGGTGAATGCAAG
 MLH1KORV: AGGAAGTCAAAGATTTCTAAGTC

yen1KO

YEN1KOFW: ACCCAAAGCAGCACGATTAG
 YEN1KORV: ACCGCCAGGAAGTGTTC

Primers used for protein tagging

Mlh1-EGFP (neo4)

Mlh1GFP5'FW: AGTAATTTGGCTTATTGAGTGAC

Mlh1GFP5'RV:

AAGTTCTTCACCCTTAGAAACATGGATCCACATCTTTCGAAGATCTTGTATGC

Mlh1GFP3'FW:

CCCGGGGGATCTGAATTCGATATCAAGCTTAACTTAATAATTTCTATTTTACCAAATA
G

Mlh1GFP3'RV: TTGTTTGTGTCAAGTTAATTTAC

Sgs1-HA (neo4)

Sgs1HA5'FW: AGAGAACTAATAAAGTAGAAGTGG

Sgs1HA5'RV:

ATAATCAGGAACATCATAAGGATAGGATCCATTTTTGCAAGTTTTGAAAGTTAAATG

Sgs1HA3'FW:

CCCGGGGGATCTGAATTCGATATCAAGCTTGTAATAAATAAATCAAAATAAATTAATT
ATTAGAAC

Sgs1HA3'RV: AGAGAAAATTAGGATTGTATAAATAAATAC

Primers for RT-PCR

SGS1

Sgs1RTPCRFW: TGGTATGGGTATCGACAAG

Sgs1RTPCRRV: GATGTCTTAGGGTTATTTGAGT

MUS81

Mus81RTPCRFW: CCAAACAGACTCTCTTCTC

Mus81RTPCRRV: CTATCACAATCAAGTTTATCCATG

MMS4

Eme1RTPCRFW: GATGAAGGAGAATAAGGGTTG

Eme1RTPCRRV: CCACTGCTTCTTTTCACTG

YEN1

Yen1RTPCRFW: GACGAAGACGAAGATGATGAAG

Yen1RTPCRRV: CTGCCTTACTTGCCCAAAC

DMC1

Dmc1RTPCRFW: AGCAGTGAGTTTGTCTCGGTAC

Dmc1RTPCRRV: TTCCGTAGTGCGAAGCTATTTAG

SPO11

Spo11RTPCRFW: TTATGGATTGAAACAACTCTGC

Spo11RTPCRRV: TGGCGATTAAGGCATCCAGC

RPL21

Rpl21RTPCRFW: AAGTTGGTTATCAACTGTTGCGTT

Rpl21RTPCRRV: GGGTCTTTCAAGGACGACGTA

RT-PCR-RNAi efficiency test

mus81i

Mus81RNAitestFW: CATGGATAAACTTGATTGTGATAG

Mus81RANitestRV: CTCCTTAATCATTCCCTTAGC

sgs1i

Sgs1RNAitestFW: CTGGACTTGTCTAAGATGGAG

Sgs1RNAitestkRV: CGATTGTTACTTTTTAGGGTATCC

RPL21-the same primers as used for RT-PCR gene expression test

Table S2. *mus81i* and *sgs1i* matings do not produce viable sexual progeny

Matings	% Sexual progeny	Number of mating pairs tested
Wild type × wild type	87,1	93
<i>mus81i</i> × <i>mus81i</i> (RNAi-induced)	0	93
<i>mus81hp</i> × <i>mus81hp</i> (uninduced)	54,8	93
<i>sgs1i</i> × <i>sgs1i</i> (RNAi-induced)	0	124
<i>sgs1hp</i> × <i>sgs1hp</i> (uninduced)	40,3	124

For viability testing, strains were mated, and individual conjugating pairs were isolated into a drop of growth medium. When colonies were growing, they were transferred to medium containing cycloheximide. Only clones that die in cycloheximide are generated by sexual reproduction, because they do not inherit cycloheximide resistance from their parents. (Conjugating cells may give rise to cycloheximide-resistant clonal progeny by return to vegetative growth.) For a detailed protocol and rationale see (1).

Strains carrying the RNAi hairpin vector, but not induced for dsRNA expression, produced less viable sexual progeny than the wild type. This is probably due to the leakiness of the Cd²⁺-inducible promoter, which causes some reduction in protein levels in the absence of cadmium.

The dramatically reduced fertility seen upon RNAi induction suggests a meiotic defect caused by *Mus81* and *Sgs1* depletion and confirms that RNAi was efficient.

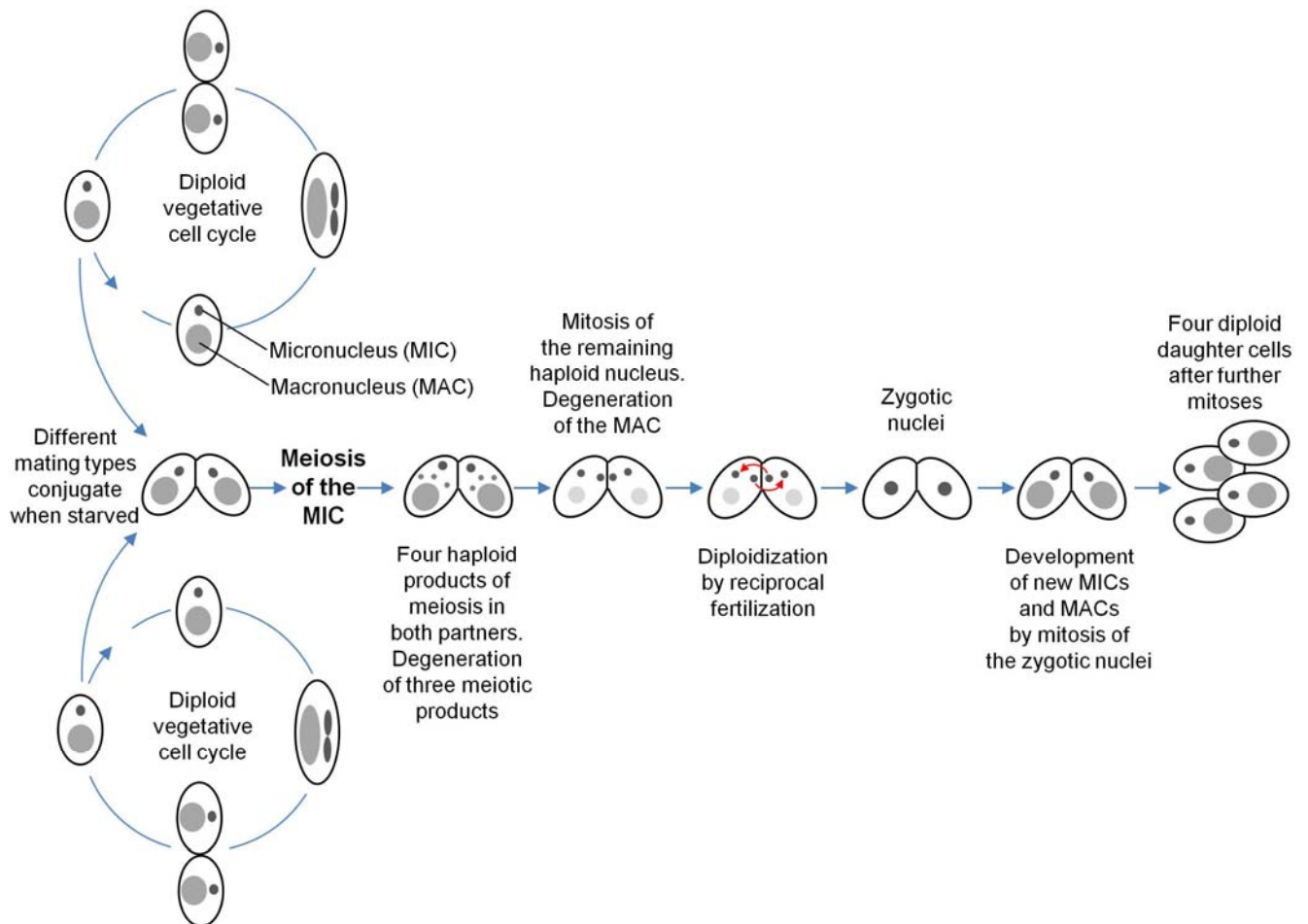


Figure S1. Vegetative and meiotic life cycles of *Tetrahymena*. Simplified from (2) and (3). Meiotic stages are shown in Figure 1. Post-zygotic mitoses are not shown.

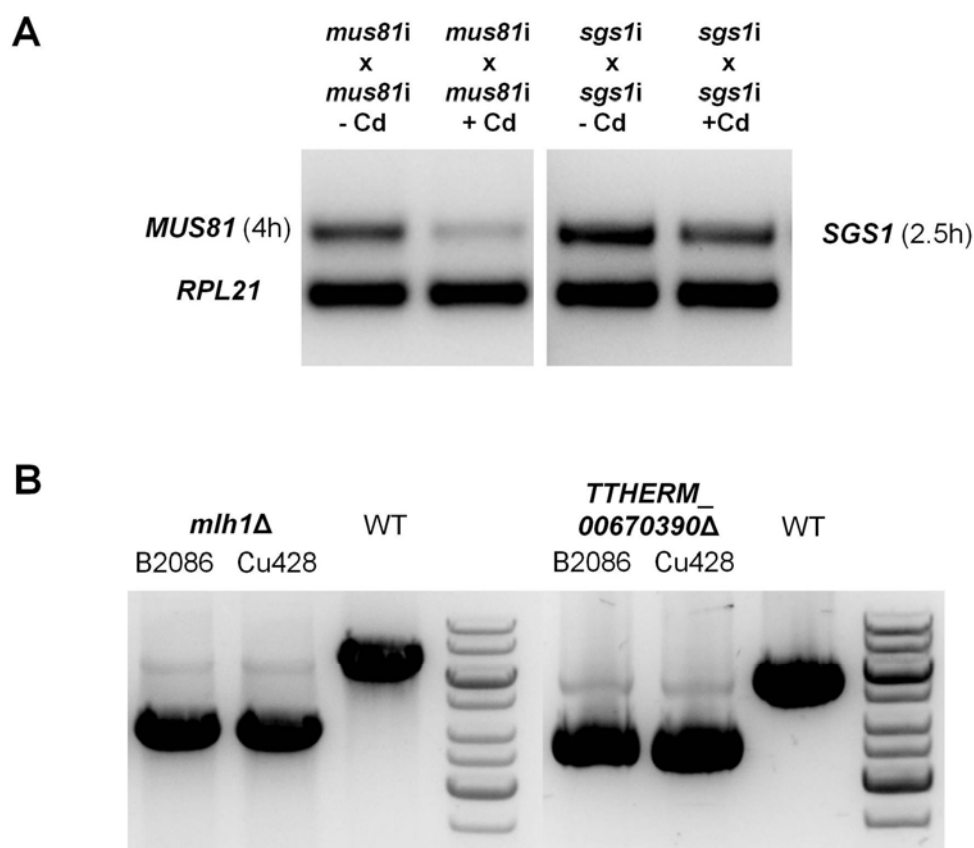


Figure S2. Reduction of mRNA by *MUS81* and *SGS1* RNAi, and extent of *MLH1* and *THERM_00670390* gene/ORF deletions. A. Matings of strains carrying the *MUS81* hairpin construct. RT-PCR shows that strains undergo an ~10-fold reduction in mRNA level upon Cd-stimulated expression of hairpin RNA, 4 h after induction of meiosis. Reduction of *SGS1* mRNA is only 2-fold. However, this is sufficient to elicit a strong phenotype. *RPL21* is an ubiquitously expressed control gene. B. The transcriptionally active macronucleus of *Tetrahymena* is ~45-ploid. (Nearly) all gene copies must be deleted to elicit a null mutant phenotype. For studying mutant meiosis in conjugating cells, genes must be deleted in both mating types. PCR with primers upstream and downstream to the respective knockout gene locus demonstrates that the deletion of *MLH1* and *THERM_00670390* copies from the macronucleus of both mating types (strains B2086 and Cu428) is complete. Residual bands at the positions of WT genes represent the non-expressing copies in the diploid micronucleus. For the primers used, see Supplementary Table S1.

