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Early developmental, meiosis-specific proteins — Spo11, Msh4-1, and Msh5 — Affect subsequent genome reorganization in *Paramecium tetraurelia*

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ABSTRACT

Developmental DNA elimination in *Paramecium tetraurelia* occurs through a trans-nuclear comparison of the genomes of two distinct types of nuclei: the germline micronucleus (MIC) and the somatic macronucleus (MAC). During sexual reproduction, which starts with meiosis of the germline nuclei, MIC-limited sequences including Internal Eliminated Sequences (IESs) and transposons are eliminated from the developing MAC in a process guided by noncoding RNAs (scnRNAs and iesRNAs). However, our current understanding of this mechanism is still very limited. Therefore, studying both genetic and epigenetic aspects of these processes is a crucial step to understand this phenomenon in more detail. Here, we describe the involvement of homologs of classical meiotic proteins, Spo11, Msh4-1, and Msh5 in this phenomenon. Based on our analyses, we propose that proper functioning of Spo11, Msh4-1, and Msh5 during *Paramecium* sexual reproduction are necessary for genome reorganization and viable progeny. Also, we show that double-strand breaks (DSBs) in DNA induced during meiosis by Spo11 are crucial for proper IESs excision. In summary, our investigations show that early sexual reproduction processes may significantly influence later somatic genome integrity.

1. Introduction

In multi-cellular eukaryotes germline cells link generations and undergo meiosis, contributing genetic as well as epigenetic information (an information not encoded in the DNA sequence, however transmissible during cell division) [1]. Since epigenetic information is inherited through many generations, it needs a mechanism for its maintenance. However, how epigenetic information is maintained within or between generations, and how stable transgenerational epigenetic information actually is, are still open questions in many organisms.

Paramecium tetraurelia is a model ciliate that allows us to study germline-soma interaction and sexual reproduction, in a unicellular context, and events that contribute to the non-Mendelian inheritance. As in other ciliates, a characteristic feature of *Paramecium* is nuclear dualism, with germline and somatic genomes existing within the same cell in distinct kinds of nuclei. During asexual reproduction, the germline, diploid nucleus (micronucleus, MIC), which possesses all the genetic information including repetitive DNA and transposable elements (TEs), stays transcriptionally silent [2]. In comparison to the MIC, the somatic nucleus (macronucleus, MAC) is transcriptionally active and highly polyploid (800n). The function of MICs becomes visible as soon as the cell enters sexual reproduction, either during autogamy or conjugation, where it undergoes meiosis, and, after fusion of two gametic nuclei, produces zygotic nuclei that transfer genetic information to the offspring. During conjugation, exchange of gametic nuclei occurs between two partner cells, whereas autogamy takes place entirely within individual cells that never pair or exchange leading to the production of two identical gametic nuclei. Once they have formed, zygotic micronuclei divide twice, mitotically, to produce four identical nuclei. Two of them remain micronuclei while the other two develop into new macronuclei. However, before the precursor micronucleus becomes a new macronucleus, it undergoes an extensive genome reorganization [2]. At

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the same time, MAC chromosomes are highly amplified and the old, parental MAC is degraded and eventually lost [3].

The genome reorganization process involves the elimination of MIC-limited sequences via two pathways: imprecise elimination of minis-atellites and transposons as well as the precise elimination of transposon-related sequences - Internal Eliminated Sequences (IESs) [4]. Excision of these sequences is carried out by a domesticated transposase PiggyMac (PGM) [5] in concert with a host of other factors [6]. The precise elimination of ~45,000 unique IESs is guided by two classes of small noncoding RNAs: 25 nt scnRNAs ("scan" RNAs) and 27+ nt iesRNAs [7,8]. Approximately a third of IESs need epigenetic information from parental cell in the form of scnRNAs for excision, and hence are known as maternally controlled IESs [9]. Briefly, during meiosis the MIC is bi-directionally transcribed producing long transcripts which then are processed to produce scnRNAs. "Scanning" of the parental MAC for complementary sequences subtracts MAC genome-matching scnRNAs and enriches MIC genome-matching ones [7]. Later, in the developing MAC, excised IESs circularize or concatenate prior to circularization to serves as a template for iesRNAs precursors. Next, produced iesRNAs ensure the elimination of most copies of IESs [8,10]. However, our knowledge of this process is still very limited. Therefore, studying the mechanisms occurring in micronuclei, which encode both genetic and epigenetic information, is crucial to fully resolving the genome reorganization processes.

Since meiosis is one of the characteristic features of micronuclei during *Paramecium's* sexual reproduction, we have investigated the function of its meiotic protein homologs: Spo11, Msh4, and Msh5. In eukaryotes in general, Spo11, Msh4, and Msh5 are known to be meiosis-restricted and have well-established functions in the meiotic recombination [11–17]. Spo11 is known to induce DSBs during meiosis in a topoisomerase-like transesterase reaction that initiates meiotic recombination and ensures proper segregation of chromosomes [18,19]. After induction of DSBs, Spo11 is covalently bound to the flanking DNA sequences. Next, once Spo11 is removed from DNA, single-stranded 3′ overhangs are formed that invade the homologous DNA to initiate strand exchange between sister chromatids [20]. The majority of DSBs are repaired via the synthesis-dependent strand-annealing (SDSA) pathway. However, in some cases, the second strand is also captured, leading to the formation of four branched joint molecules (JMs) that can be resolved by cross-over (CO) [21].

Msh4 and Msh5 are eukaryotic homologs of bacterial MutS proteins [22]. Although they contain the ATP binding domain and a helix-turn-helix structural motif present at the carboxyl terminus of all MutS homologs – neither Msh4 nor Msh5 is able to interact with other Msh proteins (i.e. Msh1-3) involved in the DNA Mismatch Repair (MMR) process [14,23]. Msh4 and Msh5 exclusively interact with each other forming a heterodimer [24,25]. Unlike other Msh proteins involved in MMR, Msh4 and Msh5 lack the motif responsible for mismatched base recognition [26]. Therefore, these proteins have evolved a new function that promotes CO formation [27].

Two classes of CO have been described in yeasts [28]. Class I is dependent on a meiosis-specific set of proteins, so-called ZMM (Zip1/2/3/4, Msh4/5, and Mer3) and is manifested by synaptonemal complex (SC) formation [29–31]. Moreover, COs produced in this pathway are interfering, i.e. they prevent each other from occurring nearby [32]. The second class produces non-interfering CO without the involvement of ZMM proteins. Instead, this pathway uses a structure-selective endonuclease Mus81-Mms4(Eme1), which is able to resolve JMs to produce COs [33]. Since the class II CO pathway is simpler and depends on proteins that are involved in DNA repair, which are not exclusive to meiosis, it is believed to be evolutionarily ancestral [34].

In this study, for the first time, we demonstrate the influence of Spo11, Msh4-1 and Msh5 proteins in genome reorganization of *Paramecium tetraurelia*. We provide evidence that the presence of DSBs induced by Spo11 during meiosis are important for further excision of maternally and non-maternally controlled IESs. We also propose that Msh4-1 and Msh5 either evolved an additional, or, totally novel function in this process, necessary for the proper excision of IESs and transposons. Moreover, we suggest that there must exist a more general process (e.g. DNA or histone modifications) occurring during early sexual reproduction in germline nuclei that is also involved in determining the DNA to be targeted for excision. However, we cannot exclude the possibility of indirect downstream effects of chromosome mis-segregation due to the depletion of meiotic proteins.

2. Material and methods

2.1. Paramecium cultivation

Experiments were performed on *Paramecium* strain *51*, mating type (mt) 7 and 8. Cells were vegetatively propagated in Wheat Grass Powder (WGP; Pines International, Lawrence, KS) medium bacterized with *Klebsiella pneumonia*, and supplemented with 0.8 mg/l of β-sitosterol (85,451, Merck). Cultivation and autogamy were performed at 27 °C as previously described [35].

2.2. Proteins identity, similarity analysis and phylogenetic trees generation

To calculate percentage sequence identity, multiple sequence alignments were performed using ClustalW (version 2.1; default parameters) [36]. Identities were calculated for full length protein sequences. Accession numbers of proteins used in the study are listed in the Supplementary Table S1.

Phylogenetic trees were generated using the Geneious Prime PhyML plugin (default parameters) [37].

2.3. Silencing experiments during autogamy and analysis of the survival of the progeny

Genes silencing during autogamy was performed by RNAi feeding, as previously described [38]. To silence candidate genes, coding sequence regions were selected and amplified from genomic DNA using the primers listed in Supplementary Table S2. Next, fragments were cloned between two inverted T7 promoters of the L4440 vector [39]. The plasmids were transformed into the feeding cells: HT115 (DE3) *Escherichia coli*. For the negative control, an empty L4440 vector expressing dsRNA was used. Additionally, a PiggyMAC (PGM) RNAi plasmid was used as a positive control [5]. RNAi off-target analyses with the tool from ParameciumDB (https://paramecium.i2bc.paris-saclay.fr/cgi/tool/rnai_off_target) suggest that cross-silencing of other *Paramecium* genes is unlikely. *Paramecium* cells were seeded into silencing medium at a density of 200 cells/ml. Next, to evaluate the survival of the progeny after autogamy, 30 single cells that completed sexual reproduction in the silencing medium were isolated into fresh bacterized medium. Cells were monitored for 3 days after their isolation and divided into three groups according to the observed phenotype (healthy, sick and dead). If the transferred cell did not divide it meant that was not able to use a new MAC which was produced during silencing of the gene. So, if the cells did not divide and disappeared from the well, they were counted as a dead. If the cell was dividing, but the number of progeny cells was below the expected number (following 4 divisions per day in 27 °C), it was marked as a sick. The healthy calls are the cells which showed the expected number of progeny cells.

