Structural components of the synaptonemal complex, SYCP1 and SYCP3, in the medaka fish *Oryzias latipes*

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Short title: Synaptonemal Complex in Fish

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Summary

The synaptonemal complex (SC) is a meiosis-specific structure essential for synapsis of homologous chromosomes. For the first time in any non-mammalian vertebrates, we have isolated cDNA clones encoding two structural components of the SC, SYCP1 and SYCP3, in the medaka, and investigated their protein expression during gametogenesis. As in the case of mammals, medaka SYCP1 and SYCP3 are expressed solely in meiotically dividing cells. In the diplotene stage, SYCP1 is diminished at desynaptic regions of chromosomes and completely lost on the chromosomes at later stages. SYCP3 is localized along the arm and centromeric regions of chromosomes at metaphase I, and its existence on the whole chromosomes persists up to anaphase I, a situation different from that reported in the mouse, in which SYCP3 is confined to the centromeric regions but lost on the arm regions at metaphase I. Thus, the expression patterns of SC components are different in mammals and fish despite the resemblance in morphological structure of the SC, suggesting divergence in the function of the SC in regulation of meiosis-specific chromosomal behavior. Since the antibody against medaka SYCP3 is cross-reactive to other fishes, it should be generally useful for a meiosis-specific marker in fish germ cells.

Key words: Protein expression, Synaptonemal complex, SYCP1, SYCP3, Medaka, Fish, Meiosis, Homologous chromosomes

Introduction

Meiosis is a cell division unique to germ cells. During meiosis, homologous chromosomes are paired and recombined, a process essential for generating genetically distinct haploid cells. This unique behavior of meiotic chromosomes has been linked to the activity of a meiosis-specific supramolecular proteinaceous structure, the synaptonemal complex (SC), which is generally observed in meiotically dividing cells in most sexually reproducing organisms [1,2,3]. The SC consists of three elements: two axial/lateral elements adjacent to each homologous chromosome, a central element between the lateral elements, and transverse filaments connecting the lateral elements [2].

SYCP1 (also known as SCP1) [4,5] and an unnamed 48-kDa protein [6] have been identified as structural components of the central element of the SC in mammals. Roles

of SYCP1 in meiosis have been deduced from the phenotypes of *Sycp1*-knock-out mice, which are sterile [7]. Chromosomes in primary spermatocytes of the knock-out mice are equipped with normal axial/lateral elements and aligned with each counterpart but do not undergo synapsis. Most *Sycp1*$^{-/-}$ spermatocytes are arrested in pachytene, although a small proportion of the cells reach metaphase I, and crossing-over is rarely seen. Importance of the central element protein in the dynamics of homologous chromosomes in meiosis has also been reported in other species. In the budding yeast *Saccharomyces cerevisiae* [8] and the worm *Caenorhabditis elegans* [9], mutant analyses have revealed that the central element protein is necessary for the synapsis and crossing-over of homologous chromosomes, although it is not necessary for the stabilization of homologous chromosomal pairing. The role of the central element protein in the occurrence of synapsis and crossing-over is conserved in all species analyzed thus far.

SYCP2 and SYCP3 (also known as SCP2 and SCP3) are structural components of the lateral element [5,10]. Male mice carrying a null-mutation in the *Sycp3* gene are sterile with complete loss of spermatocytes [11], and female mutant mice produce aneuploid oocytes through defective meiotic chromosome segregation, although they are fertile [12]. These phenotypes indicate that SYCP3 is essential for the normal progression of meiosis. Importance of the lateral elements of SC in meiosis has also been reported in *S. cerevisiae*, in which Red1 and Hop1 have been identified as structural components of the lateral elements, although these proteins have no sequence homology to counterparts found in other animals. The *red1*-mutant cannot form lateral elements [13]. In addition, several lines of evidence suggest that direct interaction between Hop1 and Red1 is required for SC formation, normal synapsis and crossing-over [14].

Despite strong resemblance in the morphological structure of the SC in all eukaryotes, the precise role of the SC in chromosomal dynamics in meiosis might vary from species to species. In *S. cerevisiae* and the mouse, for example, recombination of homologous chromosomes is initiated before SC formation and the recombination itself is prerequisite for SC formation [15,16]. In *Drosophila* and *C. elegans*, however, SC formation is not dependent on the occurrence of recombination, and the presynaptic alignment of homologous chromosomes appears to be mediated by specific chromosomal domains indispensable for SC formation at later stages [9,17]. Therefore, the requirement of the SC for the recombination of homologous chromosomes varies depending on the species. In addition, it has been reported that the timings of

recombination and synapsis in meiosis differ in flies and grasshoppers, indicating the existence of divergence in the regulatory mechanisms of chromosomal behavior in meiosis even in two species phylogenetically close to each other [18]. Molecular components of the SC reported to date are surprisingly diverged, at least at the amino acid level, suggesting the existence of species-specificity in precise roles of each component of the SC. However, detailed investigations of the SC components have been confined to only a limited number of model organisms. In vertebrates, no data on molecular aspects of the SC components for non-mammalians species are available. Therefore, more information on the SC components and their roles in various species is needed for a comprehensive understanding of the mechanisms that regulate chromosomal behavior specific to meiosis.

To investigate the roles of SC components in vertebrates other than mammals, we have paid attention to fish, since teleosts constitute the largest class of living vertebrates and show various patterns in gametogenesis [19]. In addition, there has been significant progress in the past decade toward elucidation of the regulatory mechanisms of gametogenesis and maturation using fish as experimental models [20,21,22]. The structure of the SC in several fishes has been reported [23,24,25,26], but its molecular components have not yet been identified in any fish. To study the SC in fish, we selected the medaka fish *Oryzias latipes* as an experimental system for the following reasons [27,28,29]: 1) Under artificial reproductive conditions, medaka undergoes continuous spermatogenesis and oogenesis, providing experimental materials all year round, 2) medaka spermatogenesis can be reproduced in a cell culture system [30,31,32], enabling manipulation of the genes by RNA interference and DNA transfection to spermatogenic cells, 3) the medaka genome project is proceeding, providing sequence information (Medaka Genome Initiative, http://medaka.dsp.jst.go.jp/MGI/; Medaka Genome Database, http://mbase.bioweb.ne.jp/~dclust/medaka_top.html; Medaka Genome Project, http://dolphin.lab.nig.ac.jp/medaka/index.php), and 4) inbred strains as well as many mutants have been established.