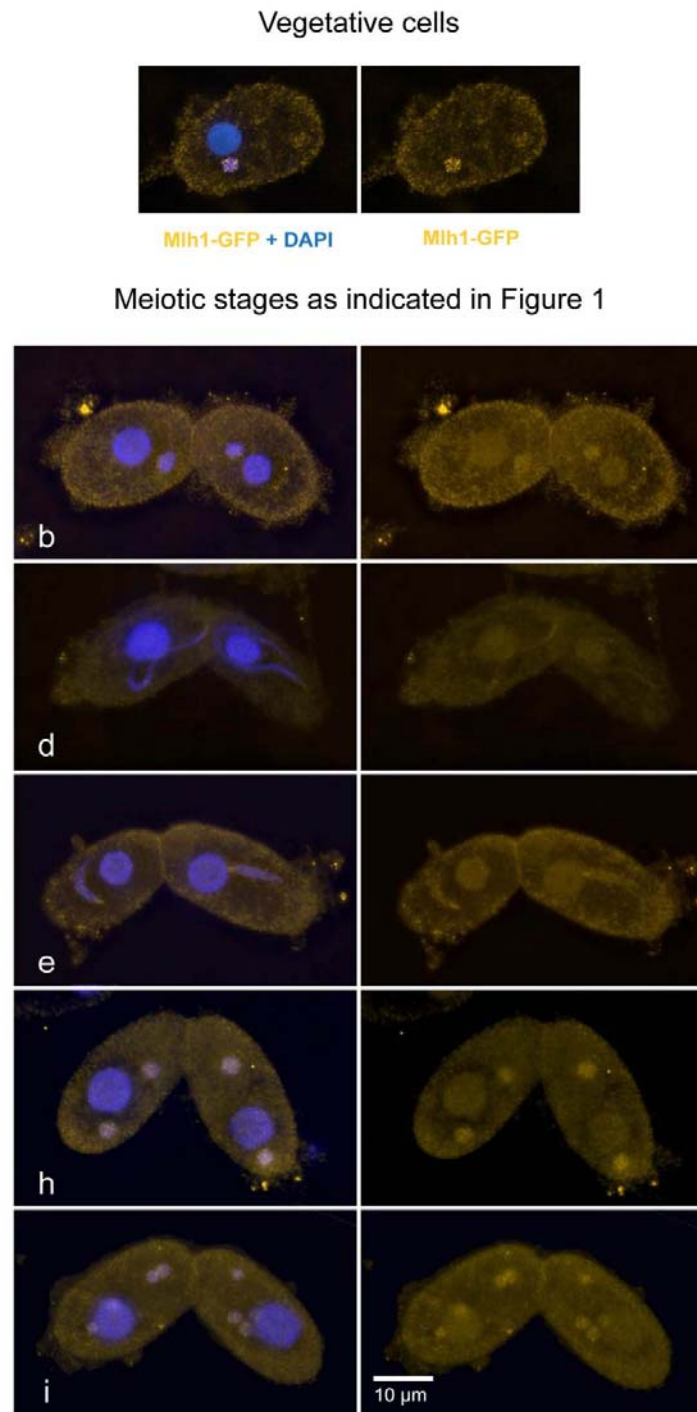


Figure S3. Mlh1 localization. Mlh1-GFP localized to micronuclei in vegetatively growing cells. It was hardly detectable in nuclei during meiotic prophase, and it produced a faint but distinct signal in meiotically dividing nuclei.

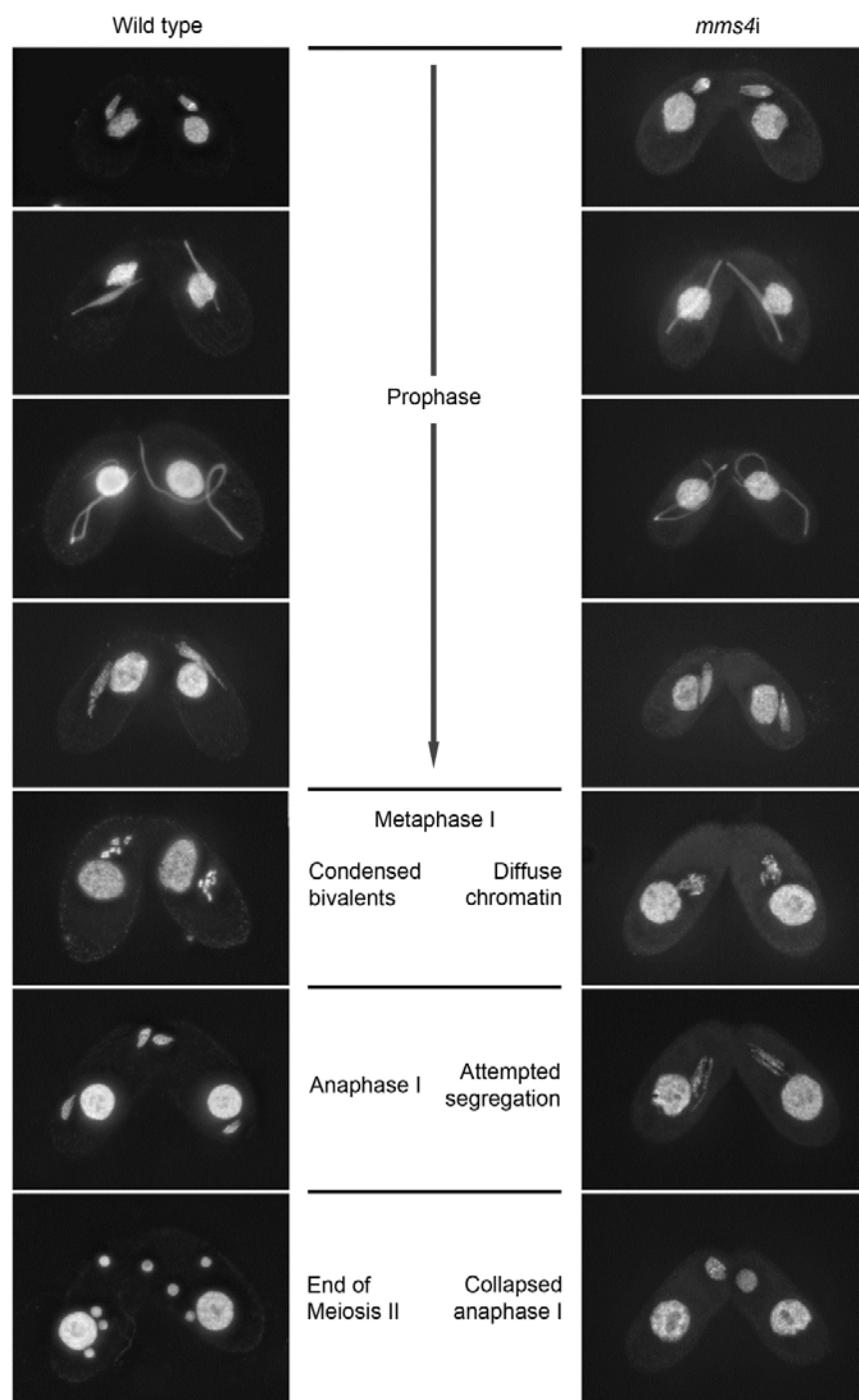


Figure S4. *mms4i* meiotic phenotype. At $t=5$ h after mixing, 192 of 200 cells (96%) were at a stage post-metaphase I, with attempted or collapsed anaphases I. The rest were escapers displaying various post-anaphase I or post-anaphase II stages. In the wild type control, all of 200 post-metaphase I nuclei were undergoing or had completed first (14%) or second (86%) anaphase 5 h after mixing. This indicates that *mms4i* arrests at anaphase I like *mus81i*.

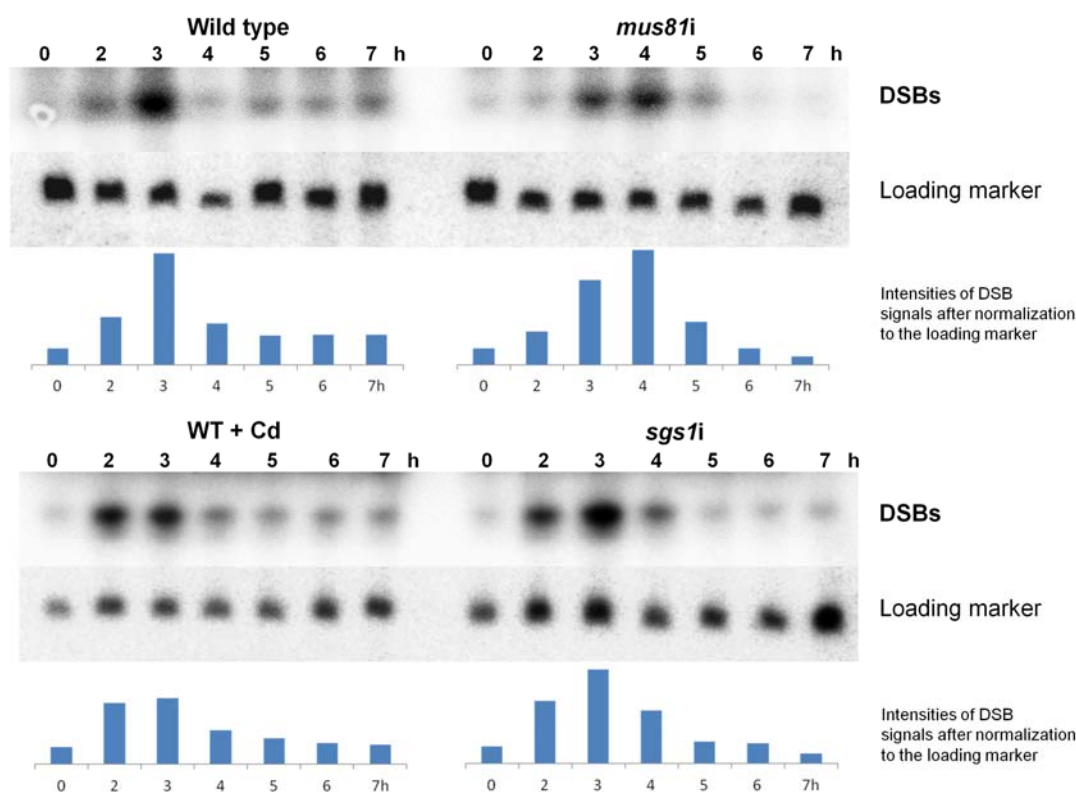


Figure S5. Timecourse of DSB formation as seen by PFGE (short-run conditions).

DSBs appear transiently during ~2-4 h after induction of meiosis in the wild type (WT) and in *mus81i* and *sgs1i*. WT meiosis took place in the presence of cadmium (Cd) to match the conditions in *mus81i* and *sgs1i* meiosis, where RNAi was induced by Cd.

Under short-run PFGE conditions, DSB-generated DNA fragments of different sizes migrate as a single band, which facilitates DSB quantification. Conditions were: 24 h run in a 1% agarose gel in 0.5× TBE at 14°C, with 6 V/cm applied at a 120° angle and a 60 to 120 second switch time. As a control for equal loading and Southern transfer, the membranes were reprobbed with a probe to a 121 kb macronuclear minichromosome shown underneath. Densities of DSB-generated bands and marker bands were measured using ImageJ (Wayne Rasband, N.I.H.; <http://rsb.info.nih.gov/ij/>). The densities of DSB-generated bands were normalized to the marker bands and indicate the relative amounts of DSBs, plotted at the bottom.