2.4. Silencing experiments during conjugation analysis of the survival of the progeny

To obtain reactive cells of both mating types of *P. tetraurelia*, bacterized medium with *K. pneumoniae* or silencing medium was added allowing five divisions. Next, reactive cells were mixed and incubated around 1 h at 27 °C to allow the first pairs to appear. Ten pairs were

transferred either to the bacterized medium with *K. pneumoniae* or silencing medium depending on the purpose of the experiment. As soon as cells stopped conjugating one of the exconjugant cells was transferred to a separate well. As for autogamy, cells were monitored for 3 days and divided into three groups according to the observed phenotype (healthy, sick and dead). Experiment was done in 2 replicates.

2.5. DNA extraction

For the MSH4-1, MSH4-2, MSH5 and SPO11 knockdowns (KDs) and empty vector control (Ctrl), total genomic DNA from 100 ml of each postautogamous culture was extracted according to GenElute Mammalian Genomic DNA Miniprep Kit (G1N70-1KT, Sigma-Aldrich). After this PCR analyses of IES retention were done with the standard GoTaq polymerase (M3001, Promega) protocol.

For EV, MSH4-1, MSH5 and SPO11 KDs, macronuclear DNA was isolated from 500 ml of postautogamous cells (two biological replicates) as previously described [40], after which standard paired-end Illumina libraries were prepared for DNA-Seq. Standard Illumina PCR-free library preparation and sequencing were performed.

2.6. Genome-wide analysis of IES retention scores (IRS) and transposons retention

After quality filtering and adapter removal, Illumina reads were mapped to the reference genomes as described in [41].

For each sample, IES retention scores (IRS) were determined for every annotated IES present in the genome using the ParTIES software [42]. The number of reads that contain the IES sequence is symbolized as IES^+ while the number of reads that correspond to the excised IES with only the macronuclear IES junction is represented as IES^-. Only unambiguously mapped read pairs were counted. To avoid overcounting due to paralogous matches, each read was counted only once. Reads were only counted at IES ends, to avoid length biases resulting from IES length variation. The retention score (RS) of an IES is then calculated as: $IRS = IES^+/(IES^+ + IES^-)$. All IESs were obtained from ParameciumDB [43].

In other to analyze transpososns retention, regions of the *Paramecium* genome containing transposons were extracted and next Illumina reads from Ctrl and knockdown samples were mapped using the read mapper from Geneious Prime (version 8; default parameters).

2.7. Dot blot

Dot blot assays were performed according to a standard protocol [44]. To analyze transposon retention, 3 µg of macronuclear DNA from postautogamous cells was fixed on Hybond N+ membrane (GE Healthcare). *Thon, Sardine,* and Actin specific probes were amplified from genomic DNA using the primers listed in Supplementary Table S2. Next, probes were labeled with α-32P dATP (3000 Ci/mmol) using the RadPrime DNA Labeling System (18,428,011, Invitrogen™) according to the manufacturer's instructions. Hybridization for each probe was performed at 60 °C. The imaged signal was quantified with ImageJ version 1.48e (https://imagej.nih.gov/ij/, U. S. National Institutes of Health, Bethesda, MD, USA) and the mean value out of two independent experiments is shown. The data represent the relative density normalized to Actin.

2.8. Small RNAs sequencing

To analyze small RNAs from control as well as SPO11, MSH4-1 and MSH5-KD samples, pair-end libraries and Illumina HiSeq2500 sequencing were used. sRNA reads were mapped, and their histograms normalized as previously described [8]. For KD experiments reads were normalized to the total RNA reads mapping to IESs, Other Eliminated Sequences (OESs) [45], the MAC genome, and the L4440 plasmid.

2.9. Analysis of small RNA population using RNA electrophoresis

2.5 µg of total RNA corresponding to early (E) and late (L) time points and 60 ng of small RNA ladder (#1090, Zymo Research) were labeled by γ phosphate transfer from ATP (γ -^{32}P-ATP, 0.4 MBq, Amersham) to the 5′ OH terminus of RNA by T4 polynucleotide kinase (EK0032, Thermo Scientific) according to manufacturer's instructions. The radioactively labeled RNA and small RNA ladder was run on a 15% acrylamide (19:1) urea gel in 0.5 x TBE buffer. A Phosphorimager screen was exposed to the gel and scanned with Typhoon FLA 7000 (GE Healthcare Life Sciences).

2.10. GFP tagging, microinjection, and GFP localization experiment

SPO11 (GenBank accession number: CR933361), MSH4-1 (GenBank accession number: XM_001441551) and MSH5 (GenBank accession number: XM_001457391) gene sequences with upstream and downstream regulatory regions extending up to the next coding sequences were amplified using Phusion DNA Polymerase (F530, Thermo Fisher Scientific). Obtained sequences were cloned into an L4440 vector (SPO11 and MSH4-1) or into pGMET (A1360, Promega) vector (MSH5). In all cases, Enhanced Green Fluorescent Protein (EGFP) optimized for *Paramecium* codon usage was inserted at the fusion construct C-terminus. Before microinjection, all plasmids carrying the fusion transgene were linearized using the *Ahd*I enzyme (R0584, New England BioLabs), filtered through 0.22 µm Ultrafree®_MC_GV filter (Z359904, Millipore), and precipitated with 96% EtOH. Next, DNA was dissolved in water to a final concentration ranging from 3 to 5 µg/ul, and linearized constructs were injected into vegetative cell MACs. Positive injection of cells was confirmed by dot blot analysis with the GFP probe at hybridization temperature of 55 °C.

Cells were collected during different autogamous stages, stored in 70% EtOH at −20 °C. Cells were then fixed in 2% paraformaldehyde in 1xPHEM buffer (10 mM EGTA, 25 mM HEPES, 2 mM MgCl₂, 60 mM PIPES pH 6.9). Cells were then washed in 5% BSA with 0.1% Triton X-100 and counterstained with 4,6-diamidino-2-2-phenylindole (DAPI) in 5% BSA with 0.1% Triton X-100. Next cells were mounted with ProLong® Gold Antifade Mountant (P36930, Thermo Fisher Scientific) and protein localization images were taken using a Leica SP8 TCS confocal microscope and a 63× oil objective. *Z*-series stacks were taken with Z-steps of 0.5 µm.

2.11. Indirect immunofluorescence

For immunofluorescence analyses, cells were collected during different autogamous time points and stored in 70% EtOH. Depending on subsequent detection purposes, cells were fixed and prepared according to one of the following methods.

To analyze meiotic progression and enhance intensity of GFP signal during Spo11 localization cells were permeabilized for 20 min in 1% Triton X-100 in 1× PHEM (10 mM EGTA, 25 mM HEPES, 2 mM MgCl₂, 60 mM PIPES pH 6.9). Next, cells were fixed in 2% paraformaldehyde in 1xPHEM for 10 min and blocked in 3% BSA + 0.1% Triton X-100 in TBSTM (10 mM EGTA, 2 mM MgCl₂, 0.15 M NaCl, 10 mM Tris pH to 7.4 + Tween 20 (1%, *v*/v)) for 1 h. The primary antibody used for analysis was rat monoclonal anti α-tubulin (ab6161, Abcam) at 1:200 or rabbit anti-GFP IgG fraction (A11122, Life Technologies) at 1:100. Incubation was performed overnight at 4 °C. After incubation with the primary antibody, cells were washed 3× for 15 min with 3% BSA + 0.1% Triton X-100 in TBSTM, incubated with secondary antibody goat anti-rat IgG H&L Alexa Fluor® 568 (ab175476, Abcam) at 1:500 or anti-rabbit IgG Alexa Fluor® 488 (A11008, Thermo Fisher Scientific) at 1:300 for 1 h at room temperature.

To detect the presence of DSBs, cells were fixed using Schaudinn fixative (2HgCl₂:1EtOH) as previously described [46]. The primary antibody used for analysis was anti-γH2A.X (phospho S139) (ab11174,

Abcam) at 1:100. Cells were incubated for 3 h at room temperature. Next, cells were washed and incubated with secondary antibody goat anti-rabbit IgG Alexa Fluor® 568 (A11011, Thermo Fisher Scientific) at 1:200 for 1 h at room temperature.

After incubation with secondary antibody, cells were washed and stained for 2 min in 3% BSA in TBSTM +0.25 μl/ml of 1 mg/ml DAPI and