In this study, we cloned medaka homologs of *Sycp1* and *Sycp3*, *MeSycp1* and *MeSycp3* (for medaka *Sycp1* and *Sycp3*, respectively). Using antibodies raised against recombinant proteins, we conducted immunoblotting and immunohistochemical analyses in the testis, ovary, and isolated spermatogenic cells. We found that the expression patterns of MeSYCP1 and MeSYCP3 are different in some respects from

those reported in mammals.

Materials and Methods

Fishes

Sexually mature *Oryzias latipes* (orange-red type) was obtained from a local fish farmer (Yatomi, Aichi, Japan). The fish were cultured in fresh water at 27˚C under artificial light conditions (14-hr light and 10-hr dark conditions) for inducing a daily reproductive cycle.

Cloning of cDNA encoding medaka *Sycp1* and *Sycp3*

Following isolation of total RNA from medaka testes with ISOGEN (Nippon Gene, Tokyo, Japan), cDNAs were produced with a first-strand cDNA synthesis kit (Gibco BRL LIFE Technology, Tokyo, Japan). A cDNA fragment of medaka *Sycp1* was obtained by RT-PCR with a primer set, 5'-TGTCAGCAGAAGATAGCAGAC-3' and 5'-CTTCTTTCTCTTTTCAAGACCC-3'. The primers were designed according to a sequence homologous to mouse SYCP1 (DDBJ/EMBL/GenBank accession number **NP_035646**), which was found by a BLAST search in the medaka genome database (Medaka Genome Project, http://dolphin.lab.nig.ac.jp/medaka/index.php). Using the fragment as a hybridization probe, a full-length cDNA was isolated from a cDNA library constructed from medaka testis (The sequence data appear in the DDBJ/EMBL/GenBank databases with the accession number **AB207974**.). The isolation of medaka *Sycp3* cDNA clones was described previously [33].

Production of antibodies

A cDNA encoding the N-terminal region (amino acids 1-453) of MeSYCP1 was amplified by PCR with a primer set introducing the *Bam*HI and *Xho*I sites (indicated by underlines) at the 5' and 3' ends, respectively (CGGGATCCATGGAGAGAGATCATGGCTT and CCGCTCGAGTGCTGTCGATACTTTGAGCTT). The amplified fragment was ligated into the expression vector pGEX-KG [34], and a recombinant protein was produced for use as an antigen, according to the method described previously [35]. Mouse antiserum raised against MeSYCP1 was affinity-purified with the antigenic protein electroblotted onto an Immobilon membrane (Millipore, Tokyo, Japan). The production of

affinity-purified guinea pig anti-MeSYCP3 antibody was described previously [33].

Protein extraction and immunoprecipitation

Proteins were extracted from tissues of adult medaka by sonication in RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, 50 mM NaF, 1 mM Na_3VO_4, 5 mM 2-mercaptoethanol, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethanelsulfonyl fluoride]. After centrifugation at 16,000 *g* for 10 min, the supernatants were collected and stored at -80˚C until use. Tissue extracts were incubated with either anti-MeSYCP1 or anti-MeSYCP3 antibody in the presence of Protein G-Sepharose or Protein A-Sepharose (Amersham Biosciences, Piscataway, NJ), respectively. After washing five times in RIPA buffer, the immunoprecipitates were separated by SDS-PAGE with 7.5% gel for MeSYCP1 and 12.5% gel for MeSYCP3, blotted onto an Immobilon membrane, and probed with primary and secondary antibodies, as described previously [33].

Immunohistochemistry and chromosome spread

Testes were processed for immunohistochemistry and chromosome spreads as described previously [33,36] with some modifications described below. The medaka spermatocytes put on poly-L-lysine-coated cover slips were treated with 85 mM NaCl for 10 min (for Fig. 4A) or 0.8% Na-citrate for 20 min (for Fig. 4B) instead of 75 mM KCl for 10 min [33] or 85 mM NaCl for 3 min [36], respectively. The samples were incubated overnight at 4˚C with primary antibodies, anti-MeSYCP1 at 1:50 dilution and anti-MeSYCP3 at 1:200 dilution. After washing, the primary antibodies were detected with appropriate secondary antibodies conjugated with either Alexa 488 or Alexa 546 (Molecular Probes, Eugene, OR), counterstained with propidium iodide (PI) when the samples were single-labeled, and mounted with Vectashield (Vector Laboratories, Burlingame, CA). The samples were observed under a Bio-Rad MicroRadiance confocal microscope.

Results

Spermatogenesis in medaka

Teleost testes are classed under two types, restricted spermatogonial type and unrestricted spermatogonial type, according to the distribution of spermatogonia in the

testis [37], and the medaka testis is classified into the former [38,39]. The testis structure and the process of spermatogenesis in the medaka are schematically shown in Fig. 1. The medaka testis consists of many tubules, in which spermatogenesis proceeds from the distal part near the wall to the proximal part at the center. Type A spermatogonia are non-proliferating stem cells, which contribute to germ cell renewal. They are localized to the most distal region of the tubule and separated from each other by Sertoli cells. Type B spermatogonia, which supply germ cells to spermatogenesis by mitotic proliferation, are situated in a space formed by a single layer of flat Sertoli cells, called a cyst. The germ cells, except for type A spermatogonia, are connected to each other by intracellular bridges and undergo spermatogenesis and spermiogenesis synchronously in each cyst. Therefore, all spermatocytes present in one cyst represent the same meiotic stage (Fig. 1).