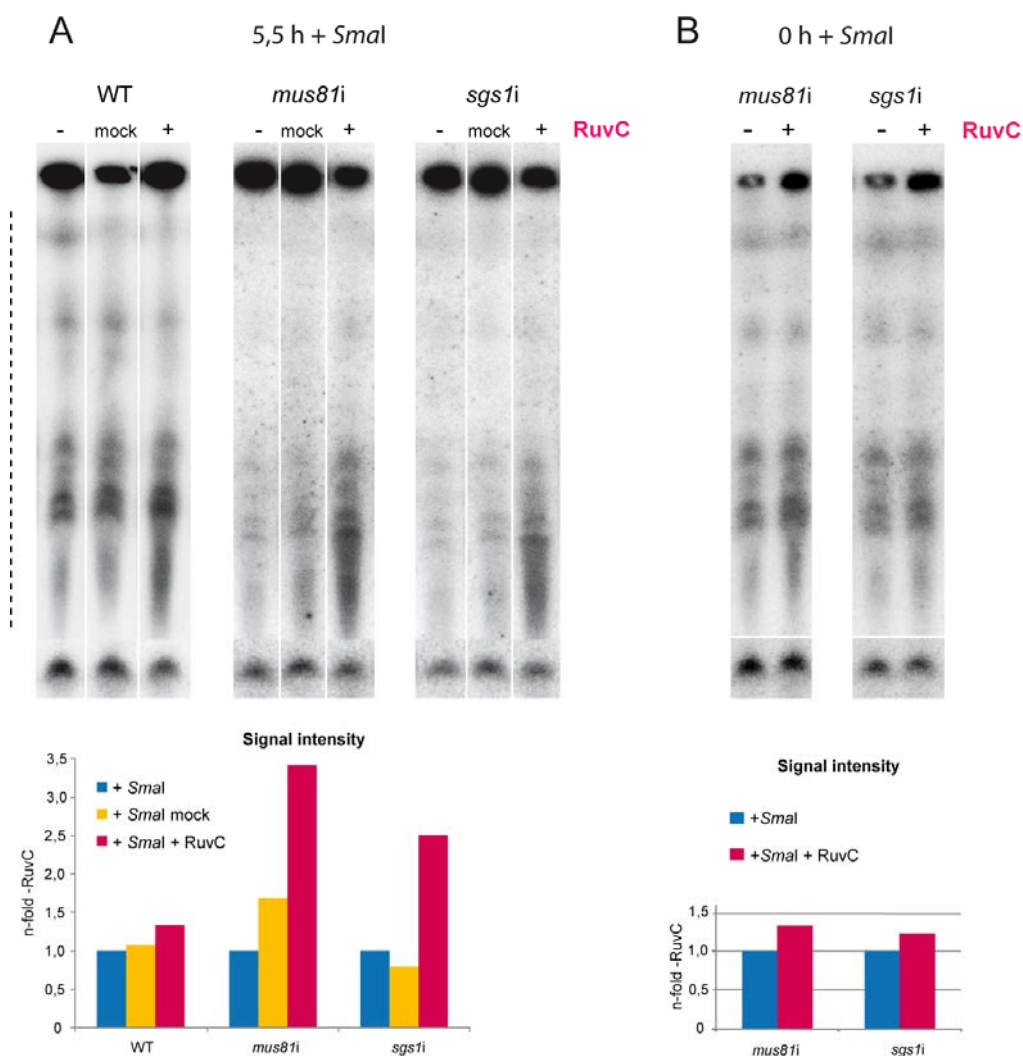


Figure S6. (A) Extended version of Figure 4C, including mock treatment to exclude the possibility of unwinding of JMs by branch migration due to high temperature during RuvC treatment. DNA samples collected at 5,5 h after induction of meiosis were digested with *SmaI* and subsequently treated with RuvC or with RuvC buffer only (mock control). RuvC cleavage of DNA accumulated in the loading well generates a smear of linear fragments that enter the gel. For quantification of DNA in the smear, see the legend to Figure 4. A substantial increase in smear intensity was detected upon RuvC digest of *mus81i* and *sgs1i*, whereas mock treatment had only a minimal effect on DNA mobility.

(B) RuvC does not release DNA in addition to *SmaI*-generated fragments at $t=0$. (The slight signal increase, seen in the quantification at the bottom, is of the same magnitude as that caused by mock treatment in A.) This confirms that the RuvC-sensitive DNA species seen in *mus81i* and *sgs1i* at 5.5 h are not generated by premeiotic DNA repair processes following leaky RNAi hairpin expression. Thus, the fragments produced by RuvC represent resolved DSB-dependent JMs.

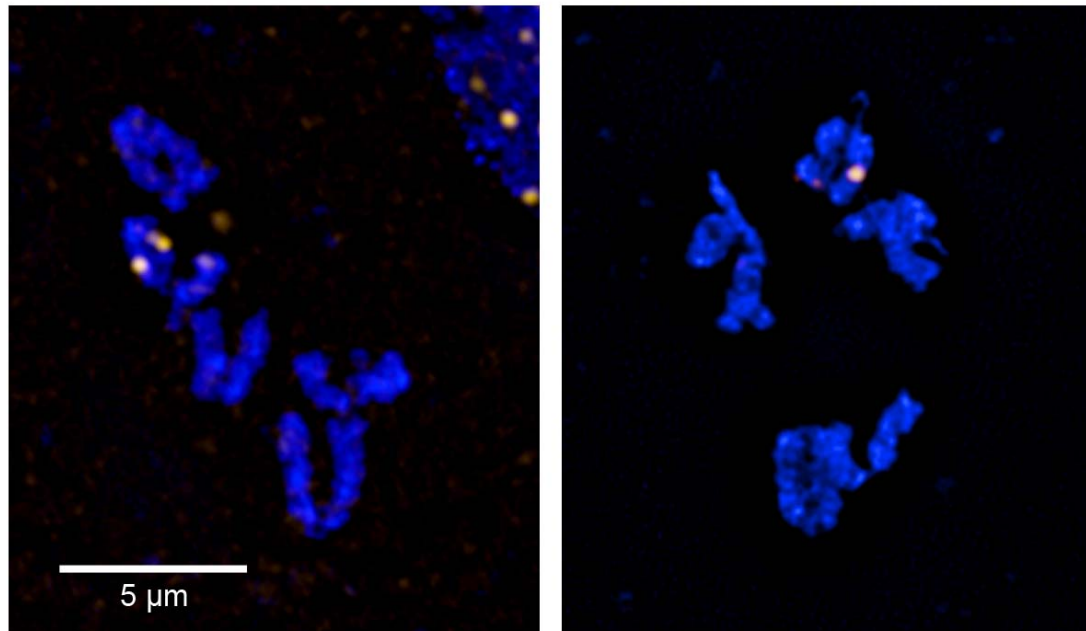


Figure S7. Examples of metaphase I nuclei with bivalents. Bivalent formation is indicated by the close association or fusion of FISH signals (orange). Slides for the evaluation of bivalent formation by FISH were prepared according to the method described in (4), aged for several days, and then subjected to the FISH procedure described in Materials and Methods.

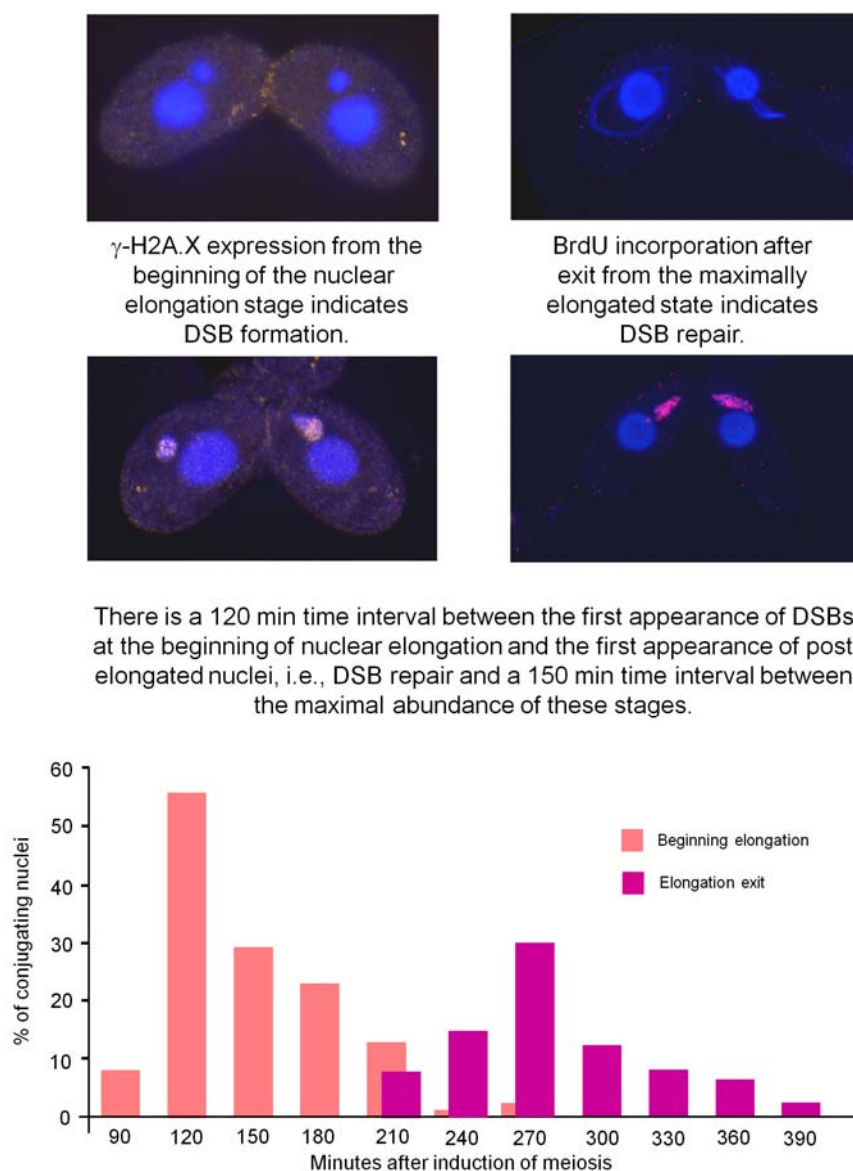


Figure S8. Meiotic DSBs undergo recombinational repair only ≥ 90 min after formation. γ -H2A.X is a reliable indicator of DSBs, it is not seen in a *spo11 Δ* mutant (5). BrdU incorporation is an indicator of recombination-related DNA synthesis. Frequencies shown in the graph are the proportions of the respective nuclear stages among all conjugating cells. Data are taken from (6). 200 nuclei were evaluated for each timepoint. For the detection of γ -H2A.X see (1,7), and for BrdU incorporation and immunodetection see (8). Cells were exposed to BrdU 2 h after induction of meiosis, i.e., 90 min before nuclei that had BrdU incorporated during meiotic recombination were detected. However, BrdU incorporation may be observed as early as 15 min after exposure. Therefore, failure of elongated nuclei to incorporate BrdU was not due to insufficient time for BrdU uptake.

Supplementary References

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2.1. Additional results

2.1.1. DSB ends are processed in *sgs1i* and *mus81i* like in wild-type meiosis

The absence of Mus81 and Sgs1 elicits a strong homolog segregation defect at anaphase I. In order to test if mutants are affected even earlier in meiosis, their ability to process DSB ends was examined.

To this end, conjugating cells of the wild type, *sgs1i* and *mus81i* mutants (suffix “i” stands for RNAi-knocked-down genes) were spread, in order to remove proteins that are not bound to chromatin, and then immunostained with an antibody recognizing Rad51 and Dmc1 (according to Lukaszewicz et al., 2010). These proteins assemble on 3′ single-stranded ends exposed by 5′ end resection and mediate the single end invasion step during DSB recombinational repair (for review see Krogh and Symington, 2004; Neale and Keeney, 2006). In *Tetrahymena*, the meiosis-specific Dmc1 recombinase forms foci in meiotic prophase nuclei, whereas the Rad51 exchange protein, although essential for meiotic DSB repair, does not produce a visible focus in the elongated pachytene-like micronucleus, and can be only observed in the non-meiotic macronucleus (Howard-Till et al., 2011).

Elongated nuclei of the wild-type, *sgs1i* and *mus81i* mutant cells did not differ with respect to chromatin-associated Dmc1 focus formation. Therefore, in both mutants, DSBs are processed and Dmc1 is normally loaded on single 3′ ends (see Fig. 6 and also discussion, chapter 3.1).

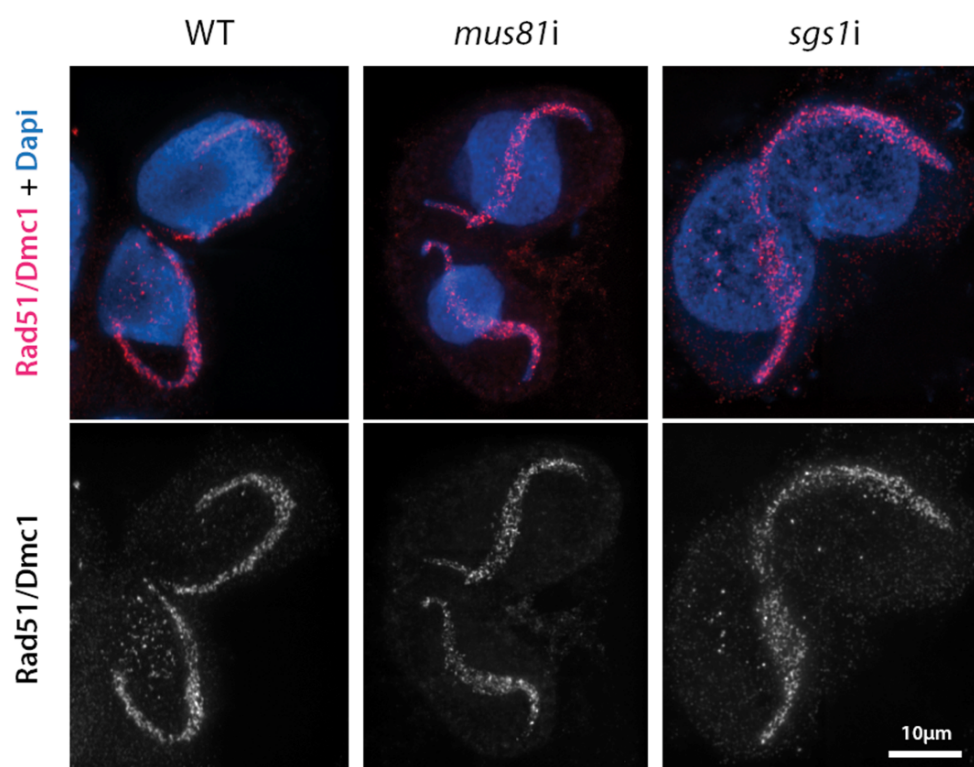


Fig. 6 Localization of Rad51/Dmc1 in spread cells. See main text for details.