mounted in ProLong® Gold Antifade Mountant (P36930, Thermo Fisher Scientific). Images were acquired using a Leica SP8 TCS confocal microscope and a 63× oil objective. *Z*-series stacks were taken with Z-steps of 0.5 μm.
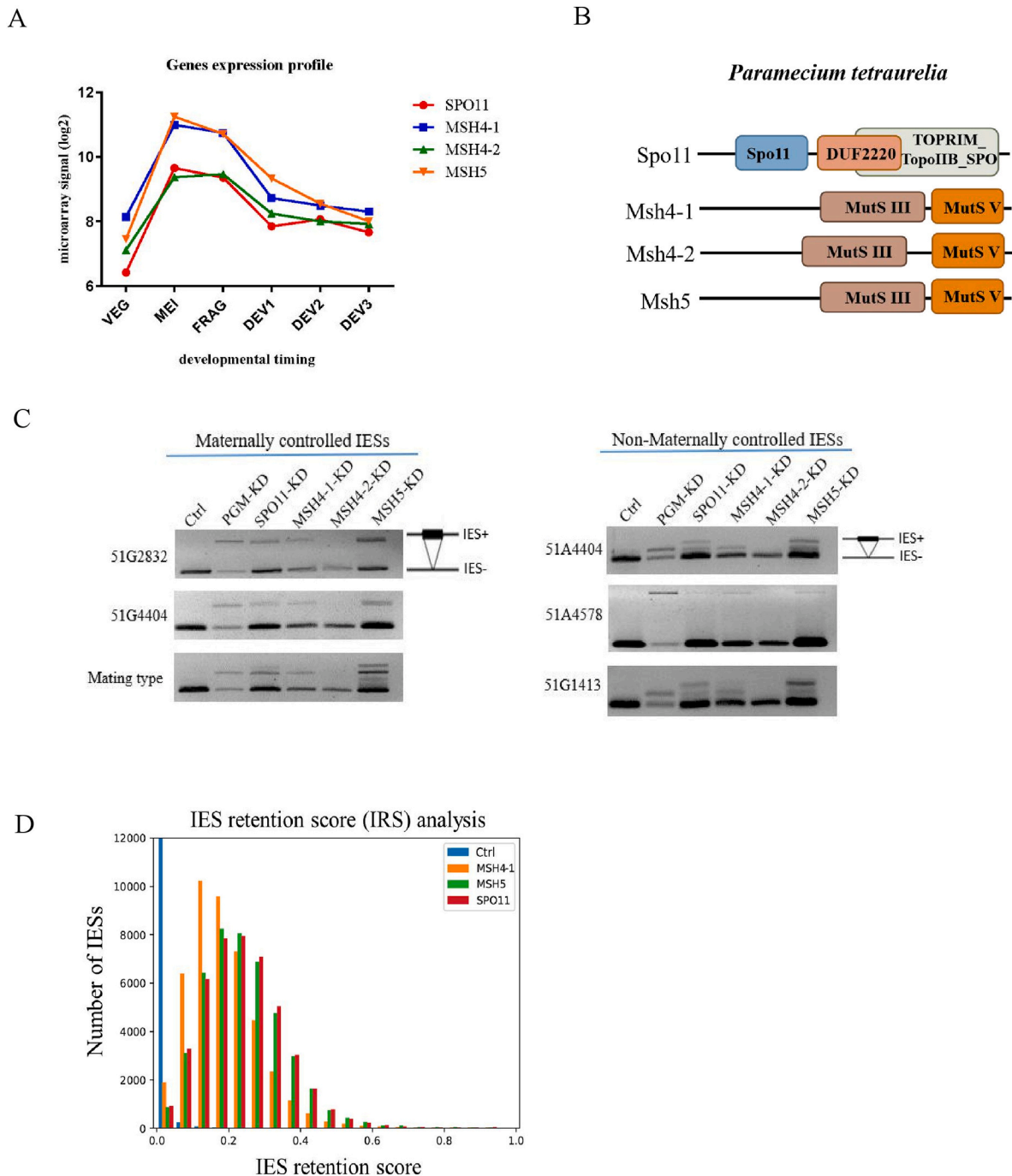

Fig. 1. SPO11, MSH4-1, MSH4-2, MSH5 in *Paramecium tetraurelia* and its knockdown effects on IES retention. (A) Autogamy time-course of gene expression for *P. tetraurelia* SPO11 (GSPATG00009108001), MSH4-1 (GSPATT00010425001), MSH4-2 (GSPATP00037600001), MSH5 (GSPATT00023204001). The Y-axis shows the log2 microarray signals [43]. VEG: vegetative cells; MEI: beginning of macronuclear fragmentation and micronuclear meiosis; FRG: population in which about 50% of cells have a fragmented old macronucleus; DEV1: earliest stage at which a significant proportion of cells has visible macronuclear anlagen; DEV2: majority of cells with macronuclear anlagen; DEV3: population of cells 10 h after DEV2. (B) Schematic drawing of predicted proteins domains in *P. tetraurelia*. (C) Effect of SPO11, MSH4-1, MSH4-2, MSH5 knockdowns on IES excision. IES retention was tested by PCR using primers flanking IESs sequences. The excised form is shown as (IES−) and the unexcised form is shown as (IES+). The IES− form is always detectable due to presence of fragments of the parental MAC in the sample. The IES+ is present only in case of IES retention in the newly developing macronuclei. (D) Retention score distribution determined by re-sequencing DNA extracted from a cell fraction enriched in new MACs, after SPO11, MSH4-1, MSH5 knockdowns.

2.12. Chemical treatments

Starved cells in the Spo11-KD medium were treated with cisplatin (CP) (232,120, Sigma-Aldrich) or methyl methanesulfonate (MMS) (129,925, Sigma-Aldrich) according to Loidl et al. [47]. As a control, we used EV and nontreated Spo11-KD cells. To investigate the presence of DSBs 100 ml of each culture was collected when cells when around 30% of cells had fragmented MACs. Collected cells were fixed in 70% EtOH and stored at -20 °C for further analysis. In order to investigate the involvement of meiotic DSBs in IESs excision, the culture treated with CP was left to finish autogamy. Next genomic DNA extraction from postautogamous cells and IES retention PCR was done as previously described.

2.13. Statistical analysis

The results represent the mean $\pm$ SD (standard deviation) from at least two independent experiments. Differences between control and knockdown samples were analyzed using one-way ANOVA and Dunnett's multiple comparison test. Statistical significance was evaluated using GraphPad Prism 8. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Selection of the candidate genes

Since investigated genes involved in the genome reorganization process in *Paramecium* have typically been upregulated during sexual reproduction, we took this characteristic feature as the main criterion to identify and investigate putative genes involved in this phenomenon. Moreover, we narrowed the selection of genes to those upregulated during the meiotic stage of sexual reproduction. Our analysis were based on transcriptome data available in the *P.tetraurelia* gene expression resource from ParameciumDB [43,48,49]. Among the selected candidates, we found SPO11, MSH4-1, MH4-2 and MSH5 genes whose expression peaks during meiosis in *Paramecium* (Fig. 1A).

Paramecium, as in *Tetrahymena*, lacks a traditional eukaryotic ortholog of Spo11 (i.e. it has low sequence identity to those described in plants and other well-described taxa (Supplementary Fig. S1A) [50,51]. *Paramecium's* Spo11 contains an additional PFAM domain of unknown function (DUF2220), abundant in bacteria and rare in eukaryotes, in the PFAM database, in addition to the canonical Type IIB DNA topoisomerase domain (Fig. 1B). However, this region aligns well among all the Spo11 homologs we inspected. Furthermore, in UniProt *P. tetraurelia* strain d4-2 Spo11 (Q3SD96) is annotated with a characteristic, eukaryotic Spo11 protein domain TOPRIM_TopoIIB_SPO1 (https://www.ncbi.nlm.nih.gov/Structure/cdd/cd00223), according to searches of NCBI's conserved domain database (CDD). Thus, there may be a distant relation between the unknown PFAM domain predominantly found in bacteria and the Spo11 domain in eukaryotes.

In the case of MSH4, *Paramecium* has two paralogs: MSH4-1 and MSH4-2 which likely arose from one of the previous whole genome duplications (WGD). Msh4-1, MSH4-2 and Msh5 in *Paramecium* possess canonical domains III and V specific for these proteins. However, the space between these two domains in MSH4-2 is greater than that of Msh4-1 and Msh5 (Fig. 1B). Overall, the *Paramecium* Spo11, Msh4-1, MSH4-2 and Msh5 are highly divergent from those found in budding yeast (Supplementary Fig. S1A, B, C, D, E, F, S2A, B, C).

3.2. SPO11, MSH4-1, and MSH5 are essential for sexual reproduction

To determine whether these developmentally upregulated candidate genes might have a role in *Paramecium* sexual reproduction we analyzed the effect of individual knockdowns (KD) by RNAi on survival of progeny from autogamy. Moreover, as negative and positive controls

respectively, we used an empty vector (EV), and a PiggyMAC (PGM) silencing construct. PGM is the main enzyme responsible for IES excision and is lethal to the sexual cell progeny after progression through sexual reproduction [5].

RNAi against SPO11, MSH4-1 and MSH5 led to severe lethality (95% for SPO11 and MSH5; and 98.3% for MSH4-1), mostly observed on the first day after the post-autogamous cells were re-fed and allowed to divide vegetatively (Supplementary Fig. S3A). In addition, our data for the SPO11-KD supports previous evidence for its impact on progeny survival by Baudry et al. [5]. Interestingly, although MSH4-1 and MSH4-2 are expressed at the same time and share 87% sequence identity on the nucleotide level they have different effects on progeny survival, with only 31.7% lethality observed from MSH4-2-KDs (Supplementary Fig. S3A). All silencing experiments were performed at least three times and were highly reproducible. We conclude that SPO11, MSH4-1, and MSH5 are necessary during sexual reproduction.

3.3. SPO11, MSH4-1, and MSH5 are needed for proper IESs and transposons excision

To investigate if the SPO11, MSH4-1, MH4-2 and MSH5 genes are involved in DNA elimination during the sexual reproduction of a new macronucleus, we first checked for maternally and non-maternally controlled IES retention. To determine this for the knockdowns of the meiotic protein homologs we performed PCR using primers flanking known IESs (Supplementary Table S2) using genomic DNA from cells that have finished sexual reproduction; DNA from PGM silenced cells was also used as a positive control. If an IES was retained (not excised) during sexual reproduction, the PCR product would be larger (IES+) than if it were excised (IES-). The sizes of tested IESs and expected IES+ and IES- bands sizes are listed in the Supplementary Table S2. The depletion of SPO11, MSH4-1, and MSH5 genes prevents the excision of both maternally and non-maternally controlled IESs, while knockdown of MSH4-2 has no influence on IES elimination (Fig. 1C). Since MSH4-2 KD did not have a strong effect on IES elimination or progeny survival, we further focused our analyses on the SPO11, MSH4-1, and MSH5 genes.

Additionally, since transposable elements are eliminated from the new MAC, we tested whether SPO11, MSH4-1 and MSH5 are also involved in their excision. In order to determine this, we used dot blot analyses using the MAC DNA from silenced or control cells (Ctrl). We also hybridized a specific probe to the *Sardine* and *Thon* transposons present in the *Paramecium* germline genome (a probe targeting the Actin gene was used as a loading control). While qualitative in nature, the experiment indicates that the depletion of the tested genes leads to the strong retention of *Sardine* and *Thon* transposons (Supplementary Fig. S3B). These observations were confirmed by mapping Illumina reads to the transposons, where they clearly are more abundant in the knockdowns (Supplementary Fig. S3C). These results were reproduced by two independent experiments, suggesting the involvement of all three genes in imprecise DNA elimination.