Immunological detection of MeSYCP1 and MeSYCP3

In immunoblotting analyses using antibodies raised against recombinant MeSYCP1 and MeSYCP3 proteins, MeSYCP1 was detected as a 104-kDa band and MeSYCP3 was detected as two bands, 27- and 30-kDa bands, in the medaka testis (Fig. 2A). Two bands of SYCP3 have been reported in the mouse and rat [10,36,40]. These signals were missing when the antibodies were preincubated with antigenic proteins, confirming the specificity of the signals (data not shown). Neither MeSYCP1 nor MeSYCP3 was found in liver extracts (Fig. 2A), consistent with the fact that SYCP1 and SYCP3 are structural components of the SC that are specifically formed in germ cells undergoing meiotic divisions.

MeSYCP1 and MeSYCP3 were not detectable in the ovary by ordinary immunoblotting analysis (Fig. 2A). We encountered a similar situation in which SMC1β, a meiosis-specific cohesin subunit, was not detectable in the ovary by immunoblotting [33]. In that case, however, we succeeded in detecting SMC1β by increasing its protein contents by precipitation with the antibody, since the ratio of germ cells to somatic cells is very low in the ovary compared to that in the testis. Therefore, the contents of MeSYCP1 and MeSYCP3 in ovarian extracts were concentrated by immunoprecipitation and then analyzed by immunoblotting. Using this technique, MeSYCP1 and MeSYCP3 were detected in the ovary, as in the testis, but not in the liver (Fig. 2B). MeSYCP3 in the ovary existed as a 27-kDa form and a 30-kDa form was

absent, in contrast to the presence of both forms in the testis (Fig. 2B).

Expression of MeSYCP1 and MeSYCP3 in the testis

The expression patterns of MeSYCP1 and MeSYCP3 in the testis were examined by immunocytological analyses of frozen sections (Fig. 3). In mammals, it has been reported that SYCP1 is specifically expressed during meiotic prophase and localized on the SC [4,5,41]. MeSYCP1 was also expressed in primary spermatocytes but not in spermatogonia, spermatids, spermatozoa and somatic cells (Fig. 3A, B). The signals of MeSYCP1 in the nuclei of spermatocytes appear as threads, probably representing the SC (Fig. 3A, B). The intensity of signals was diminished as meiosis proceeded and the signals were completely lost in secondary spermatocytes (Fig. 3A). The expression patterns of MeSYCP3 were similar to those of MeSYCP1. In frozen sections, MeSYCP3 was expressed as threads (probably representing the SC) in primary spermatocytes (Fig. 3C, D, E) as in the case of mammals [42]. The expression of MeSYCP3 was not detected in secondary spermatocytes and spermatogenic cells thereafter (Fig. 3C). In the ovary, MeSYCP1 and MeSYCP3 were also detected in oocytes but not in oogonia and somatic cells such as follicle cells (data not shown). These results clearly indicate that MeSYCP1 and MeSYCP3 are expressed solely in meiotically dividing cells.

The expression of MeSYCP1 and MeSYCP3 began in leptotene spermatocytes, starting from one side of the nucleus with distinct polarity (Fig. 3D, see also Fig. 4A-2). The oriented expression of SC components presumably reflects the polarized nuclear organization known as the meiotic bouquet structure [43,44]. Close observations using double-labeling of frozen sections with anti-MeSYCP1 and anti-MeSYCP3 antibodies revealed that some cysts of spermatocytes expressed MeSYCP3 but not MeSYCP1 (indicated by a blue arrow in Fig. 3C), demonstrating that MeSYCP3 expression starts at an earlier stage of meiosis than MeSYCP1 does. Expression of SYCP3 slightly earlier than that of SYCP1 has also been reported in the mouse [42]. In spermatocytes at the pachytene stage, MeSYCP1 and MeSYCP3 were colocalized and represented the SC (Fig. 3C, D). In spermatocytes at metaphase I, MeSYCP3 appeared as dots in the cytoplasm, while MeSYCP1 was not detected (Fig. 3C, E), indicating the disappearance of MeSYCP1 earlier than that of MeSYCP3. The dot-like signals of MeSYCP3 at metaphase I might correspond to a finding in the mouse that SYCP3 released from

chromosomes aggregates in the cytoplasm [45].

Localization of MeSYCP1 and MeSYCP3 on meiotic chromosomes in spermatocytes

We intended to investigate in detail the localization of MeSYCP1 and MeSYCP3 on meiotic chromosomes using chromosome spreads of spermatocytes. Since chromosome spreads with high resolution have already been reported in fish [46,47], we applied these methods to medaka spermatocytes. However, the resulting spreads did not react to the antibodies against MeSYCP1 and MeSYCP3, because of the loss of antigenicity due to strong spreading conditions during the sample preparation. Conversely, our previous method of medaka chromosome spreads under mild conditions allowed us to detect the SC components and cohesins on the chromosomes, but it provided relatively low resolution images [33]. To diffuse chromatins more extensively but retain the response to antibodies, we modified our methods as described in Materials and Methods. The modified methods improved the quality of images, enabling close observation of the localization of MeSYCP1 and MeSYCP3 on meiotic chromosomes (Fig. 4).

In mammals, SYCP3 is expressed from leptotene spermatocytes [42]. MeSYCP3 was not detected in pre-leptotene spermatocytes (Fig. 4A-1). Its expression started at the leptotene stage as short fine treads with distinct polarity (Fig. 4A-2). At the zygotene stage, the thread-like signals became distinct lines that marked the axes of chromosomes undergoing synapsis (Fig. 4A-3). At the pachytene stage, the MeSYCP3 signals became thicker and shorter than those found at earlier stages, appearing as lines associated with each pair of homologous chromosomes (probably representing the lateral elements of the SC) (Fig. 4A-4, B-4). At the diplotene stage, the homologous chromosomes underwent desynapsis, remaining bound to each other at the chiasmata and at the arms distal to chiasmata [48]. MeSYCP3 remained in the axial cores of chromosomes, but its signal was weaker and thinner than those in the synaptic regions (Fig. 4A-5, B-5). At metaphase I, MeSYCP3 was found on all of the 24 fully condensed bivalent chromosomes, irrespective of the regions of arms and centromeres (Fig. 4A-6, B-6). Homologous chromosomes were completely separated and pulled toward opposite poles by spindle microtubules at anaphase I, a stage at which MeSYCP3 still remained in the arm and centromere regions of all chromosomes (Fig. 4A-7). Thereafter, the intensity of MeSYCP3 signals on chromosomes was diminished (Fig. 4A-8, -9).