2.1.2. A preliminary experiment for testing epistasis of *SGS1* and *MUS81*

The *mus81i* and *sgs1i* mutants are unable to resolve recombination intermediates (Lukaszewicz et al., 2013). In order to test a possible additive effect of the lack of Mus81 and Sgs1 on the amount of unresolved and accumulated JMs, a strain for double RNA interference (RNAi)- mediated knockdown was created (also see discussion, chapter 5).

To this end, the *SGS1* RNAi construct used to generate the *sgs1i* single mutant (Lukaszewicz et al., 2013) was excised from the RNAi hairpin (hp) vector and inserted into the *Apal* and *XhoI* sites of the pDET2c vector (gift of Kazufumi Mochizuki, modified by Rachel Howard-Till). This vector carries the *MTT2* copper-inducible metallothionein promoter and the neo5 selection cassette, and targets knock-ins to the *BTU1* locus. The *NEO5* gene confers resistance to paromomycin and is expressed under the control of the constitutive histone H4.1 promoter. The new *SGS1hp* construct was introduced into starved *mus81i* cells of both sexes by biolistic transformation (Bruns and Cassidy-Hanley, 2000). The *mus81i* strain is cycloheximide resistant and *MUS81* RNAi is induced from the *MTT1* cadmium-inducible promoter (for details see Lukaszewicz et al., 2013). Transformants were selected in 120 µg/ml paromomycin and then grown in the presence of 10 µg/ml cycloheximide and in gradually increasing paromomycin concentrations (up to 15 mg/ml). Mus81 and Sgs1 depletion was performed in vegetatively growing cells for 3 h (equal to 1 mitotic cycle) in the presence of 0.25 µg/ml CdCl₂ and 50µM CuSO₄ and overnight in starved cells in the presence of 0.075 µg/ml CdCl₂ and 5µ CuSO₄, and the salts were washed out before starved cultures of cells of opposite mating types were mixed (similar to hp constructs expression in *sgs1i* and *mus81i* single mutant cells, described in Lukaszewicz et al., 2013).

The *sgs1i mus81i* double mutant strain thus obtained was examined for the presence of DSBs and JMs by pulsed-field gel electrophoresis (PFGE) (method described in Lukaszewicz et al., 2013; Lukaszewicz et al., 2010). In short, only fragments generated by DSBs enter the gel, therefore, a diagnostic band is seen from ~2-4 h after induction of meiosis. Intact chromosomes remain in the loading well. Treatment with *SmaI* causes additional fragmentation that allows DNA to enter the gel after DSBs are repaired in the WT 5,5 h after induction of meiosis. In the mutants, *SmaI*-generated fragments do not enter the gel 5,5 h after meiosis induction because of their non-linear shape (Lukaszewicz et al., 2013). The branched structure of these fragments was attributed to the persistence of JMs (Lukaszewicz et al., 2013).

The timing of meiotic DSBs in the *sgs1i mus81i* mutant was similar to that seen in *sgs1i*, *mus81i* and WT meiosis (see Fig. 7 and Supplementary Fig. S5 in Lukaszewicz et al., 2013). The quantification of the relative amount of JMs accumulated in the *sgs1i mus81i* mutant did not show an additive effect of simultaneous Sgs1 and Mus81 depletion on JM resolution, and the average amount of

accumulated JMs (a mean of 3 biological repeats) was the same as compared to the *mus81i* mutant and slightly lower than in the *sgs1i* mutant (see Fig. 8). This result might suggest that Sgs1 and Mus81 act together in processing recombination intermediates, however such a scenario is unexpected (see discussion, chapter 5). An alternative explanation of the similar intensities of the smear in the single and double mutants could be that DNA fragments that do not enter the gel may contain one or more branched molecules. Therefore, *Sma*I-generated DNA fragments in the *sgs1i mus81i* double mutant could accumulate more JMs than those in the *mus81i* or *sgs1i* single mutants, but under the present experimental conditions these possibilities can not be discriminated. Due to these possible technical limitations, the question of whether Mus81 and Sgs1 act together or in separate pathways can not be decided here.

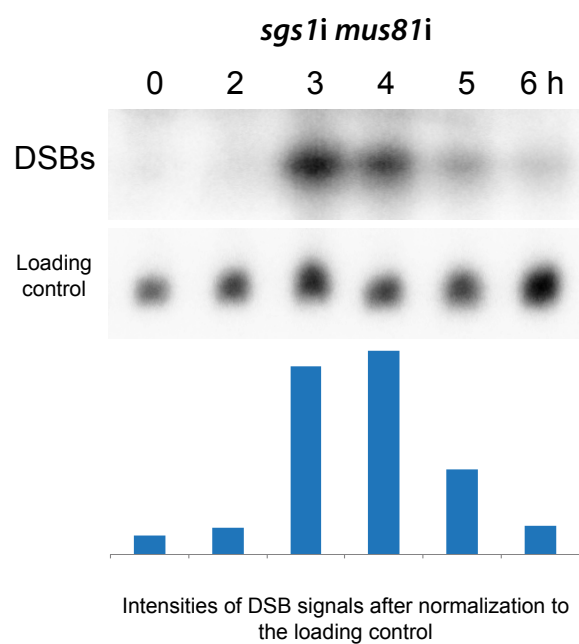


Fig. 7 Timecourse of DSB formation in the *sgs1i mus81i* mutant.

DNA samples were separated using short-run PFGE conditions (according to Lukaszewicz et al., 2010), which cause DSB-generated fragments of different length to migrate as a single band (for comparison to the WT, *sgs1i* and *mus81i* see Supplementary Fig. S5 in Lukaszewicz et al., 2013).

A

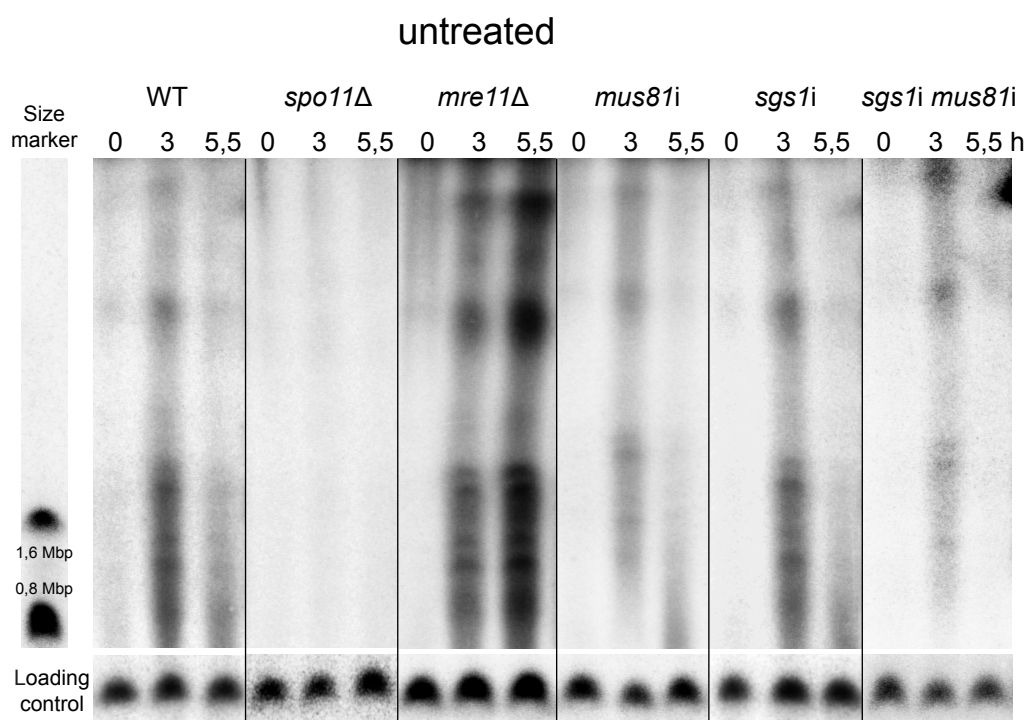
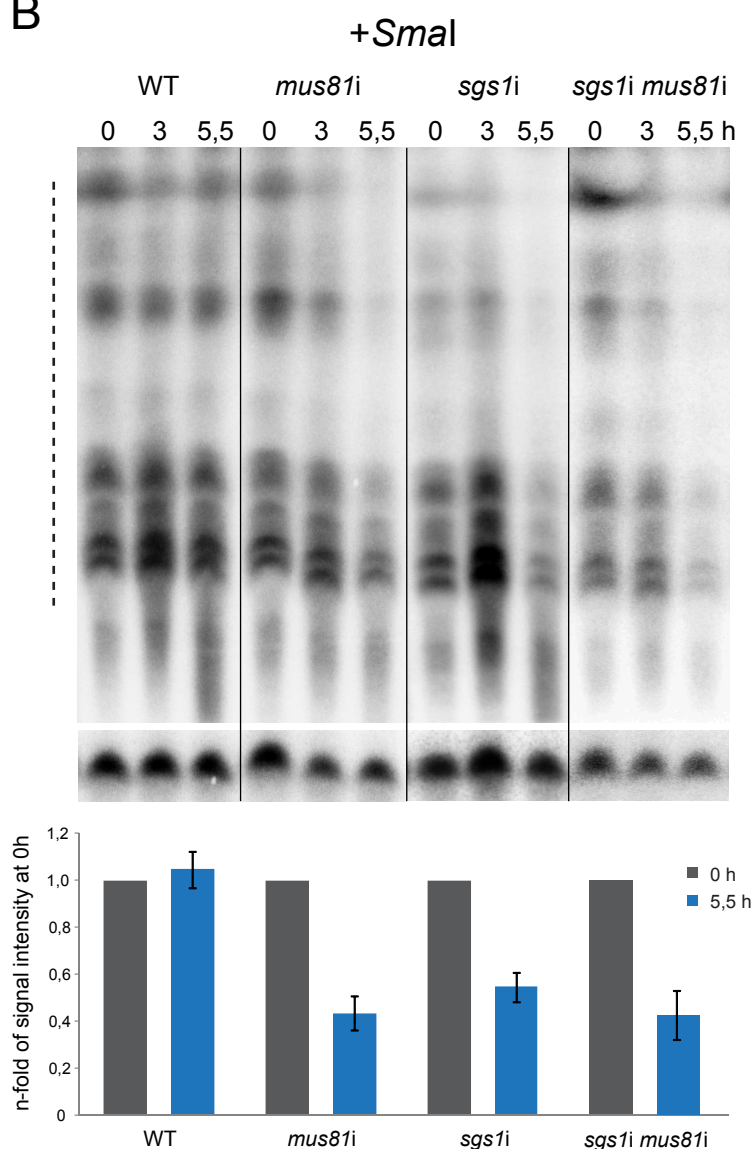


Fig. 8 Detection of DSBs and JMs by PFGE.

B



(A) Separation of micronuclear chromosomes using long-run PFGE conditions (described in Lukaszewicz et al., 2013). DSB-generated fragments are abundant at 3 h after meiosis induction and produce a smear. Intact chromosomes do not enter the gel at 0 h and 5,5 h after induction of meiosis, when DSBs are repaired. Control experiment shows no DSB formation in *spo11Δ* and DSB accumulation in *mre11Δ*.

(B) Upper panel: *Sma*I digest of DNA samples and separation under long-run PFGE conditions.

The weakening of the smear in the mutants at 5,5 after meiosis induction as compared to 0h indicates accumulation of JMs (see main text and also Lukaszewicz et al., 2013).

Lower panel: Quantification of signal intensities at 0 h and 5,5 h after meiosis induction.

Signal intensities were calculated from the area of the gel indicated by the dotted line and normalized to the loading control (for details see Lukaszewicz et al., 2013 and Fig. 4 in Lukaszewicz et al., 2013). Error bars indicate SD of the mean from three biological repeats.

DISCUSSION

1. Processing of Spo11-linked DSB ends differs among studied organisms

Most, if not all meiotic DSBs are generated by Spo11 in a topoisomerase-like reaction. A Spo11 monomer remains covalently linked to the 5' DNA end and is removed by the endonucleolytic cleavage of the 5' DNA strand close to a DSB. Release of Spo11-attached oligonucleotides is crucial for the initiation of resection. If not accomplished, broken ends remain unresected (for review see Keeney and Neale, 2006). The Mre11 complex and Sae2/Com1 play a prominent role in DSB processing in meiosis of numerous investigated eukaryotes. However, only in *S. cerevisiae* and *S. pombe* has it been directly demonstrated that both proteins are required for Spo11 removal, and also, that this cleavage requires the nuclease activity of Mre11 (for details and references see introduction, chapter 2.2). These results led to the conclusion that Mre11 acts in conjunction with Sae2/Com1. Moreover, the roles of these proteins in mitotic DSB repair provide support for their possible functional interdependence (for details and references see introduction, chapter 2.2).

