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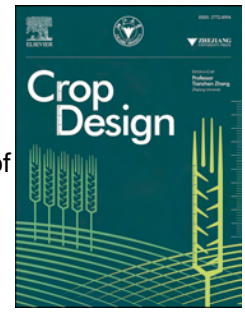


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# Aggregation of chromosome axis proteins on the chromatin and in the nucleoplasm of *Brassica oleracea* meiocytes.

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**Keyword: meiosis, chromosome axis, recombination, brassica, polycomplexes, protein aggregates.**

## Abstract

Meiotic recombination is essential for the generation of genetic diversity in natural and breeding context. The chromosome axis comprises cohesin, HORMA-domain containing proteins and coiled coil proteins and is crucial for the establishment of meiotic recombination. These proteins form a complex during meiosis of *Brassica oleracea* but information about their respective localisation and dynamic on meiotic chromosomes remain sparse. Our study reveals that the HORMA-protein ASY1 aggregates on the chromatin and forms domains of high and low abundances. The regions enriched for ASY1 are also highly enriched for the axis proteins ASY3, SMC3 and SCC3, although to varying degrees between leptotene and pachytene stages. At later stages, when most DNA double strand breaks are repaired and the chromosome axis disassemble, ASY1, ASY3, SCC3 and SMC3 co-localise and form large aggregates on the discontinuous axis structures. As the axis structures reduce in length, we found that all four axis proteins relocalise in the nucleoplasm and further aggregates. Moreover, we found that ZYP1, the transverse filament of the synaptonemal complex, forms numerous chromosomic aggregates that are sometimes associated with MLH1 and can form ectopic synaptic interactions. Overall, our study indicates that axis proteins have a high propensity to aggregate. This property is important for assembling the chromosome axis but the association of axis proteins with the chromatin must be tightly regulated to limit polycomplex formation.

## Introduction

Meiotic recombination is essential for the generation of novel genetic variation and is found in most sexually reproducing organisms. Meiotic recombination initiates with the formation of numerous DNA double strand breaks (DSBs) follows by their resection to form single stranded DNA overhangs onto which the recombinases RAD51 and DMC1 (DNA Meiotic Recombinase 1) are loaded. DMC1 promotes homology search toward the homologous chromosome for DSB repair. This process facilitates the co-alignment of chromosomes and the establishment of a tripartite proteinaceous structure, referred to as the synaptonemal complex, connecting the transverse filament protein ZYP1 with the two chromosome axes. A subset of inter-homologue recombination intermediates is stabilised and the E3 ligase HEI10 (Human Enhancer of cell Invasion No. 10) and nuclease MLH1 (MutL homolog 1) mediate their conversion into crossovers, which are points of reciprocal genetic exchanges between two chromosomes. Crossovers break genetic linkages, reshuffle allelic combinations, and provide adaptive properties to a population. Hence, meiotic recombination is pivotal for genome evolution. Meiotic recombination is also important in breeding as it breaks genetics linkages between beneficial and deleterious alleles [1].

Meiotic chromatin is organised along a chromosome axis forming arrays of loops anchored to the underlying axis. Components of the chromosome axis include cohesin, HORMA-domain (for HOP1, REV7 and MAD2) containing proteins, like ASY1 (Meiotic Asynaptic Mutant 1), and coiled-coil proteins, such as ASY3 and ASY4 [2-4]. Evidence from *Arabidopsis thaliana*

suggests that the genomic regions enriched in axis proteins are also enriched in nucleosomes [5]. In contrast, DSBs occur in regions of low nucleosome density and are thought to be formed on chromatin loops [6]. The cohesin complex comprises four subunits, which include two Structural Maintenance of Chromosomes (SMC1 and SMC3), one kleisin (REC8) and one Stromalin Antigen (STAG)/Sister Chromatid Cohesion (SCC3), that forms a ring-shaped structure which can entrap DNA molecules to form chromatin loops. In the absence of REC8 kleisin, the chromatin appears diffuse, and the formation of the chromosome axis is disrupted causing recombination defects. For instance, Arabidopsis *rec8* has fewer DSBs and shows extensive ectopic recombination and chromosome fragmentation [5]. Components of the chromosome axis also have an important role in mediating the repair of DSBs using the homologous chromosome for DNA template. Thus, the chromosome axis is crucial for initiating DSB formation and promoting their conversion into crossover.