3.4. Global effect on IES excision

To gain a more detailed picture of the silencing of SPO11, MSH4-1 and MSH5 genes on global IESs excision we performed high-throughput DNA sequencing from newly developed MACs (two biological replicates). We used genomic DNA from newly developing MACs after silencing with an empty vector as a negative control. IES retention scores (IRSs) for all three silenced genes were calculated as previously described [41]. Briefly, IRSs vary from 0.0–1.0, from complete excision (0.0) to complete retention (1.0). The histograms of SPO11, MSH4-1, and MSH5 knockdowns show similar IES retention distribution with IRS mean approximately 0.15 (Fig. 1D), suggesting that these genes may affect IES excision in a similar manner. Correlation analyses of IRS among knockdown replicates show a good correlation, particularly for

the second batch of the slightly more efficient knockdowns (Supplementary Fig. S3E; r_s = 0.80–0.83 for pairwise correlations between the MSH4.b, MSH5.b and SPO11.b knockdowns). In general, SPO11, MSH4-1, MSH5 IRSs correlate better with each other than with any other proteins known to be involved in genome reorganization (Supplementary Fig. S3D). These data are consistent with SPO11, MSH4-1 and MSH5

working in the same pathway.

3.5. SPO11, MSH4-1, and MSH5 affect iesRNA but not scnRNA production

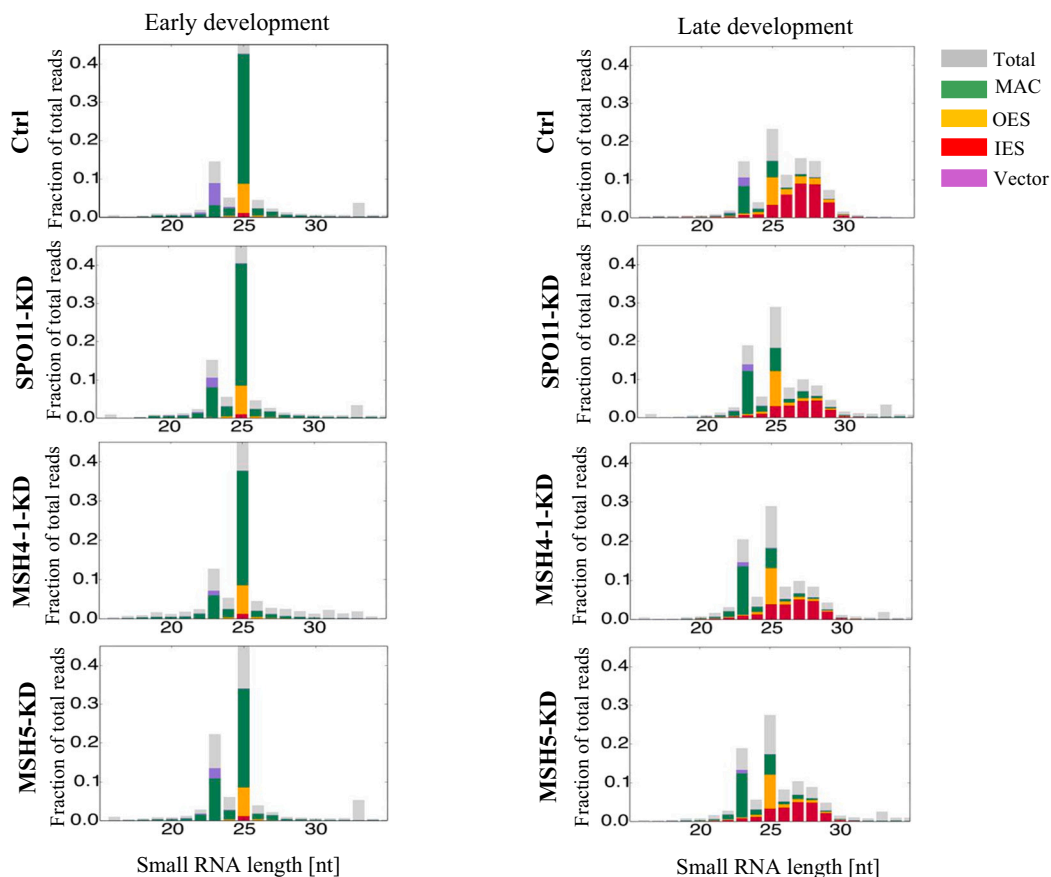Since the development-specific sRNAs, scnRNAs and iesRNAs, are

A
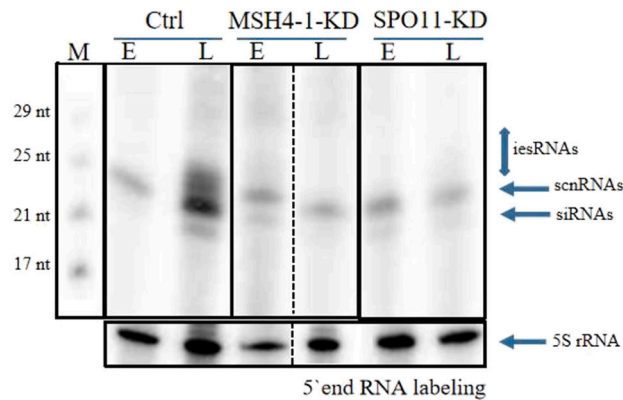
B

Fig. 2. Analysis of small RNA populations in SPO11, MSH4-1 and MSH5 silenced cells. (A) Gel electrophoresis of sRNA from SPO11, MSH4-1 silenced and control (Ctrl) cells. Total RNA samples corresponding to the early (E) (20% of the cells with the fragmented macronucleus), and late (L) (majority of cells with macronuclear anlagen) time-points were run on a denaturing 15% polyacrylamide-urea gel. After electrophoresis the samples were labeled with γ -^{32}P-ATP. M: RNA Low Molecular Weight Marker. 5S rRNA was used as a loading control. Dashed line indicates an empty lane in the gel between the MSH4-1-KD E and L samples. The complete gel can be found in Supplementary Fig. S3E. (B) Small RNA libraries from Ctrl, SPO11, MSH4-1 and MSH5 silenced cells corresponding to early and late development time-points were sequenced and mapped to the reference genomes (*Paramecium tetraurelia* MAC reference genome and MAC + IES reference genome). Stacked bar plots show the normalized number of sRNA reads that match the MAC genome (green), OESs (yellow), annotated IESs (red), L4440 vector (purple) and total reads (gray). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

proposed to guide IES excision, we assessed whether depletion of SPO11, MSH4-1, and MSH5 might affect their production, using RNA electrophoresis on a denaturing gel. RNA was isolated from the control and silenced cells for two time points: early autogamy (around 20% fragmentation), during scnRNA production, and late autogamy (100% fragmentation plus the majority of the cells with visible new MAC) timepoint, during iesRNA production. Based on the sRNA gel analysis, we observed no changes in 25-nt scnRNA production compared to the control. However, the population of the iesRNA was markedly depressed (Fig. 2A).

These observations were further analyzed by sRNA high-throughput sequencing (HTS). From early time point the 25-nt scnRNAs corresponding to the MAC matching sequences (green) slightly decreased in the knockdown samples compared to control, while there was little change of the scnRNAs mapping to the IESs and OESs (Fig. 2B). This suggest that RNA scanning is largely unaffected by the knockdowns. The reduction of iesRNAs relative to scanRNAs during late sexual reproduction for the SPO11, MSH4-1, and MSH5 knockdowns observed from HTS confirmed the iesRNA decreases observed in the sRNAs gel (Fig. 2B). Since iesRNAs are produced from excised IESs, the observed effect may be explained by the inhibition of IES excision by SPO11, MSH4-1 and MSH5 knockdown.

3.6. SPO11, MSH4-1, and MSH5 localize to the micronuclei during autogamy

To determine the localization of Spo11, Msh4-1, and Msh5 proteins during autogamy, we generated C-terminal GFP fusion constructs under the control of endogenous promoters for each of these proteins, and microinjected them into *Paramecium* MACs. Due to the low abundance of Spo11, we amplified the signal of the translated protein using an anti-GFP antibody. The localization of Spo11 GFP fusion protein was observed in the micronuclei during meiosis, with the strongest signal during prophase I of meiosis (Fig. 3 panels B, C, D). No GFP signal was detected during vegetative growth nor during later stages of autogamy (Fig. 3 panels A, E, F).

As in SPO11, the MSH4-1-GFP and MSH5-GFP signals were exclusively detected during autogamy and localized to the micronuclei (Fig. 4A and B). However, the MSH4-1-GFP and MSH5-GFP signals were present throughout meiosis and beyond, during formation of zygotic nuclei and post-zygotic mitotic divisions (Fig. 4A and B (panels B, C, D, E, F, G)). The signal disappeared during or just before macronuclear development (Fig. 4A and B panel H). No GFP signal was present during vegetative growth (Fig. 4A and B panel A).

3.7. No visible effect on meiotic and postzygotic mitotic performance due to SPO11, MSH4-1, and MSH5 knockdown

Since Spo11, Msh4-1, and Msh5 are localized to the micronuclei, with known roles in meiotic recombination in other organisms, we tested if their silencing affects the production of meiotic nuclei, i.e. meiotic performance. To analyze this, we used an α-tubulin antibody which is a specific marker for micronuclei (divide mitotically and meiotically). Macronuclei divide amitotically without an internal spindle formation allowing us to clearly distinguish both nuclei [52]. Meiotic performance was investigated by checking the presence of the correct number of micronuclei during different autogamous stages in control and silenced cells. Analyses indicated that SPO11, MSH4-1, and MSH5 knockdowns do not affect meiotic performance, showing four MICs during the first meiotic division and eight MICs after the second meiotic division (Fig. 5A, C; Supplementary Fig. S4A, C). Nevertheless, these knockdowns may have affected meiosis at the chromosomal level, which is undetectable with an α-tubulin antibody.