On the other hand, studies of meiosis in other eukaryotes show examples where these two proteins can act separately. Also, Mre11 and Sae2/Com1 activity, may be important for processes other than Spo11 removal. In *Arabidopsis*, *Atcom1* and *Atmre11* mutants display a phenotype characteristic for accumulation of unresected DSBs, namely the lack of Rad51 foci formation and fragmented chromatin in prophase nuclei (Puizina et al., 2004; Uanschou et al., 2007). These observations are consistent with the possible cooperative function of Mre11 and Sae2/Com1 in 3' overhang generation. On the other hand, studies in *C. elegans* meiosis revealed that although both proteins are essential for DSB repair, their function in the initial DSB processing step may not be overlapping or even redundant. Namely, Tijsterman and colleagues found that COM-1 is not required for Spo11 removal, but it plays an essential role in protecting DSB ends from becoming a substrate for Ku proteins. Accordingly, in the absence of COM-1, KU proteins promote the error prone NHEJ repair pathway, which often results in the formation of univalents and fusions of non-homologous chromosomes (Lemmens et al., 2013). Interestingly, in the *com-1 cku-80* double mutant, resection activity at DSBs is regained and the RAD-51 protein is loaded on chromatin as in the WT. Also, the nearly wild-type number of bivalents is restored. This suggests that SPO-11 was removed prior to resection and that COM-1 is dispensable for this process. Besides, the NHEJ-mediated repair of DSBs in the *com-1* mutant would not take place if Spo11 remained attached to the 5' end, since this would inhibit KU proteins binding. The exact mechanism by which COM-1 antagonizes Ku proteins is unclear.

Analysis of the involvement of MRE-11 in DSB processing in *C. elegans* is complicated by the fact that this protein, similar to its counterpart in budding yeast, is indispensable for DSB formation (Chin and Villeneuve, 2001). Only recently has a mutant of *mre-11* been isolated, which is proficient in DSB formation but deficient in DSB processing. Surprisingly, analysis of this separation of function allele revealed an analogous phenotype to the *com-1* mutant, namely, the activation of NHEJ, and the restoration of Rad51 foci formation after cKU-80 depletion (Yin and Smolikove, 2013). Therefore, SPO-11 is released in the absence of fully functional MRE-11. Collectively, these data suggested that MRE-11 and COM-1 can separately contribute to DSB end protection against Ku proteins. However, only simultaneous deletion of *mre-11* and *com-1* could answer whether these proteins can act separately and substitute for each other in Spo11 removal, or if another unknown factor fulfills this function in *C. elegans*.

In this PhD thesis, I studied the role of Mre11 and Sae2/Com1 in *Tetrahymena* meiosis (Lukaszewicz et al., 2010). I found that in the absence of Mre11 or Com1, highly fragmented chromosomes are visible when nuclei enter diakinesis. In addition, the accumulation of DSB-generated fragments was confirmed by pulsed-field gel electrophoresis (PFGE). Therefore, unlike in budding yeast and *C. elegans*, but similar to *Arabidopsis* and *S. pombe*, the Mre11 protein, also in this evolutionarily distant organism, is not required for meiotic break formation. Thus, it appears that the situation in yeast and *C. elegans* is rather the exception than the rule. Unexpectedly, in the *mre11Δ* and *com1Δ* mutants, the Dmc1/Rad51 protein localizes to DSBs, though, in both mutant strains, Dmc1/Rad51 foci formation is diminished by about 40% as compared to the wild type. This indicates the presence of ssDNA at DSBs. BrdU incorporation experiments demonstrated that recombination-related DNA synthesis takes place in both mutants (data not shown), which suggests that in spite of reduced Dmc1/Rad51 loading, some of the 3' single-stranded ends form stable D-loops and prime DNA synthesis. Based on these observations, two explanations of the phenotype are possible. Namely, either in the absence of Mre11 or Com1, the Spo11 protein is not efficiently removed or, these mutants are proficient in Spo11 removal but the subsequent resection is limited. Of course, it is possible that cleavage and resection functions are differently affected in *mre11Δ* and *com1Δ* cells, and yet cause the same phenotype. It will be necessary to examine a double *MRE11* and *COM1* knockout strain in the future and test if this mutant combination eliminates Dmc1/Rad51 loading. Also, testing Dmc1/Rad51 foci formation in the *mre11i spo11Δ* and *com1i spo11Δ* mutants upon irradiation will help to answer whether Mre11 or Com1 are crucial for Spo11 removal or DSB end resection. Importantly, while in *C. elegans*, COM-1 and MRE-11 may be essential for inhibition of Ku proteins binding to DSB ends (Lemmens et al., 2013; Yin and Smolikove, 2013), in *Tetrahymena*, this function is very unlikely, as the expression of Ku proteins is upregulated only after meiosis is complete (Lin et al., 2012).

Nevertheless, similar to *C. elegans*, a simple conclusion can be made, namely, that Mre11 and Com1 can work independently (discussed in chapter below). However, if Mre11 and Com1 act at the same step of DSB repair (Spo11 removal or resection), it is possible that the presence of both proteins and their joint independent activities assures maximum efficiency. This conclusion can be supported by the fact that both proteins are known to possess nuclease activity, which, in principle, would allow them to act alone (for details and references see introduction, chapter 2.2). The possible involvement of Mre11 and Com1 in DSB end resection in *Tetrahymena* meiosis is discussed in chapter 3.1.

Altogether, combined studies on the function of Mre11 and Sae2/Com1 in meiosis of different model organisms revealed their conserved role in DSB processing. However, depending on the organism, these two proteins might be involved in different processes necessary for 3' overhang formation, which are Spo11 cleavage, inhibition of NHEJ or 5' end resection. In addition, Mre11 and Sae2/Com1 may require each other to fulfill these processes or they may act separately.

It would be interesting to examine which of the Mre11 and Sae2/Com1 functions are central to execute mammalian meiosis. Unfortunately, the null mutation of any subunit of the Mre11 complex leads to embryonic lethality (see Borde, 2007), and in mice carrying hypomorphic mutations, no severe meiotic recombination defect was reported (Bender et al., 2002; Cherry et al., 2007).

2. What may determine the separate or cooperative action of Mre11 and Sae2/Com1 in different organisms

The examples of meiosis in *C. elegans* and *Tetrahymena* presented in the previous chapter suggest that Mre11 and Com1/Sae2 cooperative function may be not mandatory. This raises the question of whether there are any features of meiosis or other factors, which could influence the nature of Mre11 and Com1/Sae2 function.

Interestingly, it was shown that chromatin organization may have a considerable impact on the function of Mre11 and Sae2/Com1. For instance, the inability of DSB induction of the *mre-11* mutant in *C. elegans* can be overcome by depletion of the cohesin complex subunit, SMC-3 (Baudrimont et al., 2011). In *S. pombe* meiosis, a similar example of gain of function associated with a possible chromatin structure change was also described. Characteristically, in the *rad50S* mutant Spo11/Rec12 stays covalently attached to the break site. However, upon depletion of Mek1 or Hop1 in this mutant background, Mre11 and Ctp1-dependent Spo11 removal is partially restored (Rothenberg et al., 2009). The authors suggested that this rescue could be due to chromatin structure loosening. This, in turn, could improve Mre11 and Ctp1 interaction, which is believed to be impaired in *rad50S*. Moreover, in budding yeast, it was reported that deletion of *HOP1* and *RED1* rescues the sporulation defect of the *sae2/com1Δ* mutant, though, the authors did not discuss a

possible effect of these deletions on DSB processing (Woltering et al., 2000). Notably, SMC-3 contributes to AEs in *C. elegans*, Mek1 and Hop1 are associated with LinEs in *S. pombe*, and Hop1 and Red1 with AEs in *S. cerevisiae*. Therefore, it is conceivable that the structure of meiotic chromosomes, defined by AEs, may have an impact on the function of the Mre11 complex and Sae2/Com1. On the other hand, Hop1, Red1 and Mek1 establish a barrier to sister chromatid recombination (BSCR) in *S. cerevisiae* (for details and references see introduction, chapter 3). Also in *S. pombe*, a role for Hop1 and Mek1 in promoting interhomolog interactions was suggested (Latypov et al., 2010). Thus, these proteins may have a more direct role in the regulation of DSB processing. Kohli and colleagues proposed that the fission yeast Mek1 and Hop1 might restrain initial DSB processing by Mre11 and Ctp1. This transient inhibition would last until homologous chromosomes are aligned, to improve the chance for interhomolog crossing over (Latypov et al., 2010). Another compelling observation was made in *C. elegans*, namely, when COM-1 was depleted in the cku-80 exo-1 mutant background, Rad51 foci were still formed, but only at late pachytene (Lemmens et al., 2013). This unusually late resection by an unknown factor corresponds in time with the disappearance of the AE protein, HIM-3, which inhibits sister recombination (see Garcia-Muse and Boulton, 2007). Taking into consideration the phenotypes presented above, it would be of future interest to investigate whether chromatin compaction/organization or the AE-associated BSCR activity may influence the function of the Mre11 complex and Sae2/Com1, and if these factors may impose requirements on the cooperative action of Mre11 and Sae2/Com1, or even inhibit DSB processing by alternative nucleases. Since AEs are absent in *Tetrahymena* meiosis, it is tempting to speculate that the unusual behavior of the *mre11Δ* and *com1Δ* mutants might be related to the lack of these structures. In fact, if AE-associated proteins would cause a transient inhibitory effect on DSB processing, this would not apply to *Tetrahymena*. *Tetrahymena*'s MIC elongation is regulated by DSB-dependent ATR signaling. Spo11 cleavage and end resection, exposing 3' overhangs, is probably essential to signal a DSB to ATR (Loidl and Mochizuki, 2009).

A factor that might be related to the separate activity of Mre11 and Com1 in *Tetrahymena*, is the lack of a homolog of the third component of the Mre11 complex, Nbs1/Xrs2. It was shown in fission yeast that Ctp1 interacts with Mre11 via the Nbs1 FHA domain. In addition, collective data indicates that this interaction is important for a cooperative function of Mre11 and Com1 in vivo, although both proteins are catalytically active in vitro (for details and references see introduction, chapter 2.2). Moreover, a homolog of Nbs1/Xrs2 has not been identified in the *C. elegans* genome either (Lee et al., 2010), and the analysis of MRE-11 and COM-1 depletion phenotypes revealed that these proteins can act separately (discussed in chapter 1). The lack of Nbs1/Xrs2 homologs in these two organisms is unexpected, since the presence of this component in the Mre11 complex is thought to be a characteristic of eukaryotes. In contrast, *E. coli* possess SbcC and SbcD, which are the Rad50

and Mre11 homologs, respectively (see Krogh and Symington, 2004). Therefore, DSB repair in *C. elegans* and *Tetrahymena* might rely on the use of the Mre11/Rad50 complex or Com1, independently.

3. Which proteins are required for the 5' end resection in meiosis?

In order to form invasive 3' overhangs, 5' ends have to be processively resected. The mechanism of resection has been intensively studied in mitotic cells of budding yeast. Several proteins necessary for the 5' strand degradation have been identified. In addition to the already mentioned Mre11 and Sae2/Com1, these are Exo1, Sgs1 and Dna2. Based on their specific activity, a two step resection model was proposed (Mimitou and Symington, 2008; Zhu et al., 2008). First, the Mre11 complex and its cofactor Sae2/Com1 initiate the first step of resection, leaving short 3' ssDNA tails. Next, these early intermediates are processed by either Sgs1 helicase in collaboration with Dna2 endonuclease or Exo1 exonuclease. This second, long range resection step produces extensive 3' ssDNA ends, suitable for invading. The Exo1 and BLM (the mammalian Sgs1 ortholog) alternative resection pathways also operate in mammalian cells (Gravel et al., 2008). It was proposed that the initial trimming of DSB ends by Mre11 and Sae2/Com1 might be important for processing of DSBs with termini blocked by, for example, the NHEJ complex or topoisomerases (see Garcia et al., 2011; Krogh and Symington, 2004). This suggestion is in accordance with recent findings that resection of "clean" ends (such as I-SceI- or HO-endonuclease-induced breaks) depends less on Mre11 and Sae2/Com1 function (Westmoreland and Resnick, 2013). It was also suggested that, regardless of the nature of generated DSB ends ("clean" or blocked), it was suggested that the initial Mre11 and Sae2/Com1 activity could deliver an appropriate substrate for the long range 5' strand resection proteins. For example, the Sgs1 helicase shows higher affinity for substrates representing 3' overhangs than blunt ends (after Mimitou and Symington, 2008). Moreover, it was shown that Exo1- and Sgs1(BLM)-Dna2-dependent 5'-3' resection is enhanced by the Mre11 complex in vitro (Cejka et al., 2010a; Nimmonkar et al., 2011; Niu et al., 2010).