The localization of MeSYCP1 in early prophase I was similar to that of MeSYCP3; MeSYCP1 was absent in spermatocytes at the early leptotene stage (Fig. 4B-1), appeared at the late leptotene stage (Fig. 4B-2), increased at the zygotene stage (Fig. 4B-3), and formed 24 distinct lines that probably represent the central element of the SC at the pachytene stages (Fig. 4B-4). In contrast to MeSYCP3, MeSYCP1 was completely lost in the desynaptic regions of chromosomes at the diplotene stage (Fig. 4B-5), although it remained in the synaptic regions. At metaphase I and thereafter (Fig. 4B-6), MeSYCP1 signals were not detected on the chromosomes.

Double-labeling of chromosome spreads with anti-MeSYCP1 and anti-MeSYCP3 antibodies showed colocalization of these proteins on meiotic chromosomes. At the early leptotene stage (Fig. 4B-1), the expression of MeSYCP3 started, but MeSYCP1 was not yet expressed at this stage, consistent with results obtained by immunohistological analysis of frozen sections (Fig. 3C). At the zygotene stage (Fig. 4B-3), MeSYCP3 was detected along the SC on the entire region of all chromosomes, but MeSYCP1 was detected only partially on the SC. At the pachytene stages (Fig. 4B-4), both MeSYCP1 and MeSYCP3 were detected along the SC on the entire region of all chromosomes. At the diplotene stage (Fig. 4B-5), MeSYCP1 disappeared but MeSYCP3 still remained on the chromosomes. These results clearly indicate that MeSYCP3 appears earlier and disappears later than does MeSYCP1, being consistent with the view that SYCP3 and SYCP1 are components of axial/lateral elements and the central element, respectively, and that the formation of axial/lateral elements precedes the formation of the central element. Double-labeling of MeSYCP1 and MeSYCP3 also clearly showed that at the diplotene stage MeSYCP1 had disappeared in the desynaptic regions of homologous chromosomes, whereas MeSYCP3 was still localized to all regions of chromosomes (Fig. 4B-5).

Discussion

SC components in non-mammalian vertebrates

Formation of the SC and high levels of homologous recombination are the hallmarks of meiotic prophase. Pairing of homologous chromosomes culminates with SC formation. The SC has been found in almost all of the sexually reproducing eukaryotes analyzed so far [2,49], but its structural components exhibit considerable variations in amino acid sequence even between homologous proteins in closely related species, in spite of

evolutional conservation in their morphology. In vertebrates, studies on the SC at the molecular level have been confined to mammals to date, and we need to expand our knowledge of molecular components of the SC to other vertebrates. To study the SC in vertebrates other than mammals, we selected the medaka fish *Oryzias latipes*. The present study is the first investigation of the behavior of SC components during meiosis in non-mammalian vertebrates.

SYCP1 in the sex chromosomes of medaka

SYCP1 is the major component of transverse filaments, the structure playing an important role in connecting homologous chromosomes [4,5,41,50]. The expression patterns of MeSYCP1 are very similar to those of the mammalian homologs. The expression of MeSYCP1 starts from the late leptotene stage, and MeSYCP1 forms the SC with MeSYCP3 by the diplotene stage (Fig. 4B). The localization of MeSYCP1 is limited to the synaptic regions of homologous chromosomes in diplotene spermatocytes and is undetectable in the desynaptic regions (Fig. 4B-5). Although we need further evidence, it is likely that SYCP1 plays an important role in connecting homologous chromosomes in medaka as in the case of mammals.

In mammals, SYCP1 is localized on the pseudo-autosomal region of a paired XY chromosome that makes synapsis only at this region [36,51]. In contrast to this, SYCP1 as well as SYCP3 is present in the whole region of all chromosomes at the pachytene stage in medaka (Fig. 4B-4). It has been reported that there are no detectable morphological differences between X and Y chromosomes in medaka [52], and a paired XY chromosome should therefore make synapsis along its entire length. Thus, the presence of SYCP1 in the whole region of all chromosomes at the pachytene stage implies that all homologous chromosomes, including a paired XY chromosome, are joined together to each partner in medaka.

SYCP3 in medaka

MeSYCP3 was detected as two bands in testis extracts (Fig. 2). There are two possible explanations for the presence of two bands. One possible explanation is that the two bands are differently phosphorylated forms of MeSYCP3. In the rat, it has been reported that phosphorylated variants of SYCP3 are present during meiotic prophase [40]. Therefore, the two forms of MeSYCP3 may be the same molecule in different

phosphorylation states, although treatment with commercially available phosphatases failed to change the electrophoretic mobility of the two bands (our unpublished data). The other possible explanation is that two SYCP3 molecules have different amino acid sequences at their N-terminus because of a difference in start points of transcription, as suggested in the mouse and rat [53]. The mouse *Sycp3* gene consists of nine exons, and it has been suggested that two transcripts, one containing all of the 9 exons and the other containing only 8 exons (exclusive of exon 1) are produced from the gene, although two proteins translated from these transcripts seem to have essentially the same function [53].

In contrast to the two forms of MeSYCP3 found in testis extracts, only a 27-kDa form of MeSYCP3 was detected in ovary extracts (Fig. 1B). If the two forms of MeSYCP3 in the testis represent different phosphorylation states, the ovary would contain only a dephosphorylated form of MeSYCP3. The level of phosphorylation of SYCP3 in rat spermatocytes increases during meiotic prophase [40]. Since the medaka ovary used in this experiment did not contain maturing or mature eggs at later meiotic stages, including meiotic metaphases I and II, the ovary sample should include only cells at early meiotic stages from leptotene to diplotene, in contrast to the testis sample, which included meiotic cells of all stages. The absence of a phosphorylated form of MeSYCP3 in the ovary might be due to the absence of cells at later stages of meiosis in the extracts. On the other hand, if the two forms of MeSYCP3 are derived from different transcriptional starting points, the obtained results would imply that the transcriptional control of MeSYCP3 is different in the testis and ovary.