The formation of the chromosome axis occurs hierarchically. The deposition of cohesin on the chromosomes is the primary requirement for deposition of the other components of the chromosome axis [5]. ASY3 is then required for deposition of ASY1 whereas ASY1 is not essential for the loading of ASY3 in *Arabidopsis thaliana* [3]. In maize and *Saccharomyces cerevisiae*, desynaptic2 (DSY2) and Red1, which are the functional orthologue of ASY3, also have an important role in the recruitment of the orthologue of ASY1 [7,8]. Visualisation of Arabidopsis and *S. cerevisiae* chromosomes revealed that proteins forming the axis localise along the entire length of the chromosomes, albeit with varying amount, represented by domains of higher abundance alternating with domains of lower abundance [3,9]. The properties of axis proteins to aggregate were further confirmed by genomic studies showing patterns of peaks and valleys where axis proteins are enriched and depleted, respectively [5,10-11]. The degree of axis protein aggregation appears to be sensitive to heat stress. For instance, the signal of ASY1 and ASY4 are less continuous along the chromosomes and large abnormal punctuate foci become visible in Arabidopsis plants exposed to high temperature [12]. In rice, ZEP1 forms the transverse filaments of the synaptonemal complex and is first detected as bright polycomplexes in early meiosis. These structures are eventually resolved and ZEP1 localises on paired chromosomes at later meiotic stages. The resolution of these aberrant accumulations of ZEP1 is dependent on the E3 ubiquitin ligase DSNP1 [13]. Similarly, the homologues of ZYP1/ZEP1 in *Caenorhabditis elegans* and *S. cerevisiae* form polycomplexes in absence of function of the cullin E3 ubiquitin ligase CRL4 and Cdc53, respectively [14-15]. It is thought that the level of axis proteins is tightly regulated and, if not all the axis proteins are associated with the chromosomes, the excess of axis proteins can form aggregates in the cytoplasm or nucleoplasm. Indeed, the heterologous over-expression of rat SCP1 in COS-7 cells leads to the formation of SCP1 cytoplasmic polycomplexes [16]. Moreover, *S. cerevisiae* Zip1 forms polycomplexes in *ndt80,* as the cellular level of Zip1 is continuously rising, due to the arrest of meiotic progression at pachytene stage [17].

The Brassica genus consists of several species of agricultural relevance for oil and food production for people and livestock. Genetic improvement of Brassica relies on meiotic recombination to combine beneficial alleles together. Crossover frequency can be substantially increased, and its landscape changed, through the artificial generation of triploid lines using *Brassica oleracea*, *Brassica rapa* and *Brassica napus* [18]. This strategy has the potential to augment the genetic gain during selective breeding programs [19]. Another study showed that a change in ploidy level is associated with a change in the level of some proteins and gene transcripts, although it remains unclear how these changes in expression could be linked with changes in meiotic recombination [20]. The chromosome axis protein ASY1 forms a complex with the other axis components in *Brassica oleracea* and the functions of ASY1 and PCH2 on crossover formation and axis formation appear to be conserved between *Brassica rapa* and *Arabidopsis thaliana* [21-22]. However, our knowledge on meiosis in the Brassica genus remains limited. To address this gap in knowledge, our study aims at profiling the localisation of axis proteins on *Brassica oleracea* meiotic chromosomes. Our study revealed that axis and synaptonemal complex formation are not polarised in Brassica, similar to Arabidopsis, but in contrast to other major crops like wheat or barley. This indicates that crossover distalisation occurs independently of axis polarisation and that other factors are

involved in biasing crossovers toward chromosome ends. We also observed that the crossover marker MLH1 is recruited at ZYP1 nucleation sites at an earlier stage of meiosis in Brassica compared to other plant species, likely reflecting a different orchestration of chromosome axis and recombination. Moreover, we found that the axis proteins ASY1, ASY3, SCC3 and SMC3 have a propensity to aggregate on the chromosome axis in early and mid-prophase I. When the axis proteins progressively dissociate from the chromatin at late diplotene and diakinesis, they form large aggregates on the chromatin and re-localise in the nucleoplasm where they aggregate further. We also observed that ZYP1 regularly aggregates on the synapsed chromosomes and some of these structures lead to ectopic synaptic interactions and the recruitment of MLH1. Our work highlights the propensity of axis proteins to aggregate and the importance to tightly control their association with the chromatin to prevent formation of polycomplexes.

## Results

### Crossover distalisation occurs without prior polarisation of the chromosome axis and the synaptonemal complex.

A recent study mapped genome-wide the crossovers from 10 populations of *Brassica oleracea* using five genotypes as founders. *B oleracea* crossovers were found to form mostly at chromosome ends for all the genotypes analysed [23]. Distal crossover patterning was also reported in other major crops like barley, wheat and maize, but not in Arabidopsis where crossovers are enriched near the junction between the euchromatin and heterochromatin. In cereals, formation of the chromosome axis and nucleation of the synaptonemal complex are polarised as they form first near the chromosome ends. It is thought that polarisation of the axis and synaptonemal complex form a hub for recombination and are contributing factors to the observed distalisation of crossovers [24]. Since *Brassica oleracea* have distalised crossovers, we thought of evaluating the dynamic of axis formation. To determine if axis polarisation takes place, we immunostained the axis protein ASY1 and stained the chromatin with DAPI dye at early prophase I. We performed immunostaining of ASY1 from G2 to diplotene on meiotic chromosomes of male meiosis. ASY1 forms numerous foci at G2 while the signal appears more continuous at leptotene. By late leptotene, ASY1 signal is very bright and completely continuous. At zygotene and pachytene stages, ASY1 appears weaker on the synapsed regions. In contrast, ASY1 signal is bright but discontinuous at diplotene (Figure 1). To evaluate if the chromosome axis is polarised, we immunostained ASY1 in early meiosis and divided the region containing the chromatin into 4 proportional areas and determined if ASY1 signal was found in any of the four areas. If ASY1 signal is polarised, as seen in cereals, the signal should be present in a single region only. We observed that ASY1 is detected as punctuate foci throughout the chromatin in G2 (n=20) (Figure 1A). In early leptotene, ASY1 signals is observed across the chromatin area with no evidence of polarisation (n=18) (Figure 1B).