The process of resection in meiosis is not fully understood, however, some of the proteins involved may play similar roles in mitotic and meiotic resection.

In budding yeast meiosis, like in mitosis, Mre11 and Sae2/Com1 are essential for resection initiation of blocked ends, in this case by Spo11, but the further extensive 5' strand resection requires the presence of only Exo1, but not Sgs1 (Garcia et al., 2011; Zakharyevich et al., 2010). Similar to *S. cerevisiae*, no role for Rqh1 (Sgs1 in fission yeast) in meiotic DSB end resection was reported in *S. pombe* meiosis (Cromie et al., 2008). However, not Exo1 but Ctp1 (the *S. pombe* Sae2/Com1 ortholog) was proposed to act as the main meiotic resecting nuclease, in addition to its role in Spo11 removal (for references see introduction, chapter 2.2). According to this model, the

presence of the Mre11 complex at the DSB sites stimulates Ctp1-dependent resection, and at the same time blocks Exo1 (Farah et al., 2009; Milman et al., 2009). In contrast to meiosis, in fission yeast mitotic cells, Tainer and colleagues proposed a model in which the endonuclease activity of Mre11 is directly required for DSB resection (Williams et al., 2008).

Also, in *C. elegans*, the resection mechanism remains ambiguous. Although it was shown that EXO-1 is responsible for the 5' strand degradation in the *cku-80* mutant background when COM-1 is depleted or MRE-11 is not fully functional, this nuclease has no role in wild-type meiosis (Lemmens et al., 2013; Yin and Smolikove, 2013; also see discussion, chapter 1). Furthermore, although the involvement of HIM-6 (the worm ortholog of Sgs1) in CO promotion was extensively studied in *C. elegans* meiosis, no role of this helicase in strand resection was reported (Agostinho et al., 2013; O'Neil et al., 2013; Saito et al., 2013; Wicky et al., 2004; Yin and Smolikove, 2013).

In the mouse model, little is known about meiotic DSB processing. However, a recent study on Exo1 has revealed interesting results. Namely, whereas the catalytic function of Exo1 is necessary for DSB end resection in mitosis, it is completely dispensable in meiosis (Schaetzlein et al., 2013). Also, in BLM depleted spermatocytes Rad51 is normally loaded on chromatin, thus indicating that this helicase is not required for resection (Holloway et al., 2010).

Overall, although it seems that the roles of Mre11 and Sae2/Com1 in meiotic DSB end resection might be conserved, the extent of their function is variable among studied eukaryotes. On the other hand, none of the long range resection proteins, except the budding yeast Exo1 nuclease, plays a major role in meiosis. Interestingly, the activity of Mre11, Sae2/Com1 and Exo1 can be differently regulated in mitosis and meiosis, even within the same organism.

3.1. Do Mre11 and Com1 alone execute the 5' end resection in *Tetrahymena* meiosis?

As discussed in the chapter above, the meiotic 5' strand degradation mechanism differs among studied eukaryotes. Although this process is well characterized in budding yeast meiosis (see the bidirectional model described in introduction, chapter 2.2), a simple extrapolation of the yeast data to the role of excision proteins in other organisms, is not possible.

In this thesis, the examination of the meiotic function of Mre11 and Sae2/Com1 in *Tetrahymena* delivered some exciting results. As discussed in chapter 1, this is the first organism where, in the absence of Mre11 or Com1, DSB resection occurs, though the efficiency seems to be attenuated (see discussion, chapter 1 and Lukaszewicz et al., 2010). Also, as proposed in chapter 2 of the discussion, it is likely that Mre11 and Com1 can be active independently. In this view, limited resection might be caused by the absence of either nuclease. Alternatively, resection could be normal, but it is initiated not at all DSB sites, because only in the presence of both Mre11 and Com1, Spo11 would be

efficiently removed. This raises the question of which resection proteins may be involved in 3' overhang generation in *Tetrahymena* meiosis?

Tetrahymena possesses putative homologs of proteins implicated in long range strand resection in mitotic cells of other eukaryotes. These are Exo1, Dna2 and Sgs1, encoded by open reading frames TTHERM_01179960, TTHERM_00136030 and TTHERM_01030000, respectively. However, the gene expression peaks of the putative homologs of Exo1 and Dna2 are seen at 5 hours of meiosis (Miao et al., 2009), whereas the expected time window for DSB end resection is between 2 and 3 hours, which would correspond to Dmc1 loading (Howard-Till et al., 2011). On the other hand, the *SGS1* peak of expression matches the expected time in meiosis, but the Sgs1 depleted cells do not show a defect in Dmc1 foci formation (see additional results, Fig. 6 and Lukaszewicz et al., 2013). Based on this knowledge and the unusual phenotype observed in *mre11Δ* and *com1Δ*, it is attractive to build a hypothetical model for 3' overhangs formation in *Tetrahymena* meiosis, in which two nucleases, Mre11 and Com1, without employing long resection proteins, are sufficient for 5' nucleolytic degradation (see Fig. 9). In this model, Mre11 and Com1 contribute to resection by using their independent endonuclease activities. A similar model was proposed for Mre11-dependent resection of blocked ends in budding yeast mitosis (see Krogh and Symington, 2004) and for mitotic DSB processing in fission yeast (Williams et al., 2008). Characteristically, in both models, the authors assumed the presence of an unknown helicase, which could unwind DNA and thereby deliver an ssDNA substrate for Mre11, which in fact is a single-stranded (ss) DNA endonuclease (see Williams et al., 2007). Therefore, the model for DNA end processing in *Tetrahymena*, also accommodates such an unwinding factor. DNA unwinding would be also crucial for Com1 nuclease activity, which has been shown to be exerted on ssDNA substrates in vitro (Lengsfeld et al., 2007). This would allow Mre11 and Com1 to nick the 5' single-stranded DNA end at many positions, which has been considered in the model proposed here. Importantly, the loading of RPA on the nascent 3' overhang, which precedes Rad51/Dmc1 nucleofilament formation, protects this end from degrading and folding (according to Chen et al., 2013). In contrast to the bidirectional resection model proposed by Neale and colleagues in budding yeast meiosis (see introduction, chapter 2.2), *Tetrahymena's* model does not invoke the requirement of the Mre11 5'-3' exonuclease activity (Garcia et al., 2011). Also in *S. pombe*, the 5'-3' exonuclease activity of Mre11 was shown to be dispensable for mitotic repair (Williams et al., 2008). However, it can not be excluded that another unknown nuclease is involved in resection in *Tetrahymena*. Moreover, although putative homologs of *EXO1* and *DNA2* do not show the expected expression profile, it would be imperative to knock out these proteins in order to exclude their possible role in meiotic resection.

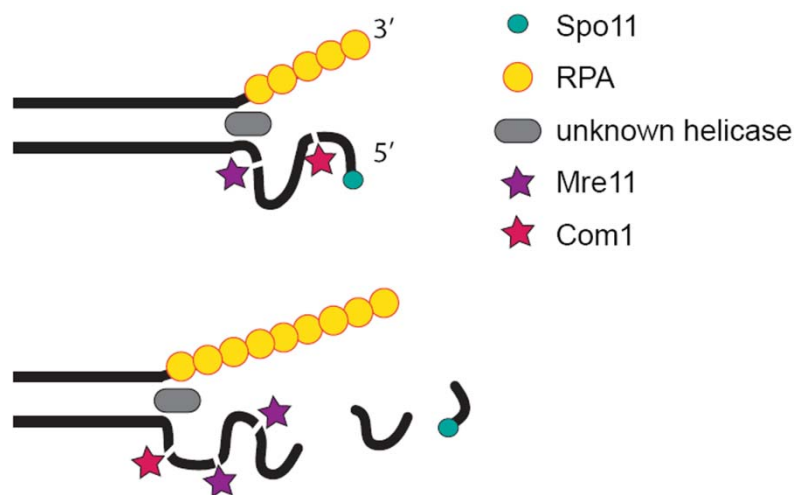


Fig. 9 Model of DSB end resection by Mre11 and Com1 in *Tetrahymena*. See main text for details.

As discussed in the chapter above, among all investigated model organisms so far, only in budding yeast, the meiotic resection mechanism depends on the long range resection protein, Exo1. Thus, in the majority of organisms, meiotic strand resection may rely on the exclusive use of Mre11 and/or Sae2/Com1 in a greater number of organisms. This possibility raises an intriguing question, namely why this manner of resection would be preferentially utilized in meiosis? One explanation of this phenomenon could be that the formation of relatively shorter 3' DNA tails is sufficient or even favored. Hunter and colleagues demonstrated in *S. cerevisiae* that mitotic and meiotic 3' overhangs differ in length, from >3 kb to ~550 bp, respectively (Zakharyevich et al., 2010). The authors suggested that excessive strand degradation, occurring at multiple DSB sites in meiosis, is unnecessary and potentially toxic. It is unnecessary because the cooperative matching of short sequences is sufficient for the recognition of homologs as it occurs at many positions along the chromosomes. Furthermore, the potential toxicity of too excessive resection derives from the fact that it can lead to the exposure of repetitive sequences present in the genome. Those, if uncovered at numerous DSB sites, may provoke their engagement in the single-strand annealing process and consequently cause deletions. On the other hand, checkpoint signaling of a single mitotic DSB likely requires exposure of a longer ssDNA track. In further support of the sufficiency of limited meiotic strand resectioning, Hunter and colleagues found that in the *exo1Δ* mutant, the 5' end is resected up to only ~250 nt, but spore viability of the nuclease-impaired *exo1* mutant is reduced only by 8% as compared to wild-type (Zakharyevich et al., 2010). The authors proposed that in the absence of functional Exo1, resection depends on Mre11 and/or Com1/Sae2. Further research by Neale and colleagues revealed that the initial cut by Mre11 is made at ~300 bp away from the DSB site (Garcia et al., 2011 and introduction, chapter 2.2), which supports the previous observation by Hunter

group. Thus, it is plausible that DSB end resection promoted by Mre11 and/or Sae2/Com1 in *exo1Δ* supports almost wild-type meiosis. In the light of these results, it would be interesting to carry out a similar study of resection rate in meiosis in other eukaryotes. Theoretically, some meiotic features such as the number of DSBs, presence of SC or dependence of homologous pairing on DSB formation, could influence the length of 3' overhangs and hence, the use of resection proteins.

4. Coordination of DSB processing and meiotic nuclear reorganization in *Tetrahymena*

Bivalent formation in *Tetrahymena* is promoted by MIC elongation and Dmc1-directed interhomolog DSB repair. These two mechanisms independently contribute to efficient homologous pairing, and thereby CO formation (for details and references see introduction, chapter 4). The present study provided better insight into recombination events that lead to precise homolog matching.

As described in Lukaszewicz et al. 2010, *MRE11* or *COM1* knockout reduces Rad51/Dmc1 foci formation but not DSB formation. This phenotype is associated with the reduction of homologous pairing efficiency. Because it is not clear if the decrease in Rad51/Dmc1 foci number reflects an inability to remove Spo11 or defective resection (for details see discussion, chapter 1), two overlapping interpretations of these results can be made. If DSB ends are blocked by Spo11 and remain unprocessed, they cannot be used for the strand invasion step. On the other hand, inefficient resection may produce 3' overhangs that are too short, also not suitable for invasion and homology search. In both cases, these DSBs may remain unproductive.

In *Tetrahymena*, over 200 DSBs are formed in meiosis (Howard-Till et al., 2011). In the *spo11Δ* mutant, upon UV treatment, only a few breaks are formed. Although this number is sufficient to trigger MIC elongation, the wild-type level of homologous pairing is not restored (Loidl and Mochizuki, 2009 and personal observations). This suggests that a certain number of DSBs is required for pairing. On the other hand, in the *dmc1Δ* mutant, although the wild-type number of DSBs is formed, homologs do not pair and breaks are repaired using the sister chromatide (Howard-Till et al., 2011). This is because, the meiosis-specific Dmc1 recombinase directs 3' single-stranded ends toward the homolog, thus it mediates interhomolog homology search and interhomolog recombination (see Gerton and Hawley, 2005). In the *mre11Δ* or *com1Δ* mutants, in the presence of the wild-type number of DSBs and Dmc1, breaks are not repaired and homologous pairing is reduced. In this case, the phenotype is caused by the impairment of the DSB end processing step (see above). Collectively, the conclusion can be made that a large number of DSBs has to be formed by Spo11 and adequately processed by Mre11 and Com1 to produce a sufficient number of 3' single-stranded tails, which become then substrates for Dmc1-mediated homology search. Therefore, these three steps of recombination are essential for homologous pairing (see Fig. 10A).