The present study revealed that MeSYCP3, the expression of which starts at the early leptotene stage, is located on the SC together with MeSYCP1 in zygotene and pachytene spermatocytes. In striking contrast to SYCP1, it has been reported in mammals that SYCP3 is localized to the entire region of paired XY chromosomes, which undergo synapsis only in the pseudo-autosomal region [36,51]. The presence of MeSYCP3 in the axial cores of homologous chromosomes was confirmed in the present study (Fig. 4B-5), demonstrating that the localization pattern of MeSYCP3 is the same as that of mammalian SYCP3 at prophase I. At metaphase I, however, SYCP3 shows different localizations in medaka and mammals. MeSYCP3 is found in the entire region of chromosomes irrespective of the arm and centromeric regions (Fig. 4A-6, B-6), whereas mammalian SYCP3 is located on the centromeric regions but not on the arm

regions at metaphase I [42]. In this respect, it should be noted that there is a report that mammalian SYCP3, like MeSYCP3, is localized to the whole chromosomes, including the arm regions, at metaphase I [45]. These apparently contradictory results reported by Eijpe et al. [42] and Parra et al. [45] might be due to the difference in the methods used in the two studies. Results of the former study were obtained by a surface-spreading method, while results of the latter study were obtained by a squashing method. Since we used a surface-spreading method in this study, it is unlikely that the difference in SYCP3 localization at metaphase I between medaka and mammals is attributable to a technical difference. We speculate that MeSYCP3 at metaphase I has a different role in cohesion of homologous chromosomes from that of SYCP3 in mammals. In agreement with this, we have previously reported that cohesin subunits, the key molecules responsible for the cohesion and separation of homologous chromosomes and sister chromatids, also show distinct expression patterns in medaka and mammals [33]. The results of the present and previous studies strongly suggest that the mechanisms regulating chromosomal behavior, especially at metaphase I, are different in medaka and mammals.

The role of SYCP3 at metaphase I remains to be elucidated. In *Sycp3*-deficient mice, meiosis is stopped at the zygotene stage, and spermatogenic cells at later stages, including metaphase I, are not present in the testis because the spermatogenesis is arrested at the checkpoint that monitors several key events, including failure to form the SC [11]. Therefore, we cannot examine the role of SYCP3 at metaphase I by using *Sycp3*-deficient mice. On the other hand, our previous study on spermatogenesis in interspecific hybrid medaka between *O. latipes* and *O. curvinotus* revealed that a defect in SC formation does not evoke the arrest of spermatogenesis, spermatogenic cells at metaphase therefore being observed in the hybrid fish [31]. This characteristic of medaka allows us to investigate the role of MeSYCP3 if the expression of MeSYCP3 can be inhibited by appropriate methods, such as antisense oligonucleotides and RNA interference.

Anti-MeSYCP1 and -MeSYCP3 as meiotic markers in medaka

Isolation and characterization of stage-specific molecules in spermatogenic cells are required for investigating the molecular mechanisms of spermatogenesis. A variety of genes, the expression of which is confined to specific stages of spermatogenesis, have

already been documented in mammals and used as stage-specific markers for meiosis. The SC components are commonly used markers for meiotic prophase I [36,42]. In contrast to mammals, there is little information on meiosis-specific molecules in medaka, despite the fact that medaka has become popular as a model organism in the field of developmental biology [54]. To date, identification of germ cells that enter meiosis in fish, including medaka, has been dependent solely on morphological criteria. Much experience is therefore needed for precise judgment of the stages of germ cells. Moreover, there is little molecular confirmation that the present morphological criteria used for staging germ cells in fish are actually appropriate. Using newly produced antibodies, we investigated the expression patterns of MeSYCP1 and MeSYCP3 in the testis and ovary and demonstrated that anti-MeSYCP1 and -MeSYCP3 antibodies can be used as meiotic markers in medaka. The anti-MeSYCP3 antibody can recognize the SC component in other fish, including zebrafish, goldfish (Iwai et al., unpublished data), tilapia (Kobayashi, T., personal communication) and eel (Miura, T., personal communication). It is therefore expected that this antibody will make a great contribution to the identification of meiotic cells in fish and promote investigations in the field of reproductive biology using fish as experimental models.

Acknowledgments

We thank Dr. Tohru Kobayashi (National Research Institute of Aquaculture, Japan) for providing a medaka testis cDNA library and sharing unpublished data on tilapia. We also thank Dr. Takeshi Miura (Ehime University, Japan) for helpful discussion with his unpublished data on the eel. This work was supported in part by the Program for Promotion of Basic Research Activities for Innovative Biosciences (PROBRAIN) and by the 21st Century COE Program “Center of Excellence for Advanced Life Science on the Base of Bioscience and Nanotechnology” to M. Y.

References

[1] D. von Wettstein, S.W. Rasmussen, P.B. Holm, The synaptonemal complex and genetic segregation, Annu. Rev. Genet. 18 (1984) 331-413.

[2] C. Heyting, Synaptonemal complexes: structure and function, Curr. Opin. Cell Biol. 8 (1996) 389-396.

[3] G.S. Roeder, Meiotic chromosomes: it takes two to tango, Genes Dev. 11 (1997) 2600-2621.

[4] R.L.J. Meuwissen, H.H. Offenberg, A.J.J. Dietrich, A. Riesewijk, M. van Iersel, C. Heyting, A coiled-coil related protein specific for synapsed regions of meiotic prophase chromosomes, EMBO J. 11 (1992) 5091-5100.

[5] M. Dobson, R.E. Pearlman, A. Karaiskakis, B. Spyropoulos, P.B. Moens, Synaptonemal complex proteins: occurrence, epitope, mapping, and chromosome disjunction, J. Cell Sci. 107 (1994) 2749-2760.

[6] A. Smith, R. Benavente, Identification of structural protein component of rat synaptonemal complexes, Exp. Cell Res. 198 (1992) 291-297.

[7] F.A.T. de Vries, E. de Boer, M. van den Bosch, W.M. Baarends, M. Ooms, L. Yuan, J. Liu, A.A. van Zeeland, C. Heyting, A. Pastink, Mouse *Sycp1* functions in synaptonemal complex assembly, meiotic recombination, and XY body formation, Genes Dev. 19 (2005) 1376-1389.