We also performed immunostaining of ZYP1 on meiocytes from leptotene to pachytene to determine if ZYP1 nucleation is polarised. Similar to ASY1, we found no evidence of polarisation for ZYP1 nucleation. When ZYP1 signal was first detected on chromatin, ZYP1 signal was not restricted to a single region of the chromatin, but instead, was found dispersed across the chromatin (n=20) (Figure 1C). At early zygotene stage, ZYP1 forms numerous foci on the chromatin but we didn't detect meiocytes with a mixture of foci and long stretches of ZYP1. This mode of ZYP1 nucleation differs from observations made in Arabidopsis, where both foci and stretches of ZYP1 are observed at the same time in zygotene [25]. These suggest that ZYP1 nucleation may be more synchronous in *Brassica olearace.* To determine if some nucleation points of ZYP1 could be associated with crossover events, we co-immunostained ZYP1 with MLH1, which is a marker of crossovers, and we confirmed that some ZYP1 nucleation sites are associated with MLH1 (Figure 1D). It also appears that MLH1 foci associate with ZYP1 foci at an early stage of meiosis. This contrasts with *Arabidopsis thaliana* where MLH1 foci is first detected on long stretches of ZYP1 at late zygotene [25]. These observations reveal that the orchestration of ZYP1 nucleation and recombination differ between *Arabidopsis thaliana* and *Brassica oleracea*. Overall, these data indicate that the

formation of the chromosome axis and synaptonemal complex occur in a disperse mode, like in Arabidopsis, and differs from the polarised mode reported in cereals. This observation also indicates that factors, other than axis and synaptonemal complex polarisation, are involved in restricting crossover formation at chromosome ends in *B. oleracea*.

**Axis proteins form domains of hyper and lower abundance on the chromosome axis.**
Further investigations of ASY1 immunostaining on meiotic chromosomes reveal that ASY1 signal forms domains of higher and lower abundance along the chromosome axis (Figure 2). A previous study showed that ASY1 forms a complex with ASY3 and the cohesin subunits SMC3 and SCC3 [21]. To determine if ASY1 is enriched with other axis proteins on meiotic chromosomes, we co-immunostained ASY1 with ASY3, SMC3 and SCC3. We observed that ASY3 signal forms domains of bright intensity and that ASY1 higher-intensity domains are highly overlapping with ASY3 higher-intensity domains at early leptotene (n=20) (Figure 2A-C) but that the two signals are less highly overlapping when the chromosomes are synapsed at pachytene (n=32) (Figure 2D). Immunostaining of SCC3 and SMC3 also show that the two proteins form domains of higher and lower abundance on chromosomes. Moreover, the brighter domains of SMC3 and SCC3 are highly overlapping with the brighter domains of ASY1 at early leptotene (n=22; n=22) (Figure 2B-C) and to a lesser extent at pachytene (n=17; n=26) (Figure 2E-F). Overall, these data reveal that axis proteins have a propensity to aggregate at similar sites on meiotic chromosomes in early prophase I, consistent with the detection of peptides for ASY3, SMC3 and SCC3 in an affinity pull-down experiment using ASY1 antibody [21].

**Axis proteins have a propensity to aggregate on chromosomes and in the nucleoplasm.**
Immunostaining of ASY1, ASY3, SCC3 and SMC3 on meiotic chromosomes from G2 through to pachytene did not reveal presence of large aggregate of proteins on chromosomes or in the nucleoplasm. However, at late diplotene, we found that the proteins forming the chromosome axes are progressively removed from the chromatin and their signals become less continuous. Moreover, large aggregates of axis proteins were frequently observed on the axes at this stage (Figure 3A-C). From a set of 53 meiocytes, ASY1, ASY3, SCC3 and SMC3 show an average number of large aggregates per cell of 22, 25, 25 and 22, respectively. In those instances, ASY1 large condensates were highly overlapping with ASY3 (99%), SCC3 (99%) and SMC3 (99%) large condensates (Figure 3A-C). These indicate that the axis proteins retain their properties to aggregate at this stage. We found that the large axis foci vary in size and intensity within and between meiocytes. We regularly observed events where two large foci were partially overlapping and those events may represent the merging of two separate aggregates into a larger one. In total, we recorded 51% of meiocytes with doublet of large foci and 8% of meiocytes with triplet of large foci (n=53).
At diakinesis, most of the continuous signals of ASY1, ASY3, SCC3 and SMC3 are no longer visible. Instead, bright and large foci of ASY1 were found overlapping with bright signals of ASY3, SCC3 and SMC3 on the chromatin (Figure 3D-F). At this stage, we also observed axis foci that localised in the nucleoplasm and not overlapping with the chromatin (Figure 3D-F). Hence, we observed the gradual removal of the axis proteins from the chromatin as they relocalise in the nucleoplasm where they retain their property to aggregate.