Thus, we sought to determine if further steps during autogamy were affected. To investigate this, we examined the number of zygotic nuclei and postzygotic mitotic products. However, as in meiosis, zygotic
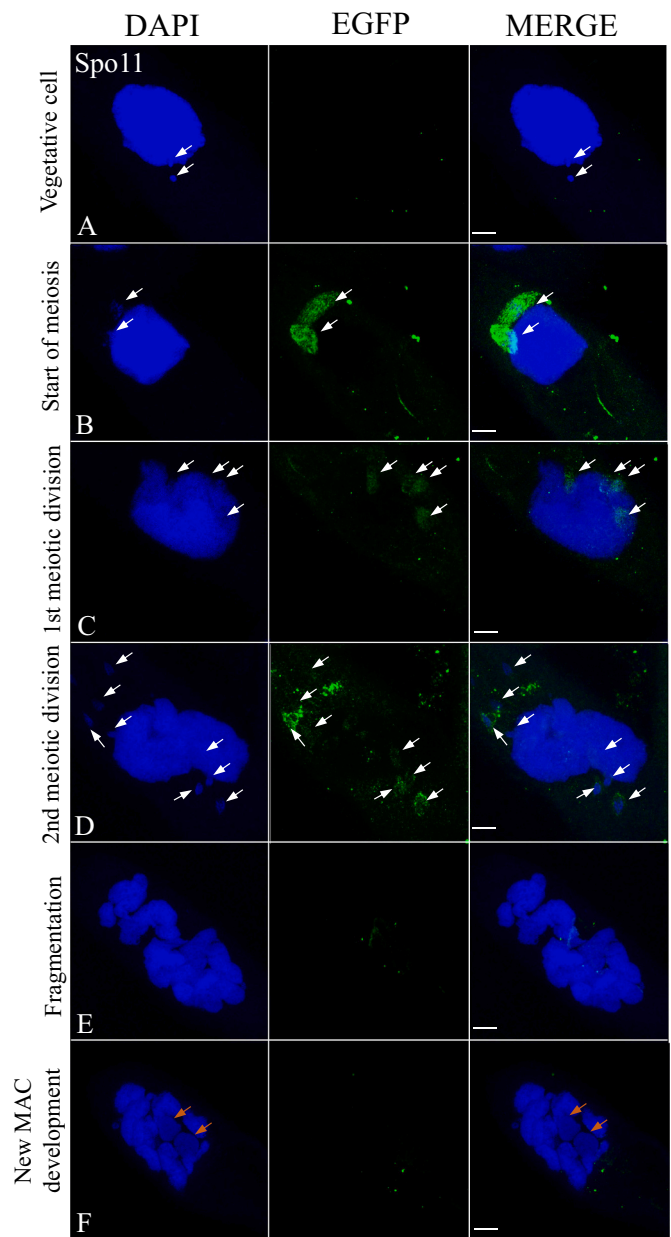

Fig. 3. Localization of Spo11 during sexual reproduction by GFP-fusion enhanced with ani-GFP antibody. Spo11 tagged C-terminally with GFP was expressed during meiosis and localized in the micronuclei. Panel A shows vegetative cells. Stages of autogamy are shown in the following panels: B: start of meiosis, C: 1st meiotic division, D: 2nd meiotic division, E: fragmentation, F: new MAC development. Blue channel: DAPI, Green channel: EGFP. Merged signals; scale bar: 5 μm. White arrows indicate cells with MICs. Orange arrows indicate new MAC. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

nuclear formation and mitotic divisions were unaffected (Fig. 5B, D; Supplementary Fig. S4B, D). This suggests either that: 1) meiosis is affected on the chromosomal level without substantially affecting nuclear morphology or development, though this is difficult to determine in *Paramecium* due to the large number of micronuclear chromosomes; 2) meiosis is not affected during silencing of SPO11, MSH4-1 and MSH5, in which case these proteins may have evolved new or additional functions necessary for genome reorganization in *Paramecium*.
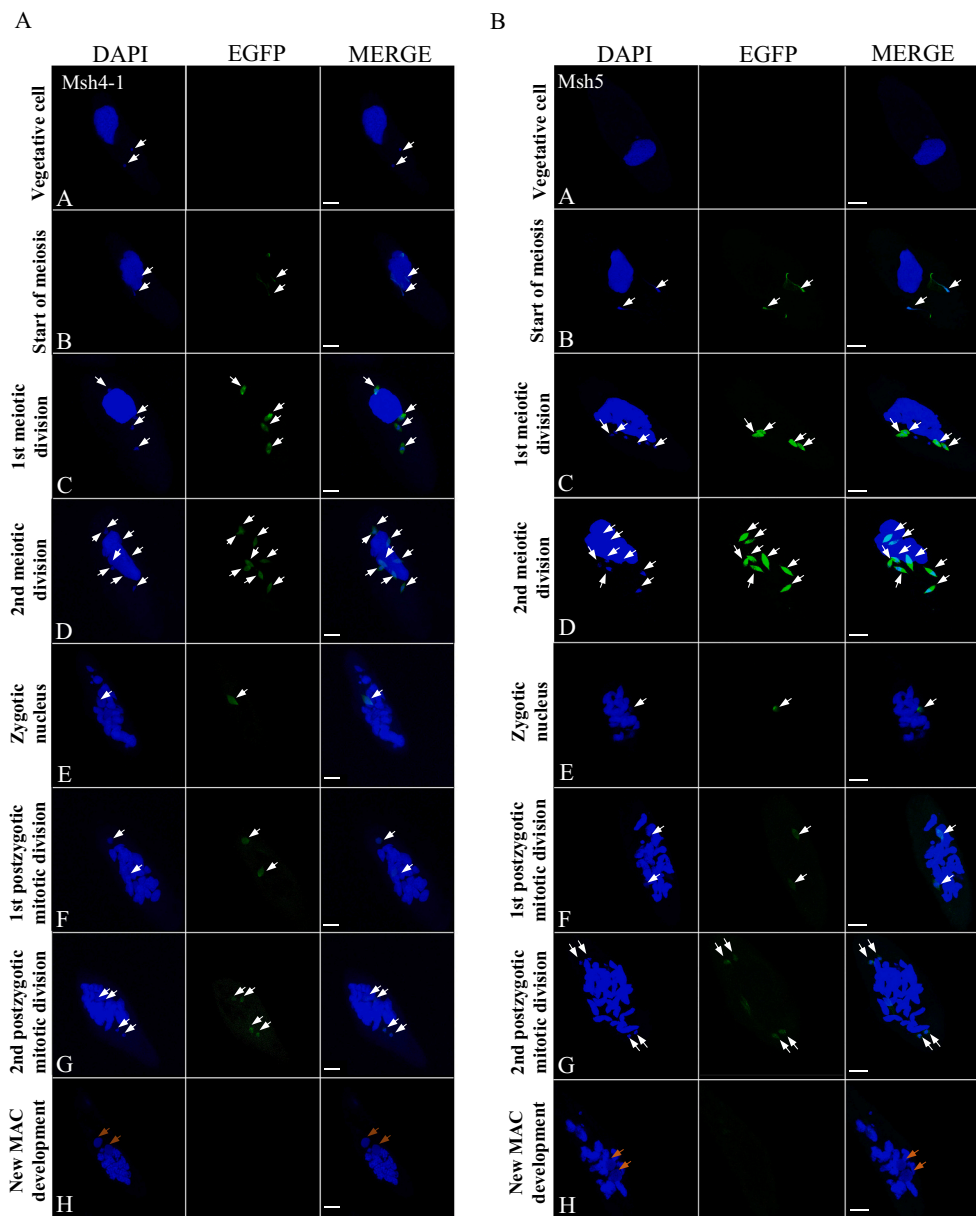
Fig. 4. Localization of Msh4-1 and Msh5 during sexual reproduction by GFP-fusion. (A) Msh4-1 tagged C-terminally with GFP was expressed during meiosis and post-zygotic mitotic divisions and localized in the micronuclei and zygotic nucleus. Panel A shows vegetative cells. Stages of autogamy are shown in the following panels: B: start of meiosis, C: 1st meiotic division, D: 2nd meiotic division, E: zygotic nucleus, F: 1st postzygotic mitotic division, G: 2nd post-zygotic mitotic division, H: New MAC development. Blue channel: DAPI, Green channel: EGFP. Merged signals; scale bar: 5 μm. White arrows indicate cells with MICs. Orange arrows indicate new MACs. (B) Msh5 tagged C-terminally with GFP was expressed during meiosis and postzygotic mitotic divisions and localized in the micronuclei and zygotic nucleus. Panel A shows vegetative cells. Stages of autogamy are shown in the following panels: B: start of meiosis, C: 1st meiotic division, D: 2nd meiotic division, E: zygotic nucleus, F: 1st postzygotic mitotic division, G: 2nd post-zygotic mitotic division, H: New MAC development. Blue channel: DAPI, Green channel: EGFP. Merged signals; scale bar: 5 μm. White arrows indicate MICs. Orange arrows indicate new MACs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.8. Potential post-meiotic roles for Msh4-1 and Msh5 in Paramecium

Since Msh4-1 and Msh5 localize to the micronuclei during mitosis we sought to determine if Spo11, Msh4-1 and Msh5 play a role in new MAC development just after this, despite their gene knockdowns having no visible impact on meiosis or mitosis. To assess this, we took advantage of conjugation process. Conjugation allows us to distinguish two events happening during sexual reproduction namely meiosis and MAC development. During conjugation meiosis occurs when *Paramecium* cells are mating, whereas macronuclear development starts right after the cells separate [53]. While the cells are mating and meiosis takes place the oral apparatus is blocked; as soon as they separate, they are able to consume food [54]. For this reason, gene silencing after meiosis occurred during conjugation was best to assess if Spo11, Msh4-1, and Msh5 might have an additional later function during MAC development.