[8] M. Sym, G.S. Roeder, Crossover interference is abolished in the absence of synaptonemal complex protein, Cell 79 (1994) 283-292.

[9] A.J. MacQueen, M.P. Colaiacovo, K. McDonald, A.M. Villeneuve, Synapsis-dependent and –independent mechanisms stabilize homolog pairing during meiotic prophase in *C. elegans*, Genes Dev. 16 (2002) 2428-2442.

[10] J.H.M. Lammers, H.H. Offenberg, M. van Aalderen, A.C.G. Vink, A.J.J. Dietrich, C. Heyting, The gene encoding a major component of the lateral elements of synaptonemal complexes of the rat is related to X-linked lymphocyte-regulated genes, Mol. Cell. Biol. 14 (1994) 1137-1146.

[11] L. Yuan, J.G. Liu, J. Zhao, E. Brundell, B. Daneholt, C. Höög, The murine SCP3 gene is required for synaptonemal complex assembly, chromosome synapsis, and male fertility, Mol. Cell 5 (2000) 73-83.

[12] L. Yuan, J.G. Liu, M.R. Hoja, J. Wilbertz, K. Nordqvist, C. Höög, Female germ cell aneuploidy and embryo death in mice lacking the meiosis-specific protein SCP3, Science 296 (2002) 1115-1118.

[13] B. Rockmill, G.S. Roeder, Meiosis in asynaptic yeast, Genetics 126 (1990) 563-574.

[14] N.M. Hollingsworth, L. Goetsch, B. Byers, The HOP1 gene encodes a meiosis-specific component of yeast chromosomes, Cell 61 (1990) 73-84.

[15] D. Zickler, N. Kleckner, Meiotic chromosomes: Integrating structure and function, Annu. Rev. Genet. 33 (1999) 603–754.

[16] S.K. Mahadevaiah, J.M.A. Turner, F. Baudat, E.P. Rogakou, P. de Boer, J. Blanco-Rodriguez, M. Jasin, S. Keeney, W.M. Bonner, P.S. Burgoyne, Recombinational DNA double-strand breaks in mice precede synapsis, Nature Genet. 27 (2001) 271-276.

[17] K.S. McKim, J.K. Jang, E.A. Manheim, Meiotic recombination and chromosome segregation in *Drosophila* female, Annu. Rev. Genet. 36 (2002) 205-232.

[18] A. Viera, J.L. Santos, J. Page, M.T. Parra, A. Calvente, M. Cifuentes, R. Gomez, R. Lira, J.A. Suja, J.S. Rufa, DNA double-strand breaks, recombination and synapsis: the timing of meiosis differs in grasshoppers and flies, EMBO Rep. 5 (2004) 385-391.

[19] S.S. Nelson, Fishes of the world, John Wiley & Sons, New York, 1994.

[20] Y. Nagahama, M. Yoshikuni, M. Yamashita, T. Tokumoto, Y. Katsu, Regulation of oocyte growth and maturation in fish, in: R.A. Pedersen and G.P. Schatten (Eds.), Current Topics of Developmental Biology, Academic Press, San Diego, 1995, vol. 30, pp. 103-145.

[21] M. Yamashita, K. Mita, N. Yoshida, T. Kondo, Molecular mechanisms of the initiation of oocyte maturation: general and species-specific aspects, in: L. Meijer, A. Jézéquel and B. Ducommum (Eds.), Progress in Cell Cycle Research, Kluwer Academic Publishers, 2000, vol. 4, pp. 115-129.

[22] T. Miura, C. Miura, Japanese eel: A model for analysis of spermatogenesis, Zool. Sci. 18 (2001) 1055-1063.

[23] C. Oliveira, F. Foresti, M.C. Rigolino, Y.A. Tabata, Synaptonemal complex analysis in spermatocytes and oocytes of rainbow trout, *Oncorhynchus mykiss* (Pisces, Salmonidae): the process of autosome and sex chromosome synapsis, Chromosome Res. 3 (1995) 182-190.

[24] A.L. van Eenennaam, J.D. Murray, J.F. Mediano, Synaptonemal complex analysis in spermatocytes of white sturgeon, *Acipenser transmontanus* Richardson (Pisces, Acipenseridae), a fish with a very high chromosome number, Genome 41 (1998) 51-61.

[25] N. Cuñado, M.A. Garrido-Ramos, R. de la Herrán, C. Ruíz-Rejón, M. Ruíz-Rejón, J.L. Santos, Organization of repetitive DNA sequences at pachytene chromosomes of gilthead seabream *Sparus aurata* (Pisces, Perciformes), Chromosome Res. 8 (2000) 67-72.

[26] N. Cuñado, J. Terrones, L. Sánchez, P. Martínez, J.L. Santos, Synaptonemal complex analysis in spermatocytes and oocytes of turbot, *Scophthalmus maximus* (Pisces, Scophthalmidae), Genome 44 (2001) 1143-1147.

[27] K. Naruse, M. Sakaizumi, A. Shima, Medaka as a model organism for research in experimental biology, Fish Biol. J. Medaka 6 (1994) 47-52.

[28] J. Wittbrodt, A. Shima, M. Schartl, Medaka – A model organisms from the Far East, Nature Rev. Genet. 3 (2002) 53-64.

[29] A. Shima, H. Mitani, Medaka as a research organism: past, present and future, Mech. Dev. 121 (2004) 599-604.

[30] A. Saiki, M. Tamura, M. Matsumoto, J. Katowgi, A. Watanabe, K. Onitake, Establishment of *in vitro* spermatogenesis from spermatocytes in the medaka, *Oryzias latipes*, Dev. Growth Differ. 39 (1997) 337-344.

[31] Y. Shimizu, N. Shibata, M. Yamashita, Spermiogenesis without proceeding meiosis in the hybrid medaka between *Oryzias latipes* and *O. curvinotus*, J. Exp. Zool. 279 (1997) 102-112.