**Chromosomic ZYP1 aggregates are prevalent, associate with MLH1 and form inter-chromosomic synaptic interactions.**
ZYP1 is recruited on paired chromosomes to establish the synaptonemal complex. Staining of ZYP1 on meiotic chromosomes at pachytene stage shows that ZYP1 forms a continuous signal along the chromosomes. Unexpectedly, in 71% of the meiocytes (n=31), we observed some regions where ZYP1 forms aggregates on chromosomes and other regions where the ZYP1 signal was distorted, forming a structure resembling to a loop. In some instances, two separate synaptic regions were joined together by two ZYP1 aggregates (Figure 4A-B).
We co-immunostained ZYP1 with MLH1 to determine if the bright and distorted signals of ZYP1 overlap with MLH1 (Figure 4C-D). We found that the meiocytes with aggregates of ZYP1

have an average of 10 aggregates per cell and MLH1 foci co-localise with 18% of these aggregates (n=9). Furthermore, a mean of 13.4 MLH1 foci were detected on chromosomes and not overlapping with ZYP1 aggregates. This number of MLH1 foci is close to the expected number of crossovers based on genomic crossover count (mean average is between 12 and 14 crossovers, depending on the genotype) [23]. Hence, the aggregates of ZYP1 are unlikely to represent crossover maturation sites, and instead, more likely represent abnormal accumulation of the proteins that can lead to ectopic synaptic interactions.

**Discussion**

Our study shows that meiotic axis proteins have a propensity to enrich at similar sites on the unsynapsed regions of chromosomes in early meiosis. Similar observations were made in *S. cerevisiae* and Arabidopsis and it was proposed that those sites represent axis sites onto which the chromatin loops are anchored [3,5,9-11]. We found that ASY1 is less highly overlapping with the regions that are enriched for ASY3, SMC3 and SCC3 on the synapsed regions at pachytene. ASY1 is actively removed by PCH2 from the synapsed regions and our data indicate that the association of ASY1 with the other axis proteins is remodelled at this stage [22,26]. It remains to be determined if this new profile of axis proteins is important for the progression of meiotic recombination.

Orthologues of ZYP1 have been reported to aggregate under certain situations in rice, *C. elegans* and *S. cerevisiae* [13-15]. In this study, we show that ZYP1 frequently forms aggregates on the synapsed chromosomes. Some of these aggregates form ectopic synaptic interactions that likely need to be resolved to prevent segregation defects. Other aggregates of ZYP1 were found overlapping with MLH1 foci. Only a small proportion of ZYP1 aggregates are associated with MLH1 and the majority of MLH1 foci are not associated with ZYP1 aggregates. Hence, it is unlikely that ZYP1 aggregates represent crossover maturation sites. Instead, MLH1 may have a role in resolving ZYP1 aggregates and inter-chromosomic interactions through its nuclease function. Indeed, Mlh1 was reported to have a role in interlock resolution in *Sordaria macrospora* [27].

Following the disassembly of the synaptonemal complex, axis proteins are gradually removed from the chromatin at diplotene and diakinesis. During this process, axis proteins appear to form larger aggregates on the chromosomes before their relocation to the nucleoplasm where they further aggregate. This relocation of axis proteins inside the nucleus may represent a first step toward their degradation. The signal of ASY1 on these large structures was found to overlap with ASY3, SMC3 and SCC3 signals. These observations suggest that the property of ASY1 to interact with the other axis proteins to form the chromosome axis persists at late stages of meiosis I when the axis is disassembling. Our study also indicates that the axis proteins tend to form aggregates in the nucleoplasm if they are not associated with the chromatin. Similar observations were reported in *S. cerevisiae.* These suggest that the expression of axis protein and their binding to the chromatin must be tightly regulated to prevent formation of abnormal aggregates. Indeed, Arabidopsis *ASY1* is over-expressed and the protein forms aggregates on the chromosome axis in meiocytes exposed to heat stress [12, 28]. Overall, our study demonstrates that axis proteins have a propensity to aggregate, which is an important feature to support axis formation, but if deregulated, can lead to polycomplex formation and inter-chromosomic interactions. The prevalence of axis proteins and ZYP1 to aggregate under optimal growth conditions could make *B. oleracea* meiocytes susceptible to heat stress. This necessitates further investigations in future experiments.