To investigate if Spo11, Msh4-1 and Msh5 functions are meiosis-specific, we silenced these genes prior to meiosis. First, post-autogamous cells were fed with silencing medium allowing five asexual divisions. Next, conjugation between two mating types (mt7 and mt8)

was induced, then single pairs were transferred to separate wells with medium bacterized with *K. pneumoniae*. As soon as cells finished conjugation one of the exconjugants was transferred to a new well to monitor cell growth (division) for three subsequent days.

To investigate the possible function of Spo11, Msh4-1 and Msh5 during MAC development *Paramecium* cells were first fed with medium bacterized with *K. pneumoniae* alone. After they started conjugation the cells were transferred to the silencing medium. In addition, to determine if the observed phenotype on IES retention and cell survival due to silencing of these genes is transferred through meiosis to the next generation we performed a conjugation experiment between mt7 wild-type (WT) cells, and mt8 silenced cells. To ensure that gene silencing worked, we performed silencing during the whole conjugation process. As negative controls, we used non-silenced cells. As an additional positive control, we silenced PGM whose expression is known to be restricted to the late stage of sexual reproduction [5].

We observed death of the majority of the SPO11 knockdown cells when they were silenced during meiosis, whereas the cells were able to divide when SPO11 was silenced during MAC development (Fig. 6A). In
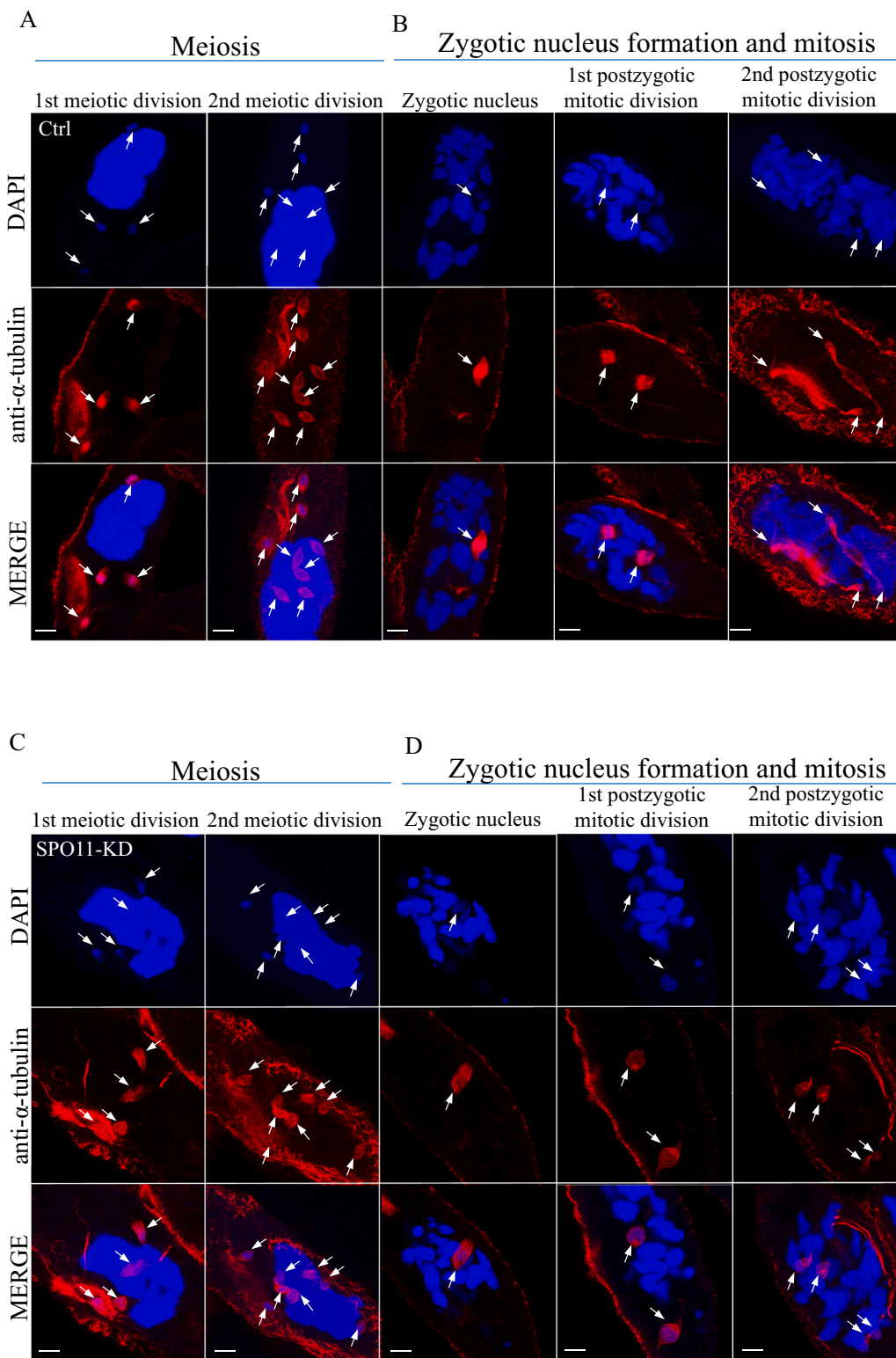
A **Meiosis**

B **Zygotic nucleus formation and mitosis**

Fig. 5. Comparison of α-tubulin signals between control and SPO11 knockdowns cells. Staining with α-tubulin aim to visualize micronuclei during sexual reproduction in *P. tetraurelia*. (A) Meiosis stage in control cells indicating 4 micronuclei at 1st meiotic division and 8 micronuclei at 2nd meiotic division. (B) Zygotic nuclei formation and mitosis in control cells indicating one zygotic nucleus, 2 micronuclei at 1st postzygotic mitotic division and 4 micronuclei at 2nd postzygotic mitotic division. (C) Meiosis stage in SPO11-KD cells indicating 4 micronuclei at 1st meiotic division and 8 micronuclei at 2nd meiotic division. (D) Zygotic nucleus formation and mitosis in SPO11-KD cells indicating one zygotic nucleus, 2 micronuclei at 1st postzygotic mitotic division and 4 micronuclei at 2nd postzygotic mitotic division. Blue channel: DAPI. Red channel: anti-α-tubulin. Merged signals; scale bar: 10 μm. White arrows indicate MICs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

C **Meiosis**

D **Zygotic nucleus formation and mitosis**

the case of MSH4-1 and MSH5 the cells were mostly sick or dead while silenced during meiosis (Fig. 6C). Consistent with the GFP localization experiments showing these proteins present in micronuclei beyond the meiotic stage of development, there is a notable effect on cell health and survival when the genes encoding these proteins are silenced after meiosis (Fig. 6C).

These results suggest Spo11 specifically performs its function during the meiotic stage of sexual reproduction, while Msh4-1 and Msh5 are needed for a longer period afterwards. Additionally, our analyses show that the observed effect on IES retention and cell survival can be largely or wholey rescued by conjugating WT (mt7) and silenced (mt8) cells (Fig. 6B, D). Overall, our data suggesting early developmental process in micronuclei, including during meiosis, can substantially influence downstream IES excision.
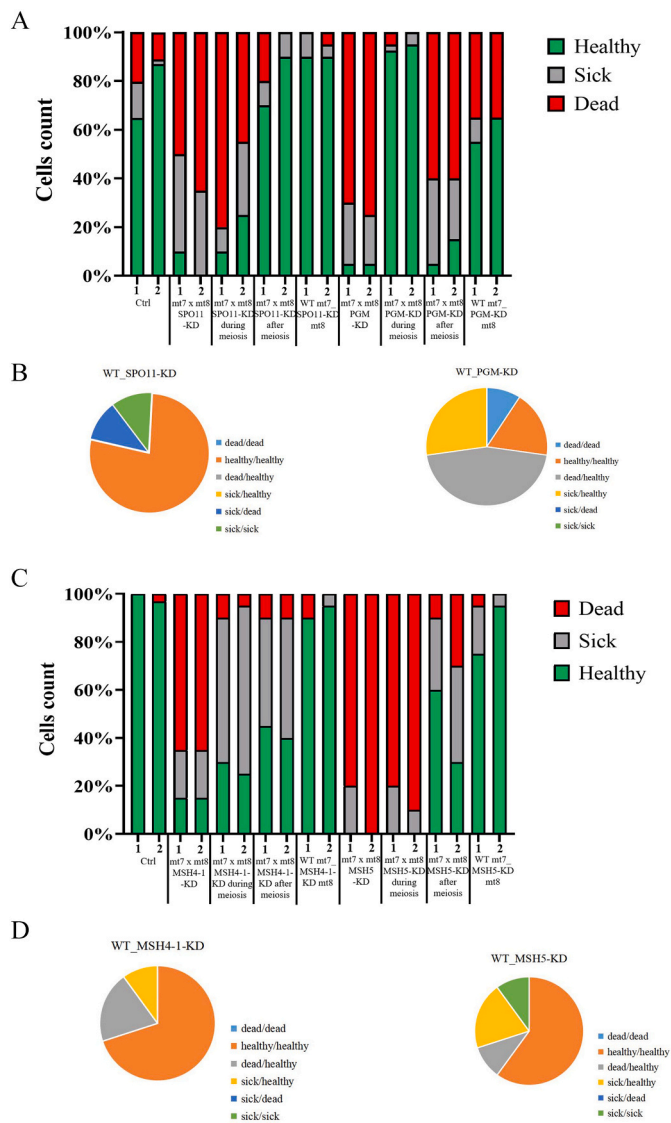
Fig. 6. Effect of SPO11, MSH4-1 and MSH5 knockdowns on cell survival during meiosis and new MAC development. (A, C) Effect of SPO11-KD (A) and MSH4-1 and MSH5-KD (C) on cell survival. The graphs show the cell survival after SPO11, MSH4-1 and MSH5-KD during: the whole autogamy process, meiosis, new MAC development and after conjugation between mt7 WT cells and mt8 knockdown cells. The dead, sick and healthy cells are shown in different colors. Red: percentage of dead cells; gray: percentage of sick cells (altered number of divisions or behavior); green: healthy cells (cell growing at a normal rate). PGM-KD was used as a positive control. L4440 vector without insert was used as negative control. For each bar $n = 20$ cells. (B, D) Cells counts showing a rescue effect of SPO11, MSH4-1 and MSH5 knockdowns on IES retention and cell survival while conjugating WT (mt7) and silenced (mt8) cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.9. DSBs induced by Spo11 during meiosis are necessary for IES excision

To get more insight into the function of Spo11 in *Paramecium* development, specifically its canonical function in other organisms to induce DSBs during meiosis, we examined the induction of DSBs measured by anti-γH2A.X antibodies. H2A.X is a variant of histone H2A that gets phosphorylated in response to DSBs [55], and is a good marker to investigate these lesions. We observed γH2A.X positive cells in our negative control cells while the staining was suppressed in SPO11-KD cells (Fig. 7A). Thus, we propose Spo11, as in other organisms, is necessary for DSB induction during meiosis in *Paramecium*.