[32] T. Sasaki, A. Watanabe, E. Takayama-Watanabe, M. Suzuki, H. Abe, K. Onitake, Ordered progress of spermiogenesis to the fertilizable sperm of the medaka fish, *Oryzias latipes*, in cell culture, Dev. Growth Differ. 47 (2005) 87-97.

[33] T. Iwai, J. Lee, A. Yoshii, T. Yokota, K. Mita, M. Yamashita, Changes in the expression and localization of cohesin subunits during meiosis in a non-mammalian vertebrate, the medaka fish, Gene Expression Patterns 4 (2004) 495-504.

[34] K.L. Guan, J.E. Dixson, Eukaryotic proteins expressed in *Escherichia coli*: An improved thrombin cleavage and purification procedure of fusion proteins with glutathione S-transferase, Anal. Biochem. 192 (1991) 262-267.

[35] T. Hirai, M. Yamashita, M. Yoshikuni, Y.H. Lou, Y. Nagahama, Cyclin B in fish oocytes: Its cDNA and amino acid sequences, appearance during maturation, and induction of $p34^{cdc2}$ activation, Mol. Reprod. Dev. 33 (1992) 131-140.

[36] J. Lee, T. Iwai, T. Yokota, M. Yamashita, Temporally and spatially selective loss of Rec8 protein from meiotic chromosomes during mammalian meiosis, J. Cell Sci. 116 (2003) 2781-2790.

[37] H. J. Grier, Cellular organization of the testis and spermatogenesis in fishes, Amer. Zool. 21 (1981) 345-357.

[38] E.W. Gresik, G.B. Quirk, G.B. Hamilton, Fine structure of the Sertoli cell of the testis of the teleost *Oryzias latipes*, Gen. Comp. Endocrinol. 21 (1973) 341-352.

[39] N. Shibata, S. Hamaguchi, Electron microscopic study of the blood-testis barrier in the teleost, *Oryzias latipes*, Zool. Sci. 3 (1986) 331-338.

[40] J.H.M. Lammars, M. van Aalderen, A.H.F.M. Peters, A.A.M. van Pelt, I.C. Gaemers, D.G. de Rooij, P. de Boer, H.H. Offenberg, A.J.J. Dietrich, C. Heyting, A change in the phosphorylation pattern of the 30000-33000 *Mr* synaptonemal complex proteins of the rat between early and mid-pachytene, Chromosoma 104 (1995) 154-163.

[41] J.G. Liu, L. Yuan, E. Brundell, B. Bjorkroth, B. Daneholt, C. Höög, Localization of the N-terminus of SCP1 to the central element of the synaptonemal complex and evidence for direct interactions between the N-termini of SCP1 molecules organized head-to-head, Exp. Cell Res. 226 (1996) 11-19.

[42] M. Eijpe, H. Offenberg, R. Jessberger, E. Revenkova, C. Heyting, Meiotic cohesin Rec8 marks the axial elements of rat synaptonemal complexes before cohesins SMC1β and SMC3, J. Cell Biol. 160 (2003) 657-670.

[43] H. Scherthan, Knockout mice provide novel insights into meiotic chromosome and telomere dynamics, Cytogenet. Genome Res. 103 (2003) 235-244.

[44] B. Liebe, M. Alsheimer, C. Höög, R. Benavente, H. Scherthan, Telomere attachment, meiotic chromosome condensation, pairing, and bouquet stage duration are

modified in spermatocytes lacking axial elements, Mol. Biol. Cell 15 (2004) 827-37.

[45] M.T. Parra, A. Viera, R. Gómez, J. Page, R. Benavente, J.L. Santos, J.S. Rufas, J.A. Suja, Involvement of the cohesin Rad21 and SCP3 in monopolar attachment of sister kinetochores during mouse meiosis I, J. Cell Sci. 117 (2004) 1221-1234.

[46] W. Traut, H. Winking, Meiotic chromosomes and stages of sex chromosome evolution in fish: zebrafish, platyfish and guppy, Chromosome Res. 9 (2001) 659-672.

[47] B.M.N. Wallace, H. Wallace, Synaptonemal complex karyotype of zebrafish, Heredity 90 (2003) 136-140.

[48] W.Y. Miyazaki, T.L. Orr-Weaver, Sister-chromatid cohesion in mitosis and meiosis, Annu. Rev. Genet. 28 (1994) 167-187.

[49] J. Loidl, Cytological aspects of meiotic recombination, Experientia 50 (1994) 285-294.

[50] K. Schmekel, R.L. Meuwissen, A.J. Dietrich, A.C. Vink, J. van Marle, H. van Veen, C. Heyting, Organization of SCP1 protein molecules within synaptonemal complexes of the rat, Exp. Cell Res. 226 (1996) 20-30.

[51] P.B. Moens, K.K. Kolas, M. Tarsounas, E. Marcon, P.E. Cohen, B. Spyropoulos, The time course and chromosomal localization of recombination-related proteins at meiosis in the mouse are compatible with models that can resolve the early DNA-DNA interactions without reciprocal recombination, J. Cell Sci. 115 (2002) 1611-1622.

[52] H. Uwa, Y. Ojima, Detailed and banding karyotype analysis of the medaka, *Oryzias latipes*, in cultured cells, Proc. Jpn Acad. B 57 (1981) 39-43.

[53] R.J. Botelho, L. DiNicolo, N. Tsao, A. Karaiskakis, M. Tarsounas, P.B. Moens, R.E. Pearlman, The genomic structure of *SYCP3*, a meiosis-specific gene encoding a protein of the chromosome core, Biochim. Biophys. Acta 1518 (2001) 294-299.

[54] M. Furutani-Seiki, J.W. Wittbrodt, Medaka and zebrafish, an evolutionary twin study, Mech. Dev. 121 (2004) 629-637.

Figure legends

Fig. 1. Schematic drawing of spermatogenesis in medaka testis. The medaka testis consists of many tubules, one of which is shown in the figure (The distal part of the tubule near the testicular wall is on the left and the proximal part to the central efferent duct is on the right.). Type A spermatogonia are situated in the most peripheral region of the tubule and are associated with Sertoli cells. Type B spermatogonia are located in the cyst formed by Sertoli cells. The spermatogenesis and spermiogenesis of male germ cells proceed synchronously in each cyst. Mature spermatozoa are released from the cyst into the efferent ducts at the center of the testis.