**Materials and methods**

**Plant material**

Brassica oleracea var. alboglabra A12 DHd plants were grown in greenhouses at 18$^{o}$C under 16h light and 8h dark each day. Plants were grown until unopened floral buds become visible. Floral buds of stage 9 as defined in [29] contain male meiocytes and were selected for immunocytology.

**Immunocytology**

Floral buds were dissected on damp filter paper and 20 anthers at meiotic stages were isolated and transferred to a microscope cavity slide containing 20μl of enzyme digestion solution (0.4% cytohelicase, 1.5% sucrose, 1% polyvinylpyrrolidone). The slide was incubated in a moist box for 4 min at 37°C, and the anthers were gently opened with a brass rod to release the meiocytes. Anther debris were removed and 10μl of enzyme digestion solution was added on to the cavity slide and the slide was incubated in a moist box for 3 min at 37°C. 4μl of the digested solution was moved to a microscope slide and 10μl of 1% lipsol was added. The mixture of meiocytes was mixed with a needle for 1 min. 20μl of 4% paraformaldehyde was added and the solution was mixed and left to dry for 2 h. Following this, the slides were incubated in PBST (1x PBS with 0.1% Triton X100) for 5 min at room temperature. A solution of primary antibody diluted in 1% BSA was added to the slides and the slides were incubated in a moist box overnight at 4°C. The slides were washed in PBST for 10 min and 5 min at room temperature before adding a solution of secondary antibody diluted in 1% BSA. The slides were incubated in a moist box for 30 min at 37°C. The slides were then washed in PBST for 10 min and 5 min at room temperature before adding DAPI DNA dye. The following antibodies were used: α-ASY1 (rabbit/rat, 1/500 dilution) [2], α-ZYP1 (rabbit/rat, 1/500 dilution) [25], α-SCC3 (rabbit, 1/500 dilution) [3], α-ASY3 (rabbit, 1/500 dilution) [3], α-SMC3 (rat, 1/500 dilution) [3], α-MLH1 (rabbit, 1/200 dilution) [30]. Image deconvolution was performed using the function “Mexican hat” as described in [3]. Images were analysed using ImageJ.

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**Figure 1. Establishment of the chromosome axis and synaptonemal complex without polarisation.**
A-B. Immunostaining of ASY1 (green) at G2 (A), early leptotene (B), late leptotene (C), zygotene (D), pachytene (E) and diplotene (F). Chromatin is stained with DAPI (blue). G-H. Co-immunostaining of MLH1 (green) and ZYP1 (red) at zygotene. Chromatin is stained with DAPI (blue). Scale bar = 10μM.

**Figure 2. Co-immunostaining of ASY1 with ASY3, SCC3 and SMC3 in early leptotene and pachytene.**
Co-immunostaining of ASY1 (green) with ASY3, SCC3 or SMC3 (red) in early leptotene (A-C) and pachytene (D-F). Chromatin is stained with DAPI (blue). White dashed boxes mark the sections that are magnified. All images were deconvolved. Scale bar = 10μM.

**Figure 3. Co-immunostaining of ASY1 with ASY3, SCC3 and SMC3 in late diplotene and diakinesis.**
Co-immunostaining of ASY1 (red) with ASY3, SCC3 or SMC3 (green) in late diplotene (A-C) and diakinesis (D-F). Chromatin is stained with DAPI (blue). White arrows represent doublet of axis aggregates. Orange arrows represent aggregates in the nucleoplasm. Scale bar = 10μM.

**Figure 4. ZYP1 forms aggregates on meiotic chromosomes at pachytene.**
A. Immunostaining of ZYP1 (green) at pachytene. Chromatin is stained with DAPI (blue). Arrows represents an inter-chromosomic synaptic interaction between two ZYP1 aggregates. B. Deconvolved image from (A). C-D. Co-immunostaining of MLH1 (green) and ZYP1 (red) at pachytene. Chromatin is stained with DAPI (blue). Arrows represent ZYP1 aggregates overlapping with MLH1 foci. Scale bar = 10μM.