Next, to test the importance of meiotic DSBs in IES excision, we silenced SPO11 and then treated cells with the DSB-inducing chemicals; cisplatin or MMS. H2A.X positive meiotic micronuclei indicate both cisplatin and MMS restore DSBs (Fig. 7A). We also isolated genomic DNA from cisplatin-treated SPO11-KD cells and analyzed IES retention by PCR to investigate if artificial DSB induction during meiosis counteracts the IES retention due to SPO11-KD. We observed DSB induction by cisplatin could partially rescue IES excision (Fig. 7B). Together, these observations suggest an important role for SPO11 in inducing meiotic DSBs, with a pronounced influence on downstream IES elimination. Interestingly, Akematsu et al. [56] have recently shown the importance of Spo11 function in post-meiotic induction of DSBs during *Tetrahymenta thermophila* development.

3.10. Cross-over with Mus81, but not Msh4 and Msh5, is essential for MAC development in Paramecium

It is well established that crossing-over (CO) in eukaryotes can be generated by two independent pathways: either the Class I, interfering pathway involving Msh4 and Msh5 or Class II, non-interfering pathway involving Mus81 [30,33]. Thus, we investigated which pathway is responsible for CO in *Paramecium*. Since, we observed that MSH4-1 and MSH5 knockdowns do not affect meiotic performance and led to the successful production of a new MAC, we wondered if knockdown of the *Paramecium* MUS81 gene, would result in a similar effect. To investigate this, we individually silenced MSH5 and MUS81 to separately silence the two CO pathways, and co-silenced MSH5 and MUS81 to simultaneously block both CO pathways. First, we investigated cell survival after knockdown of these genes (Supplementary Fig. S5A). We observed a complete blocking of meiosis, and consequent failure of MAC production due to MSH5/MUS81 co-silencing, consistent with CO being essential for subsequent MAC development. However, the single knockdown of MUS81 also resulted in no MAC production (Fig. 7C and Supplementary Fig. S5B), suggesting that a non-interfering pathway involving Mus81 is essential, if not the only, for crossing-over in *Paramecium*. Interestingly, we also observed that silencing of other homologs of genes involved in meiotic recombination, including Rad51, Hop2 and Mnd1, led to no new MAC production (data not shown). Since individual MSH4 and MSH5 silencing do not affect meiotic performance or subsequent MAC development, whereas individual MUS81 does, it appears that CO by the non-interfering pathway and not the interfering pathway is critical for this in *Paramecium*. Furthermore, we propose Msh4-1 and Msh5 may have an additional new function in genome reorganization during sexual reproduction in *Paramecium*.

3.11. SPO11 knockdown does not affect Msh5 localization during sexual reproduction

Since it is widely known that Spo11 works at the beginning of the meiotic recombination process [18,19], we tested whether the silencing of SPO11 affects Msh5 localization. We injected the previously described MSH5-GFP construct into *Paramecium* MACs and then silenced SPO11. As a negative control we used non-silenced cells. First we performed a survival test and examined IES retention by PCR, confirming that SPO11 knockdown was efficient (Supplementary Fig. S5C and D). Analyzes indicated that SPO11-KD does not affect Msh5 localization, showing GFP signals during meiosis, zygotic nuclei formation and post-zygotic mitotic divisions (Supplementary Fig. S5E).

4. Discussion

The developmental genome reorganization processes in *Paramecium tetraurelia* involve the elimination of thousands of IESs (maternally controlled and non-maternally controlled) as well as numerous transposons. Since longer IESs and those with degenerate consensus-like ends are not easily excised [40,41], the cell benefits from a process which uses
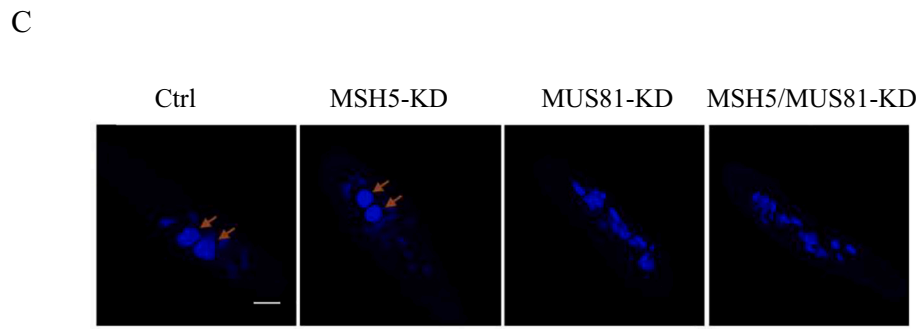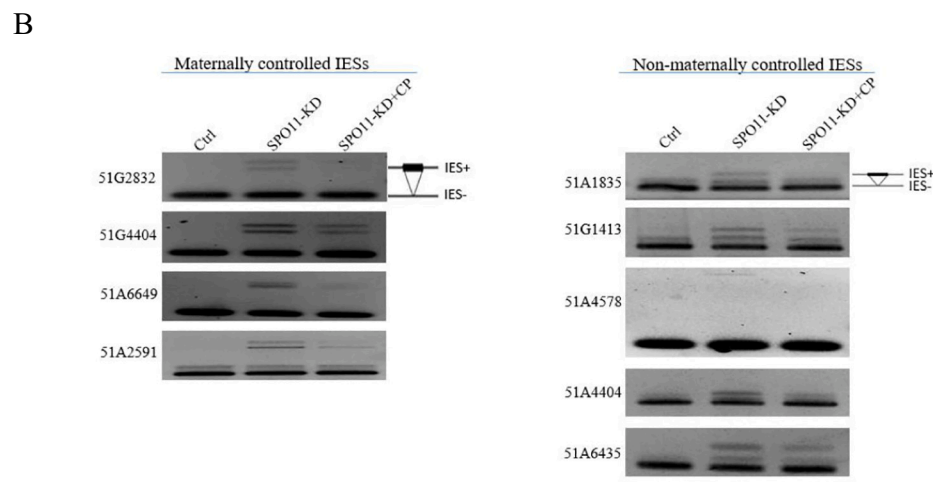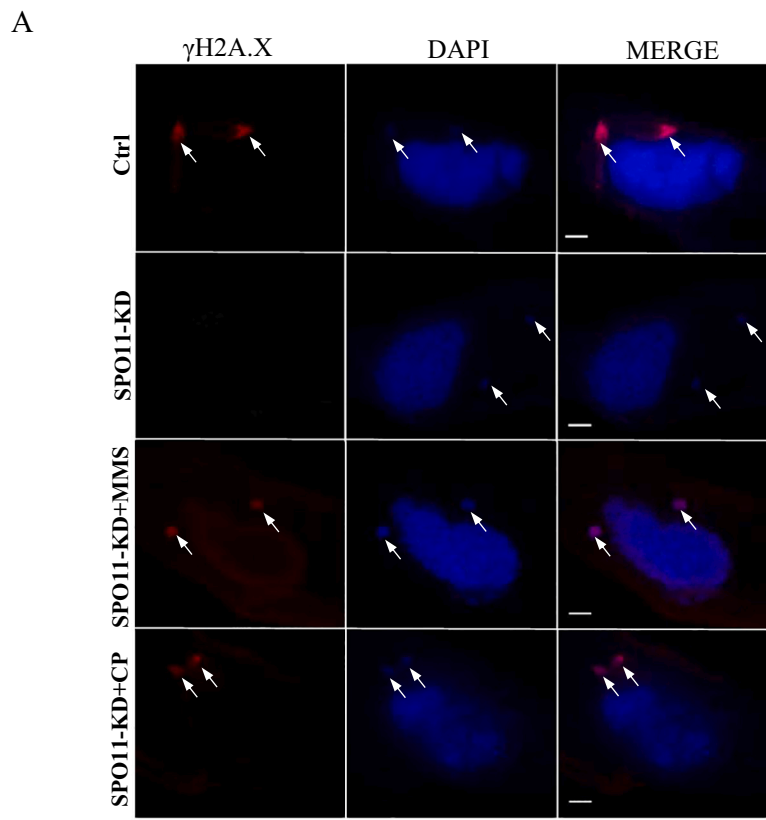
Fig. 7. Effect of SPO11-KD on DSBs formation during meiosis and investigation of which pathway is responsible for CO in *Paramecium*. (A) yH2A.X positive cells indicating presence of DSBs during meiosis in control cells, lack of the yH2A.X signal in SPO11-KD cells and restoration of yH2A.X signal in SPO11-KD cells using chemicals: methyl methanesulfonate (MMS) and cisplatin (CP) inducing DSBs during meiosis. Blue channel: DAPI. Red channel: anti-yH2A.X. Merged signals; scale bar: 10 μm. White arrows indicate MICs. (B) The importance of meiotic DSBs in IES excision. SPO11-KD, thus lack of DSBs during meiosis lead to IES retention. Restoration of DSBs in SPO11-KD cells using cisplatin (CP) lead to partial rescue of IESs retention. The excised form is shown as (IES−) and the unexcised form is shown as (IES+). The IES-form is always detectable due to presence of fragments of the parental MAC in the sample. The IES+ is present only in case of IES retention in the newly developing macronuclei. (C) Cells representing an effect of MSH5, MUS81 knockdowns and MSH5/MUS81 co-silencing on new MAC formation. L4440 vector without insert was used as negative control. Orange arrows indicates cells with a new MAC. Blue channel: DAPI. Merged signals; scale bar: 5 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