Surprisingly, another factor studied in this PhD thesis, the Sgs1 helicase, turns out to positively regulate meiotic pairing in *Tetrahymena*. The obtained results suggest that Sgs1 promotes interhomolog over intersister recombination early in prophase I, during crescent formation (see experimental details in Lukaszewicz et al., 2013). This early function likely involves disassembly of early recombination intermediates, such as SEIs, and the reuse of released 3' single-stranded ends in homology search (see Fig. 10A). This proposed function is supported by the fact that in *Tetrahymena*, recombination-related DNA synthesis takes place only after exit from the elongated stage (Loidl et al., 2012; Lukaszewicz et al., 2013). Thus, unwound 3' overhangs cannot enter the SDSA pathway to form NCOs. Similarly, dHJs, which could be subjected to Sgs1-mediated dissolution, are not formed at the time of MIC elongation. Moreover, such DSB recycling might be particularly important before the MIC achieves full elongation. At this stage, homologous chromosomes are not yet aligned, therefore, DSB ends would be prone to invade sister chromatids. This could happen particularly frequently in the absence of the AE proteins in *Tetrahymena*, which are known for their role in establishing a BSCR in other organisms (for references see introduction, chapter 3). In budding yeast, Lichten and colleagues showed that deletion of the BSCR-related *MEK1* kinase causes an increase in intersister JM formation, but also that this excess changes over time, namely, while meiosis progresses, the number of intersister JMs decreases, whereas interhomolog JMs increases. The authors suggested that this shift in favor of interhomolog connections could be due to the destabilizing role of Sgs1, which could preferentially disrupt intersister JMs (Goldfarb and Lichten, 2010). This scenario is supported by the observation that in the absence of Sgs1, intersister JM formation is elevated (Oh et al., 2007). Therefore, the function of Sgs1 in promoting interhomolog recombination by disengaging intersister connections in early prophase might be conserved.

It would be interesting to find out, whether there are any factors that could stabilize SEI intermediates in *Tetrahymena* meiosis, either in a time-dependent manner (such as after MIC elongation) or by protecting only interhomolog SEIs. Such a protective role of early recombination intermediates is assigned to the Msh4-Msh5 complex in *S. cerevisiae* (Jessop et al., 2006; Snowden et al., 2004) and the MCM complex in flies (Kohl et al., 2012). Interestingly, in *S. pombe*, which like *Tetrahymena* lacks an SC, the Rad51/Dmc1-mediator Swi5-Sfr1 was proposed to stabilize SEIs (Lorenz et al., 2012). On the other hand, Sgs1 activity could be regulated by its abundance throughout meiosis. In this case, it would be of interest to examine if the Sgs1 protein level reflects the gene expression profile, which shows a peak at the time point corresponding to MIC elongation. Alternatively, the Sgs1 protein could be subjected to posttranslational modifications modulating its function. Sumoylation of Sgs1/BLM was found to play a role in the repair of damaged replication forks by HR. In this case, SUMO modification was proposed to cause a switch between pro- and anti-

recombinogenic properties of Sgs1, via regulation of Rad51 function (Branzei et al., 2006; Ouyang et al., 2009).

Summarizing, DSB processing by Mre11 and Com1 is required for precise homologous pairing, and the Sgs1 helicase might ensure that generated single-stranded ends are available when the MIC is maximally elongated. Such coordination could support the efficient use of DSBs in favor of interhomolog interactions.

5. Mus81 nuclease and Sgs1 helicase define the meiotic recombination pathway in *Tetrahymena*

CO and NCO formation is highly regulated during DSB repair by HR, and several nucleases and helicases have been implicated in meiotic recombination intermediate metabolism in different model eukaryotes (for details and references see introduction, chapter 2.3 and 2.4).

In my PhD study, I found that Mus81 nuclease and Sgs1 helicase are essential for meiotic recombination in *Tetrahymena*. Conversely, I did not find evidence for a role of the MutLγ resolving complex or Yen1/GEN1 nuclease (Lukaszewicz et al., 2013).

In the absence of either Mus81 or Sgs1, unresolved JMs, which likely contain HJs, accumulate. This leads to a failure of homologous chromosome segregation and complete sterility. The *mus81i* mutant phenotype resembles that observed in fission yeast meiosis (Boddy et al., 2001). This indicates that, similar to *S. pombe*, *Tetrahymena* may rely on the exclusive use of Mus81 as a HJ resolvase. On the contrary, the *sgs1i* meiotic catastrophe is unique for *Tetrahymena*, and the proposed early role of Sgs1 in discouraging intersister recombination (described in the previous chapter) does not explain the observed meiotic defect. This suggests another role of Sgs1 in meiotic recombination.

In fission yeast, although the Sgs1/BLM ortholog Rqh1 promotes meiotic recombination, JMs do not accumulate in its absence, and spore viability is only moderately reduced. The mechanism of Rqh1 pro-recombinogenic activity is not clear (Cromie et al., 2008). In budding yeast meiosis, Sgs1 plays multiple, somehow antagonistic roles. On the one hand, its anti-recombination activity prevents the formation of multichromatid JMs and it is required for the generation of NCOs early in prophase I. On the other hand, Sgs1 turns to positively promoting MutLγ-dependent CO formation, though how this function is fulfilled remains to be explained. In the absence of Sgs1, COs are still generated, but their formation relies on the Mus81 resolvase (De Muyt et al., 2012; Zakharyevich et al., 2012). This is why the *sgs1Δ* mutant produces viable spores and only double deletion of *SGS1* and *MUS81* displays a synergistic lethal effect (Jessop and Lichten, 2008; Jessop et al., 2006; Oh et al.,

2008; Rockmill et al., 2003). In *Tetrahymena*, the present study suggests that Mus81 represents the only CO pathway, thus the involvement of Sgs1 in the control of recombination pathway choice is rather unlikely. However, it is conceivable that Sgs1 may have a role in NCO generation later in meiosis, after the MIC exits from the elongated stage. At this phase of meiosis, invading 3' ends start to be extended by DNA synthesis, therefore, if rejected from the D-loop by Sgs1, they could enter the SDSA repair pathway (see Fig. 10B). Alternatively, Sgs1 could mediate dHJ dissolution. Both anti-recombination activities of Sgs1 could be important to avoid the deleterious effect of excessive COs. Importantly, since Sgs1-dependent NCO formation would possibly operate in parallel to Mus81-mediated JM resolution, in the absence of Sgs1, the number of JMs could overwhelm the Mus81 resolving capacity, which would explain the *sgs1i* phenotype. Moreover, recombination intermediates that are supposed to be processed in a Sgs1-dependent pathway—may not be a preferred substrate for Mus81. (By the way, meiotic catastrophe observed in *mus81i*, could be similarly explained by the inability of Sgs1 to compensate for its lack.).

Another explanation of both *mus81i* and *sgs1i* phenotypes would be that these two proteins act together in the same pathway. It was shown that BLM has a stimulatory effect on Mus81 activity in vitro, and it was also suggested by Cohen and colleagues that in mouse meiosis, BLM could interact with and regulate MUS81-dependent recombination intermediate processing (after Holloway et al., 2010). However, there is no evidence for such a functional and physical relationship. In order to discriminate whether Mus81 and Sgs1 can partially substitute each other, act in separate pathways or together, a double *mus81i sgs1i* mutant was analyzed. Unfortunately, the obtained result of the quantification of JMs on PFGE gels in the *sgs1i*, *mus81i* and *sgs1i mus81i* mutants, did not allow a clear conclusion (Lukaszewicz et al., 2013 and additional results, Fig. 8). Nevertheless, taking into consideration the well-known Sgs1 anti-recombinogenic activity, the model of meiotic recombination pathway in *Tetrahymena* proposed here suggests a late role of Sgs1 in NCO formation by SDSA or dissolution, and the Mus81 endonuclease as the main HJ resolving factor (see Fig. 10B). Furthermore, based on the biochemical properties of Mus81 in vitro and deletion studies in other organisms, a possible mode of Mus81 endonucleolytic activity on the specific substrate is implemented (see below).

It has been shown that the Mus81 endonuclease preferentially cleaves nicked recombination intermediates in vitro, such as D-loops or nicked/unligated HJs (Gaskell et al., 2007; Osman et al., 2003; Schwartz et al., 2012). Accordingly, in meiotic HR, Mus81 may sequentially or concomitantly cleave two nicked HJs that are present after second end capture and before they are ligated to form a dHJ (see Fig. 10B). This asymmetric mechanism of cleavage generates exclusively CO products (Osman et al., 2003; Schwartz and Heyer, 2011). Hypothetically, if the two nicked HJs escape the resolution by Mus81, they could form a fully ligated dHJ, which in turn could be dissolved by Sgs1 to

a NCO. In this case Sgs1 could act as a backup mechanism. However, since *in vivo* studies in fission and budding yeast show that Mus81-mediated resolution can lead to an unbiased formation of CO and NCO products, which would occur if Mus81 processes only fully ligated dHJs (Dayani et al., 2011; Lorenz et al., 2010), this alternative is also presented in *Tetrahymena*'s model of meiotic recombination (see Fig. 10B).

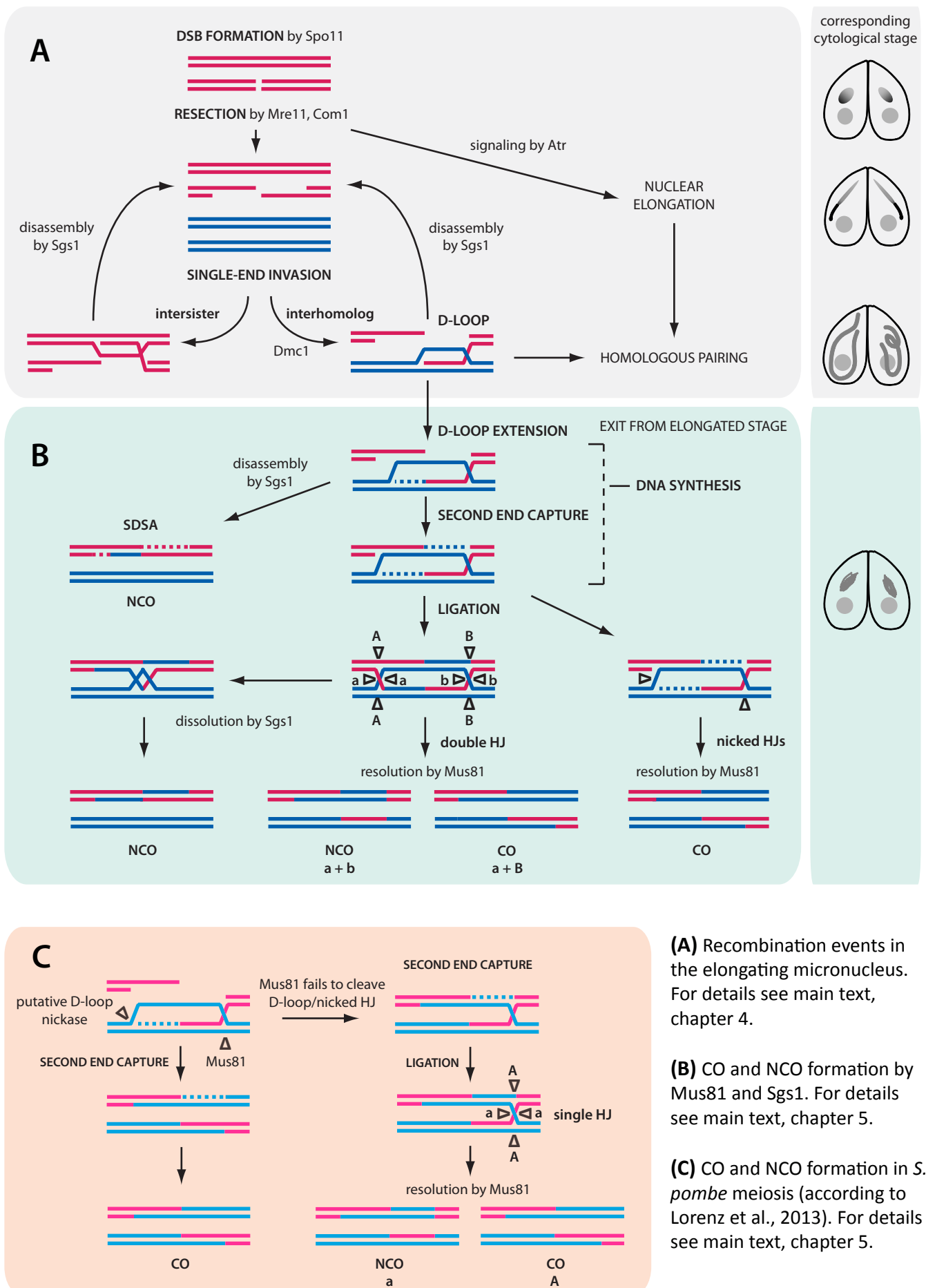
The current *S. pombe* meiotic recombination model postulates the existence of nicked HJs as predominant substrates for Mus81-dependent resolution. In addition, it assumes that when Mus81 fails to process strand exchange intermediates prior to ligation, fully ligated sHJs are formed (Lorenz et al., 2012; Lorenz et al., 2010). This is because electron microscopy analysis of the fission yeast *mus81Δ* mutant revealed the accumulation of sHJ-like JMs (Cromie et al., 2006). Moreover, the model predicts that the first nick is introduced by a putative D-loop nickase, which acts before the second end capture step, and only the following cut is exerted by the Mus81 endonuclease (see Fig. 10C). It would be of interest to check if in *Tetrahymena* meiosis, single or double HJs are generated. Of course, the presence of dHJs in *Tetrahymena* would be a precondition for a role of Sgs1 in NCO formation by dissolution.