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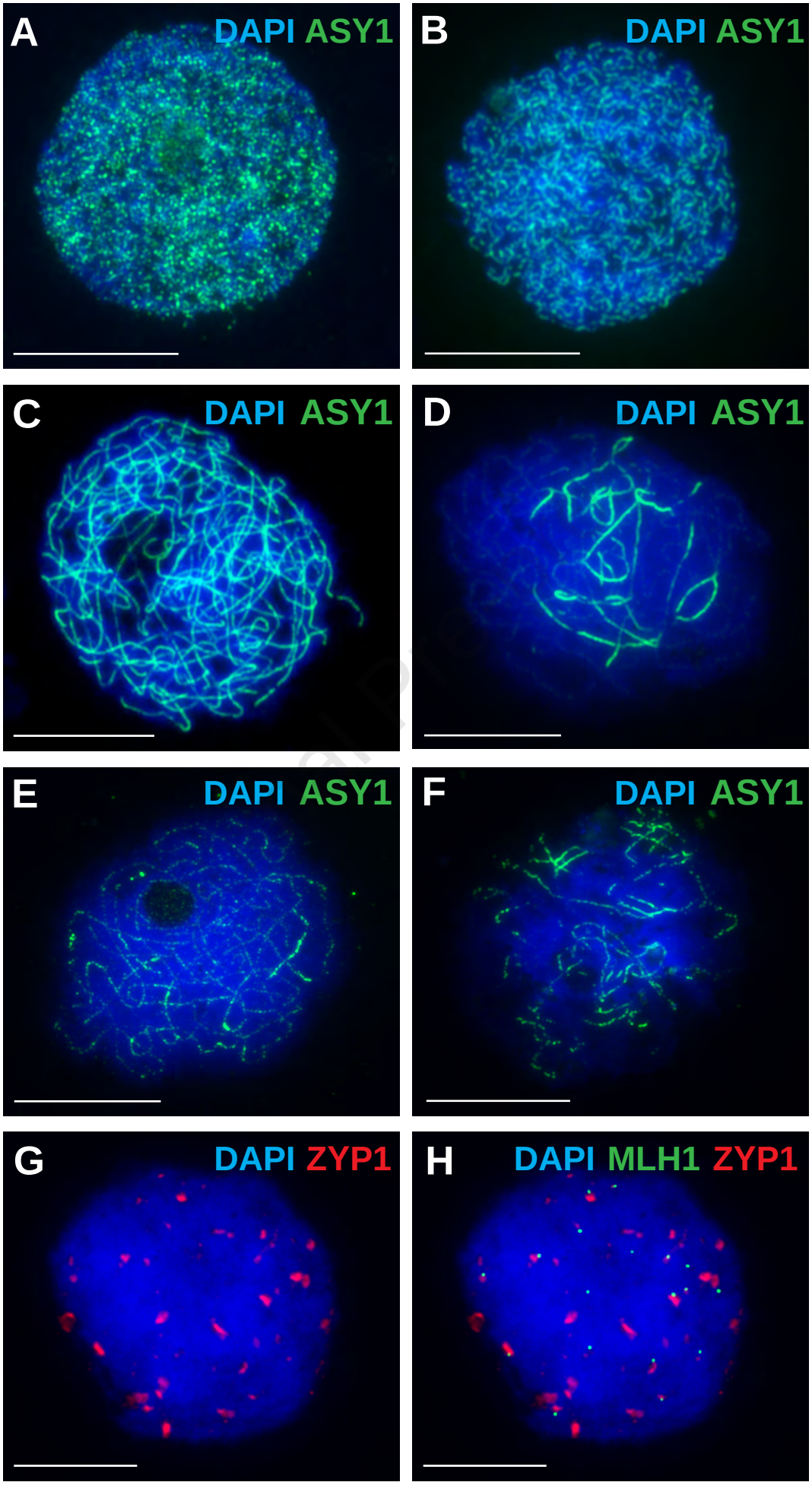
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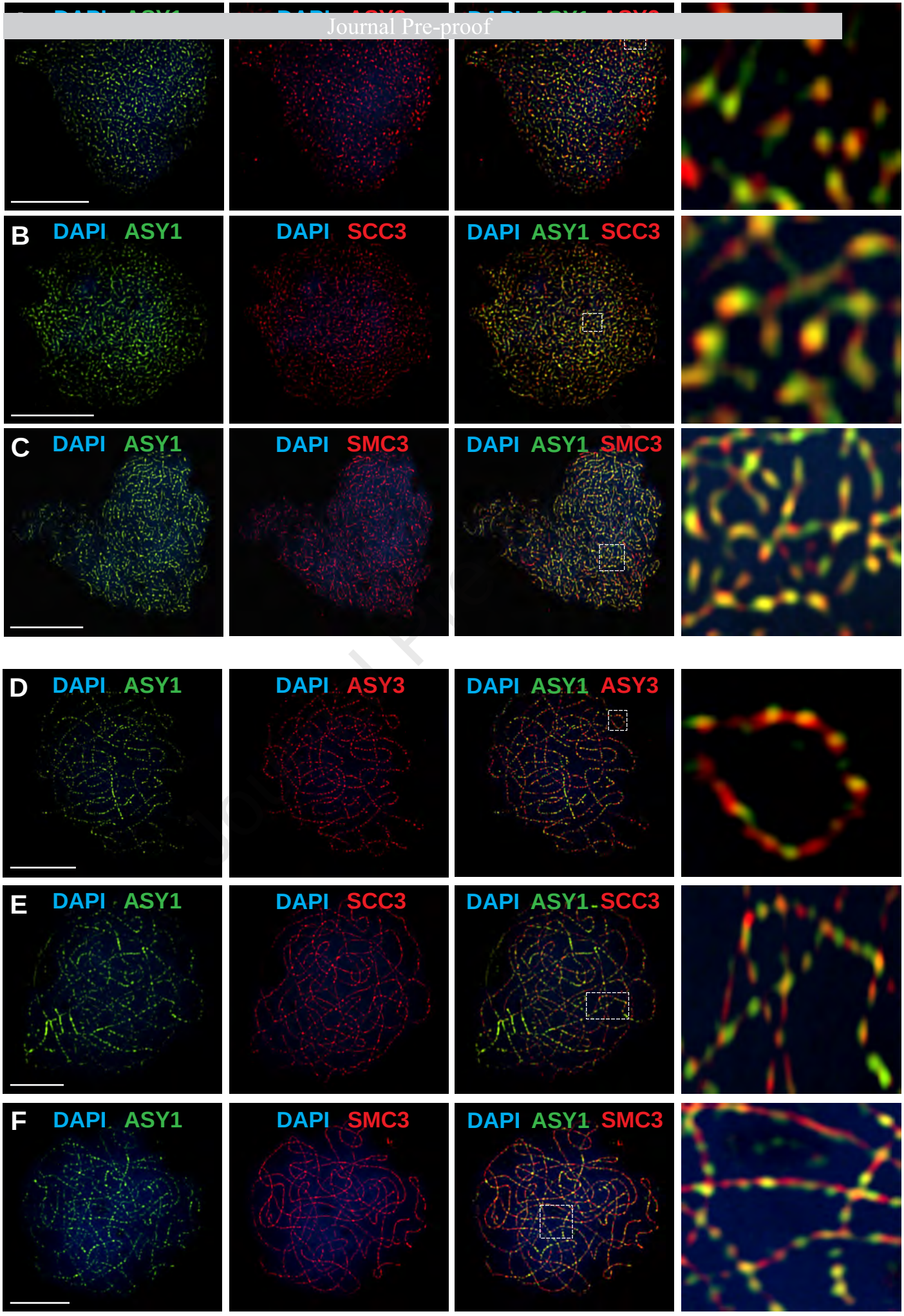