additional information that derive from the micronuclei of the parental cells in the form of small RNAs, called scanRNAs [7]. In addition, ensuring elimination of all IES copies present in the genome, *Paramecium* have evolved a second small RNA class (iesRNAs), produced in the new macronucleus [8]. Here, we describe the unexpected influence of Spo11, Msh4 and Msh5 proteins on genome reorganization. In this study, we showed that knockdown of these genes is lethal and that though scnRNAs are not notably affected by the knockdowns of these genes, the effect on iesRNAs is pronounced. IESs and transposons (irrespective of whether they are maternally controlled or not) are generally affected more strongly by the knockdowns of these genes than those involved in scanRNA and iesRNA production. The correlation of IES retention scores between the focal proteins and already known ones involved in genome reorganization suggest that Spo11, Msh4-1, and Msh5 form a separate group of proteins, consistent with an ancient shared pathway in eukaryotes. Furthermore, this also suggests the manner in which they influence DNA elimination is distinct from that of other characterized molecules.

Studies from a wide range of organisms have revealed that the activity of Spo11, Msh4-1 and Msh5 function are specific to the germline during meiosis [14,16,22,57,58]. This is also true for Spo11 in *Paramecium*. However, in *Paramecium* Msh4-1 and Msh5 may have extended roles beyond meiosis. Localization of GFP tagged versions of these proteins showed that they are also present during the postzygotic mitotic divisions, which lead to the formation of four identical nuclei, two of which are precursors of the new macronucleus. Additionally, studies performed during conjugation, where cells were silenced either during meiosis or subsequent anlagen development, showed that MSH4-1 and MSH5 perform their function not only during meiosis but also during the early onset of macronuclear development.

While Spo11's expression is restricted to the meiotic stage, Msh4-1 and Msh5 seem to be active during meiosis and post-meiotic mitosis. Our observations of the number of micronuclei during genome reorganization show, at the level of nuclear morphology, that meiotic and mitotic events are unaffected. This is unusual given that mice lacking Spo11, Msh4 and Msh5 experience meiotic catastrophes [17,59–61]. Furthermore, the testes in animals with Msh5 knocked out are smaller in size [17,62], while female mice lacking either Msh4 or Msh5 lose their oocytes completely, with ovaries becoming rudimentary after the birth [63]. In yeast, loss of Spo11 or Msh4 factor causes defects in synapsis and meiotic recombination [64,65]. Also, both mutants (*msh4Δ* and *msh5Δ*) in *S. cerevisiae* show reduction in meiotic viability and CO defects [66,67]. Overall, our data suggest that Spo11, Msh4-1 and Msh5 proteins in *Paramecium* have preserved their functions to micronuceli. However, in addition to these, we propose that they have evolved an additional function to the canonical one. Supporting this proposal is the observation of Spo11 in other eukaryotic species that lack a defined meiosis (e.g., *Giardia*, *Candida albicans*, *Entoamoeba histolytica*, *Acanthamoeba castellani*) [68].

In general, the canonical involvement of Msh4-1 and Msh5 during crossing-over in *Paramecium* seem to be unlikely since we could also show that Spo11 knockdown did not affect MSH5 localization during sexual reproduction, as well as that crossing-over in *Paramecium* is not dependent on these proteins. These observations are bolstered by the fact that though Msh4 and Msh5 are well-known ZMM proteins involved in the Class I crossing-over pathway in yeast, manifested by the presence of the synaptonemal complex (SC), none of the specific proteins related to CO or the synaptonemal complex, like Zip1, Hop1 or Red1, have been identified in the *Paramecium* genome [69]. Similarly, close related ciliate *Tetrahymena thermophila* which also does not possess any ZMM (beside Msh4 and Msh5) or SC-related proteins produces COs using a Mus81-Mms4 dependent pathway [70]. In addition to this, a Mer3 helicase, with which the Msh4-Msh5 heterodimer stabilizes Joint Molecules (JMs) to produce interfering COs, is missing as well [29,69]. It has also been previously suggested that Msh4 and Msh5 in *Tetrahymena thermophila* and *Sordaria macrospora* may work outside the usual ZMM

complex during meiosis [71,72].

We have also shown that, as in other organisms [18,20,50], Spo11 in *Paramecium* is needed for DSB induction during meiosis. However, these lesions present during meiosis in *Paramecium* micronuclei may have an additional function, as SPO11 knockdown has a substantial effect on IES excision during sexual reproduction. This raises the question: how does the presence of DSBs in the micronuclei influence later IES excision? One explanation could be that PiggyMac, the main transposase responsible for IES excision, is not sufficient alone to perform all DNA excision. It may also rely upon additional information from Spo11 encoded in the form of chromatin or DNA modification left after the induction of DSBs. It is known that Spo11 is covalently bound to the cleavage site and resected together with a small piece of the oligonucleotide [73]. Interestingly, Choi et al. [74] indicated high SPO11-1-oligo levels in nucleosome-depleted Helitron/Pogo/Tc1/Mariner DNA transposons in *Arabidopsis thaliana*. Since IESs are the relicts of transposons (39), one may hypothesize that this may be the case in *Paramecium* as well. Therefore, in future, identifying the cut sites of Spo11 in *Paramecium* may help to understand the role of Spo11 in influencing the genome reorganization process. With these data we could also investigate the relation of these sites to the cleavage sites of PiggyMac. Furthermore, H3K4me3 or H3K36me3 are known to be present around DSBs [75]. Therefore, identifying those histone modifications around the excision sites using Chromatin Immunoprecipitation (ChIP) would help to support the hypothesis of a more direct involvement in genome reorganization.

A more general explanation is that epigenetic information is metastable and needs a mechanism for its preservation. Since it is known that meiotic chromosomes undergo dramatic changes to chromatin structure, it is possible, that these germline events may interfere with transgenerational epigenetic memory or can be altered by it, thus influencing IES excision.

The presence of additional epigenetic information in the form of DNA or histone modifications in the micronuclei that help to recognize DNA for elimination may be possible, as both maternally and non-maternally controlled IESs, as well as transposons, were retained during silencing of SPO11, MSH4-1, and MSH5 even though scnRNAs were produced. Furthermore, cell survival was rescued following SPO11, MSH4-1 or MSH5 knockdown by conjugating wild type cells with silenced ones, suggesting that the information affecting DNA for elimination is present in the micronuclei already at the earliest stages of sexual reproduction.

Moreover, we cannot exclude the possibility that SPO11, MSH4-1 and MSH5 knockdowns lead to the chromosome mis-segregation that consequently affect genome reorganization in *Paramecium*. The ability to test this phenomenon at the chromosomal level in the micronuclei as well as through single-cell sequencing and identification of the missing segments during gene knockdowns compared to the control is still challenging, but may become feasible in future with continued technological improvements. Although the possibility of chromosome mis-segragation definitely exists, is rather unlikely to be causing the observed effect on the genome rearrangement process. Chromosome mis-segregation is a random process and therefore would affect random chromosome(s) and in some cells probably no mis-segregation would occur. Depending on which chromosome is affected, some cells might show problems with genome rearrangements and others would not. Also, not every single cell would display exactly the same phenotype. In our case, however, the effect of silencing of meiosis-specific genes is very homogeneous. Every cell seems to be affected in exactly the same way. Also, our replicates show exactly the same effects on DNA elimination, i. e., the same DNA elements are retained after development, so it would be very difficult to explain these effects by random chromosome mis-segregation.

In conclusion, we have shown for the first time that proteins traditionally associated with meiosis, Spo11, Msh4-1, and Msh5, are crucial for proper genome reorganization in *Paramecium tetraurelia*. Moreover,

we suggest that the mechanisms determining which DNA will be eliminated may be influenced by processes occurring in the micronuclei in the early stages of sexual reproduction, potentially involving histone modifications such as H3K4me3 or H3K36me3. We also provide evidence that the presence of DSBs induced by Spo11 during meiosis is important for further excision of both maternally and non-maternally controlled IESs. As a followup, precipitating and sequencing the oligonucleotides attached to Spo11 after cleavage would give us a better idea as to how Spo11 influences genome reorganization process. Therefore, future work is needed to determine the exact mechanism that directs DNA excision in *Paramecium* and how these proteins influence the subsequent genome reorganization.

Availability

The following reference genomes were used in the IES analyses and read mapping:

- MAC: http://paramecium.cgm.cnrs-gif.fr/download/fasta/ptetraurelia_mac_51.fa
- MAC + IES:http://paramecium.cgm.cnrsgif.fr/download/fasta/ptetraurelia_mac_51_with_ies.fa

Accession numbers

Sequencing data are deposited in the European Nucleotide Archive (ENA):

- DNA Seq for EV and MSH4-1, MSH5, SPO11 knockdowns: ERS2705073-ERS2705075, ERS3389991.
- DNA Seq: EV and MSH4-1, MSH5, SPO11 knockdowns (replicates): ERS3390186, RES3390253, ERS3390142, ERS3391508
- sRNA Seq: ERS3396490-ERS3396497.

CRediT author contribution statement

IR and MN designed experiments. IR wrote the paper and performed most of the laboratory experiments. ECS performed bioinformatic analysis. IR and ES edited the manuscript. SP cloned the full gene of the SPO11 construct. AR began investigating localization of the Msh5 protein and performed RNAi trials. MN supervised the project.

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Declaration of competing interest

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbamcr.2022.119239.

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