Interestingly, it has been shown recently that MUS81-EME1 activity can be coordinated with SLX1-SLX4 activity for efficient HJ resolution in mitotic human cells (Wyatt et al., 2013). Also in *C. elegans* meiosis, the SLX-1 endonuclease was proposed to nick HJs in order to deliver preferred substrates for MUS-81 (Agostinho et al., 2013). However, Slx1 plays no role in fission yeast meiosis, and in budding yeast, it has a minor role in JM resolution (De Muyt et al., 2012; Lorenz et al., 2012; Zakharyevich et al., 2012). In *Tetrahymena*, no good homologs of Slx1 or Slx4 have been identified (Lukaszewicz et al., 2013), which rather precludes a similar mode of Mus81 action in this organism.

As already mentioned, Sgs1 has a minor role in *S. pombe* meiosis and no specific function in NCO formation. Instead, this is performed by the Fml1 helicase, which redirects DSB repair to SDSA (Lorenz et al., 2012). In *Tetrahymena*'s genome, a putative homolog of Fml1 (TTHERM_00535470) is present and it is expressed at the time corresponding to CO formation (Miao et al., 2009). Therefore, this is another candidate that could play a role in the NCO pathway in *Tetrahymena* meiosis.

Summarizing, Sgs1 helicase and Mus81 nuclease are essential factors defining the meiotic recombination pathway in *Tetrahymena*. Moreover, next to fission yeast, this ciliate represents another example of a eukaryote where, in the absence of a synaptonemal complex, mitotic repair proteins are efficiently used for meiotic recombination intermediate processing and executing meiosis.

Fig. 10 Model of meiotic recombination pathway in *Tetrahymena*



5.1. Do Mus81-dependent COs display interference in *Tetrahymena* meiosis?

Taking into consideration the proposed late role of Sgs1 in NCO formation raises the intriguing question whether NCO versus CO formation is regulated in *Tetrahymena*, and moreover, if COs are interfering. In general, Mus81-dependent COs are thought to be non-interfering, and this is true for those that are formed via the minor Mus81 pathway operating in *S. cerevisiae*, *Arabidopsis* and mouse, and also for COs of the exclusive Mus81 pathway in *S. pombe*. Conversely, interference is characteristic for the ZMM-MutLy-dependent COs (for review see Berchowitz and Copenhaver, 2010; also see introduction, chapter 2.3 and 2.4).

It was previously suggested that SC assembly might be required for CO interference (Novak et al., 2001). Later experiments in budding yeast showed that it is not the case (Borner et al., 2004; Fung et al., 2004). On the other hand, it has been reported in *C. elegans* that the discontinuity of the axial or central element decreases CO interference level (Hillers and Villeneuve, 2003; Libuda et al., 2013; Nabeshima et al., 2004). This could explain the lack of interference in the SC-less meiosis of fission yeast, where linear elements (LinEs) do not form continuous tracks (after Loidl, 2006). In contrast in mouse, neither a continuous axial element structure nor SC formation plays such a role (de Boer et al., 2007). Therefore, the function of the SC in mediating CO interference is still unclear. In this view, the absence of an SC in *Tetrahymena* does not exclude the presence of interference mechanism.

Interestingly, in mouse meiosis, there seem to exist two levels of interference. Namely, all Msh4-Msh5-stabilized recombination intermediates formed early in meiosis show interference, but only a subset of those are processed by the Mlh1-Mlh3 complex to become final COs later in meiosis. Consequently, COs show stronger interference than the Msh4-Msh5-CO/NCO-precursors (de Boer et al., 2006). Since Msh4-Msh5 early recombination foci co-localize with RAD51/DMC1 foci, it is thought that the first level of interference may occur at the DSB formation step (see Berchowitz and Copenhaver, 2010). On the other hand, the secondary selection depends on the recently identified RNF212 protein, which is the Zip3 ortholog, one of the ZMM proteins in *S. cerevisiae*, associated with the SC (Reynolds et al., 2013). In contrast, in budding yeast, all Msh4-Msh5-intermediates are destined to form COs (see Lynn et al., 2007). Moreover, whereas SC assembly in yeast depends on Zip3, RNF212 in mouse is not required for synapsis (after Reynolds et al., 2013). The difference between these two meioses could be related to the fact that although in both organisms, interference is independent of synapsis (Borner et al., 2004; de Boer et al., 2007), the NCO/CO decision implementation seems to differ in time. Namely, in budding yeast, NCOs are formed early in prophase I preceding CO formation in late pachytene (Borner et al., 2004; De Muyt et al., 2012), whereas in mouse both CO and NCO are believed to be generated in parallel, from middle to late

pachytene (Guillon et al., 2005). In addition, in contrast to *S. cerevisiae*, in the absence of Msh4 in mouse meiosis, not only synapsis (like in yeast), but also homologous pairing is affected (Kneitz et al., 2000; Novak et al., 2001). Therefore, Msh4 and Msh5 proteins might contribute to pairing or synapsis independently of their function in the CO-promoting ZMM pathway and further, the mode of pairing may influence the timing of NCO/CO differentiation (also see Baudat and de Massy, 2007).

Interestingly, in *C.elegans*, where pairing and synapsis is DSB-independent and interference is strong (one CO per bivalent), Mus81 has been shown to be one of the main CO resolvases (for review see Bellendir and Sekelsky, 2013; Garcia-Muse and Boulton, 2007). Also, although the Msh4-Msh5 complex (HIM-14 and HIM-20 in *C.elegans*) is essential for CO generation in worms (Colaiacono et al., 2003; Zalevsky et al., 1999), a role for Mlh1-Mlh3 has been not described (see Garcia-Muse and Boulton, 2007).

Fission yeast lacks the SC-associated ZMM proteins. *Tetrahymena*, except for weak homologs of Msh4 (TTHERM_00857890) and Msh5 (TTHERM_00763040), does not possess the other proteins of the ZMM group. Therefore, it would be interesting to learn if, in these two organisms, the predominant (if not exclusive) Mus81-dependent COs similarly do not display interference. Alternatively, if Msh4 and Msh5 had a function in *Tetrahymena* meiosis, it would be exciting to test if Msh4 and Msh5 foci display a low level of interference and if they undergo a similar CO-destined selection like in mouse, or if they are not interfering but still are important for homologous pairing. Theoretically, since no meiotic role of the Mlh1 protein was found in *Tetrahymena* (Lukaszewicz et al., 2013), similar to *C. elegans* (see above), Mus81 nuclease could work in the pathway with Msh4 and Msh5.

Altogether, *Tetrahymena* is an attractive model system for studying meiosis and investigating the basic function of recombination proteins outside of the highly complex SC.

ABBREVIATIONS

AE - axial element

BSCR - barrier to sister chromatid recombination

CO – crossover

CE -central element

DSB - DNA double-strand breaks

D-loop - displacement loop

dHJ - double Holliday junction

HR - homologous recombination

JM - joint molecule

LE - lateral element

LinEs - linear elements

MAC - macronucleus

MIC - micronucleus

NCO - noncrossover

NHEJ - nonhomologous end-joining

PFGE - pulsed-field gel electrophoresis

SC - synaptonemal complex

SDSA - synthesis-dependent strand annealing

sHJ - single Holliday junction

SEI - single-end invasion

ssDNA - single-stranded DNA

TFs - transverse filament

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AGNIESZKA LUKASZEWICZ

Curriculum Vitae

PERSONAL DETAILS

Born: 13.09.1983

Nationality: Polish

Work address:

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EDUCATION

- **PhD studies**

University of Vienna (Austria), Genetics and Microbiology 2008-2013

Doctoral program: „Functional Organization of the Cell Nucleus” 2008-2011

Thesis: Studies of meiotic recombination in the ciliate *Tetrahymena thermophila*

Adviser: Dr. Josef Loidl

- **MSc studies**

Agricultural University of Poznan (Poland), Biotechnology 2002-2007

Specialization: Animal Biotechnology 2005-2007

Thesis: Synaptic behavior of B chromosomes at pachytene stage of meiotic prophase I in the Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*)

Adviser: Dr. Jaroslaw Sosnowski

RESEARCH EXPERIENCE

- University of Vienna

Department of Chromosome Biology, adviser: Dr. Josef Loidl

Project: The role of Mus81 and Sgs1 in meiotic recombination in *Tetrahymena*. 2010-present

Project: The role of Mre11 and Com1 in meiotic double-strand break repair (DSB) in *Tetrahymena*.
2008-2010

- Agriculture University of Poznan

Department of Genetics and Animal Breeding, adviser: Dr. Jaroslaw Sosnowski

Project: Pairing and recombination of B chromosomes in spermatocytes of the Chinese raccoon dog.
2005-2008

RESEARCH INTEREST

- DNA double-strand break (DSB) repair pathways in mitosis and meiosis
- control of crossover and non-crossover formation in mitosis and meiosis
- homology search during DSB repair
- structure-specific nucleases
- chromatin remodeling at the DSB and its reassembly after repair
- evolutionary conservation of meiotic recombination
- repair of stalled or collapsed replication forks
- telomere protection

TEACHING EXPERIENCE

- University course tutor:

Genetics and Microbiology	06.2011 and 06.2012
Cytogenetics and Developmental Genetics	02.2009

- PhD student supervisor:

Anura Shodhan	10.2012-present
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- Internship student supervisor:

Christiane Brugger	02.2011-04.2011
Simon Brandstetter	06.2009-08.2009

PROFESSIONAL SERVICE

- Symposium Co-Organizer

“Meeting, Pairing and Propagation” (The Molecular Biology of Sexual Reproduction)
Campus Vienna Biocenter, Austria, 13.09.2010

PUBLICATIONS

Lukaszewicz, A., Howard-Till, R.A., and Loidl, J. (2013). Mus81 nuclease and Sgs1 helicase are essential for meiotic recombination in a protist lacking a synaptonemal complex. *Nucleic Acids Res.* doi: 10.1093/nar/gkt703.

Howard-Till, R.A., Lukaszewicz, A., Novatchkova, M., and Loidl, J. (2013). A single cohesin complex performs mitotic and meiotic functions in the protist tetrahymena. *PLoS Genet* 9, e1003418.

Loidl, J., Lukaszewicz, A., Howard-Till, R.A., and Koestler, T. (2012). The Tetrahymena meiotic chromosome bouquet is organized by centromeres and promotes interhomolog recombination. *J Cell Sci* 125, 5873-5880.

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.

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.

Sosnowski, J., Lukaszewicz, A, Migalska, L, Wojnowska, M and Polanski, Z. (2011). Different levels of a lack of X-Y chromosome pairing in pachytene spermatocytes of red fox (*Vulpes vulpes*) and Chinese raccoon dog (*Nyctereutes procyonoides procyonoides*). *Ann. Anim. Sci.* 11(1):71–81.

CONFERENCES

- EMBO Workshop, **Structure-specific nucleases in DNA replication and repair**, 16-20.09.2012, Côte d'Azur, France

Poster title: Mus81 resolvase and Sgs1 helicase collaborate to ensure CO formation in the SC-less meiosis of *Tetrahymena*

- EMBO Workshop, **Recombination mechanisms and genome instability**, 21-25.05.2012, Jerez de la Frontera, Spain

Poster title: Mus81 resolvase and Sgs1 helicase collaborate to ensure CO formation in the SC-less meiosis of *Tetrahymena*

- EMBO conference series, **Meiosis**, 17-21.09.2011, Capaccio/Paestum, Italy

Poster title: Mus81 is a key resolvase of recombination intermediates in SC-less meiosis

- The 10th Gordon Research Conference on **Meiosis**, 13-18.06.2010, Colby-Sawyer College, USA

Poster title: The interplay of Rad51 and Dmc1 in meiotic recombination partner choice in the protist *Tetrahymena*

- **DSB repair** meeting, 26.03.2010, Masaryk University, Brno, Czech Republic

Talk title: Detection of DSBs and recombination intermediates by a PFGE-based assay in the protist *Tetrahymena*

- EMBO conference series, **Meiosis**, 19-23.09.2009, Isle sur la Sorgue, France

Poster title: Detection of meiotic DNA double-strand breaks (DSBs) and DSB-dependent DNA repair synthesis in wild-type and mutant *Tetrahymena*

LANGUAGES

- English: fluent
- German: intermediate
- French: intermediate