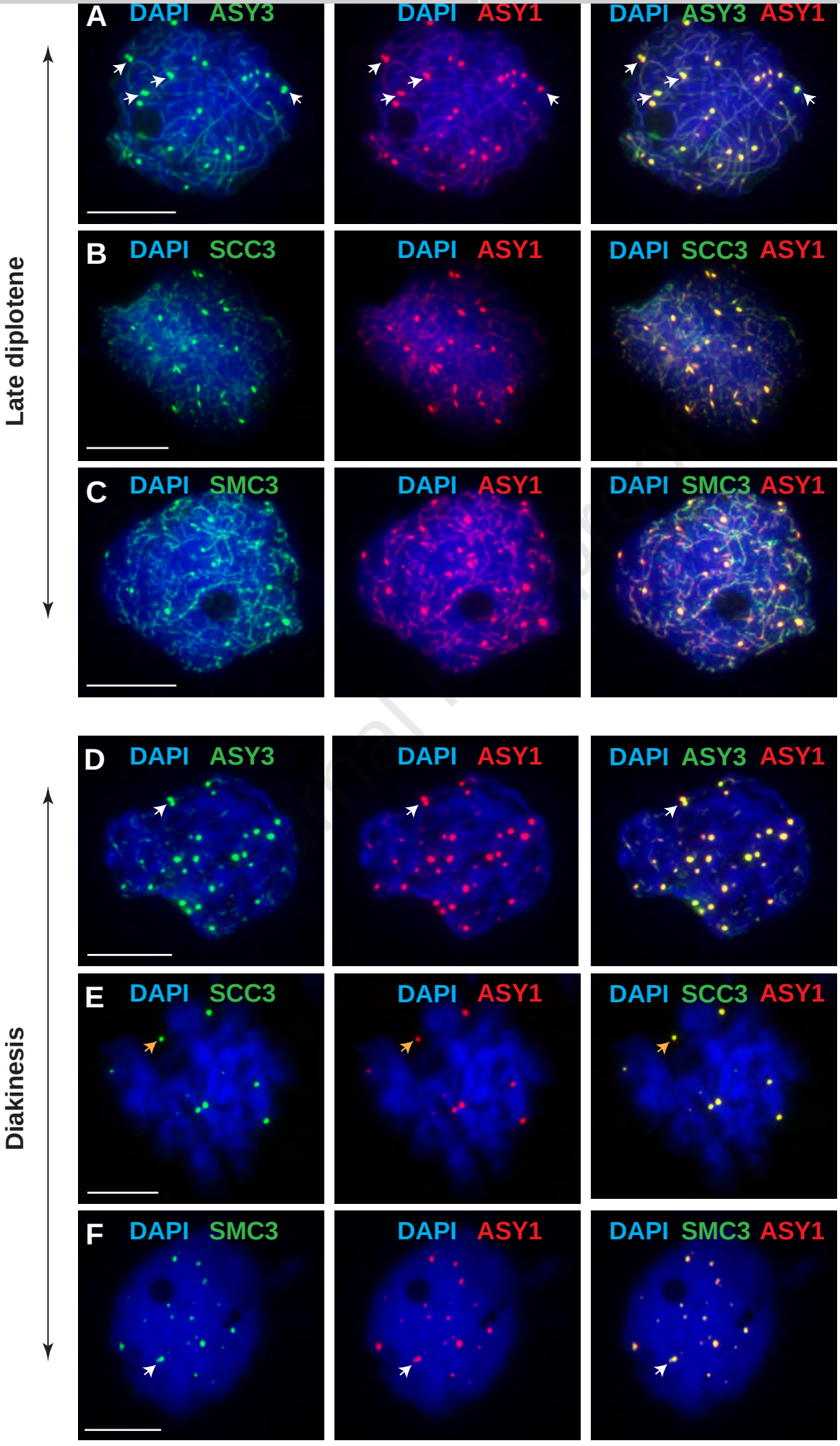
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C
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Late diplotene
D
DAPI ASY3
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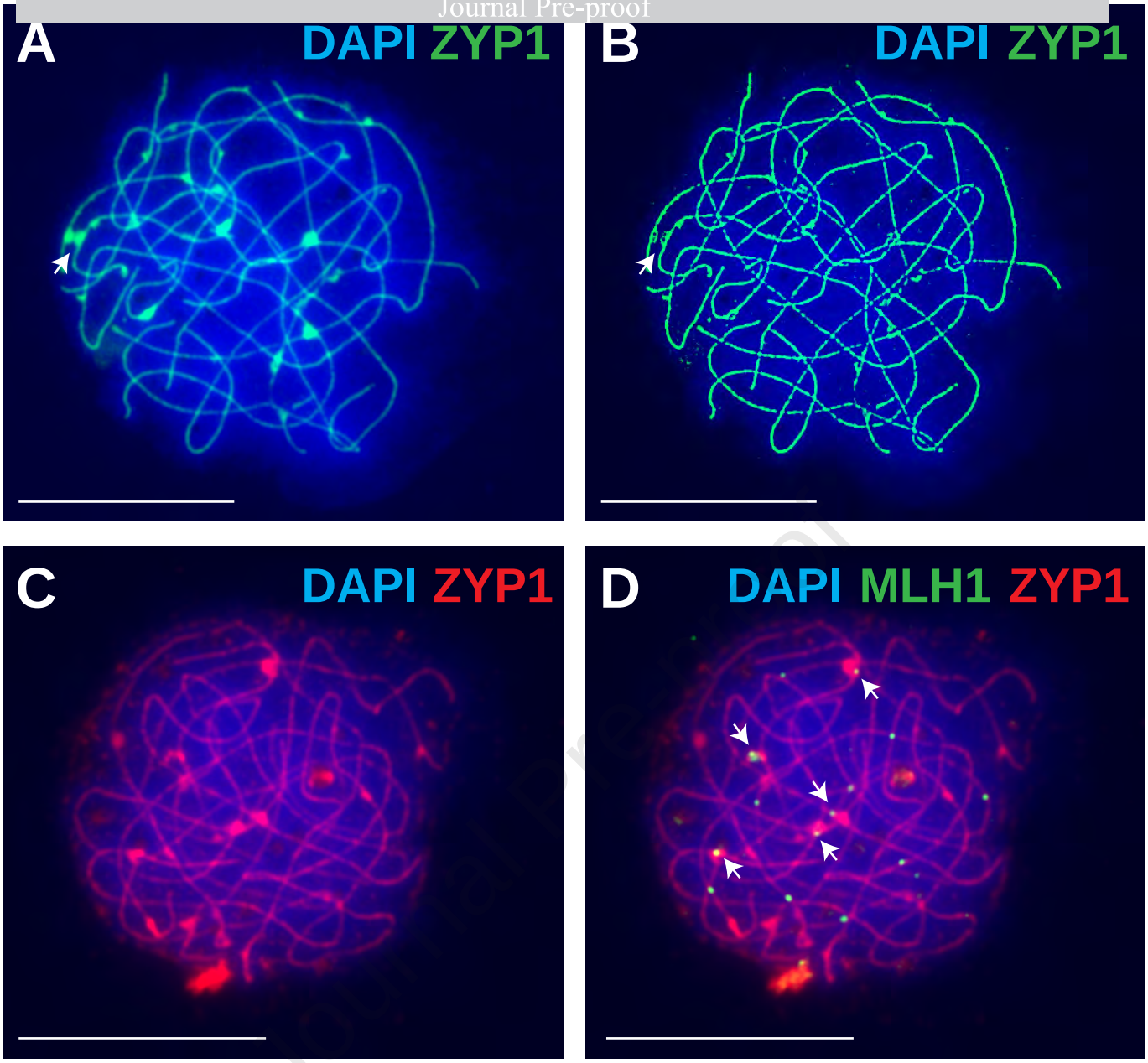

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ROTHAMSTED RESEARCH

Dear Editorial Board,

We declare that there is no conflict of interest.

Yours sincerely,

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