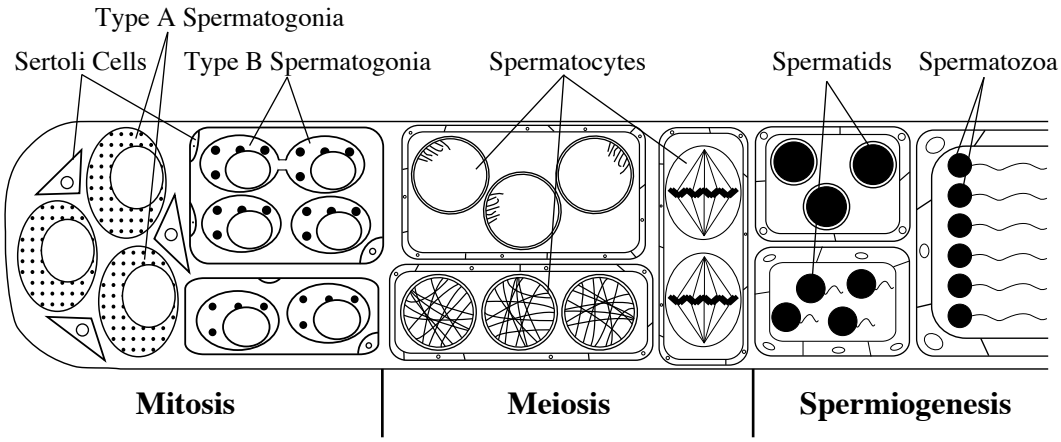
Fig. 2. Immunoblotting analyses of two structural components of the SC, SYCP1 and SYCP3, in medaka. (A) Detection of MeSYCP1 and MeSYCP3 (indicated by triangles). Protein extracts from the ovary (O), testis (T) and liver (L) were blotted with anti-MeSYCP1 antibody (SYCP1) and anti-MeSYCP3 antibody (SYCP3). (B) Detection of MeSYCP1 and MeSYCP3 by immunoblotting following immunoprecipitation. Anti-MeSYCP1 or anti-MeSYCP3 immunoprecipitates from the ovary (O), testis (T) and liver (L) were blotted with the same antibodies. SYCP1 and SYCP3 were detected in the ovary and testis containing meiotic cells but not in the liver.

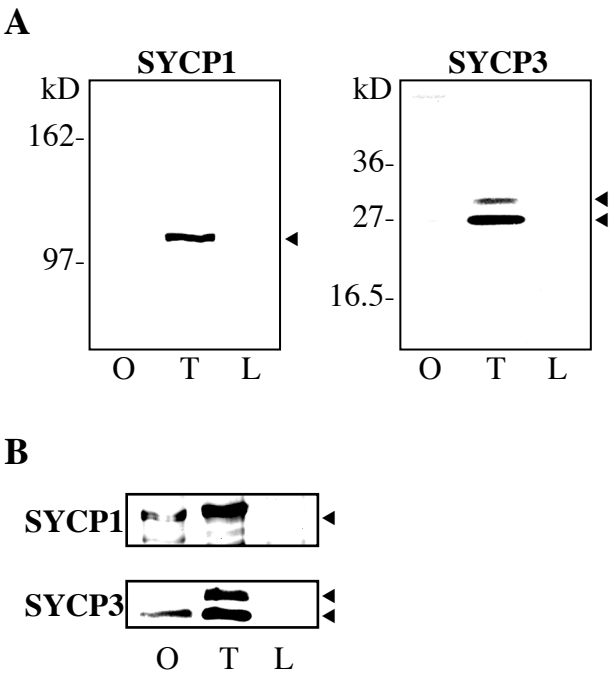
Fig. 3. Immunocytochemical analyses of SYCP1 and SYCP3 in medaka testis. Frozen sections were incubated with anti-MeSYCP1 antibody (A, B) or a mixture of anti-MeSYCP1 and anti-MeSYCP3 antibodies (C, D, E). The primary antibodies were then detected with Alexa 488 (green)-conjugated anti-mouse IgG (for MeSYCP1) or Alexa 546 (red)-conjugated anti-guinea pig IgG (for MeSYCP3). DNA was stained with propidium iodide (red) in A and B. The regions indicated in A are magnified in B, and the two regions indicated in C are magnified in D and E. The blue arrow in C indicates a SYCP1-negative but SYCP3-positive cyst. SG, spermatogonium; PS, primary spermatocyte; SS, secondary spermatocyte; L, leptotene spermatocyte; P, pachytene spermatocyte; MI, metaphase I spermatocyte. Bar, 30 μm (A, C), 10 μm (B, D, E).

Fig. 4. Immunocytochemical analyses of SYCP1 and SYCP3 on meiotic chromosomes in medaka spermatocytes. Chromosome spreads were stained with anti-MeSYCP3

antibody (A) or a mixture of anti-MeSYCP1 and anti-MeSYCP3 antibodies (B). The primary antibodies were then detected with Alexa 488 (green)-conjugated anti-mouse IgG (for MeSYCP1) or anti-guinea pig IgG (for MeSYCP3 in A) or Alexa 546 (red)-conjugated anti-guinea pig IgG (for MeSYCP3 in B). DNA was stained with propidium iodide (red) in A. (A) Localization of MeSYCP3 on meiotic chromosomes at the pre-leptotene (1), leptotene (2), zygotene (3), pachytene (4), diplotene (5), metaphase I (6), anaphase I (7), metaphase II (8) and anaphase II (9) stages. (B) Colocalization of MeSYCP1 (green) and MeSYCP3 (red) on meiotic chromosomes at the early leptotene (1), late leptotene (2), zygotene (3), pachytene (4), diplotene (5) and metaphase I (6) stages. The insets in (B-5) are enlargement (3 times) of a chromosome, demonstrating the localization of MeSYCP3 but not MeSYCP1 in the axial cores. Bar, 10 μm (A, B).

Fig. 1 T. Iwai et al.,

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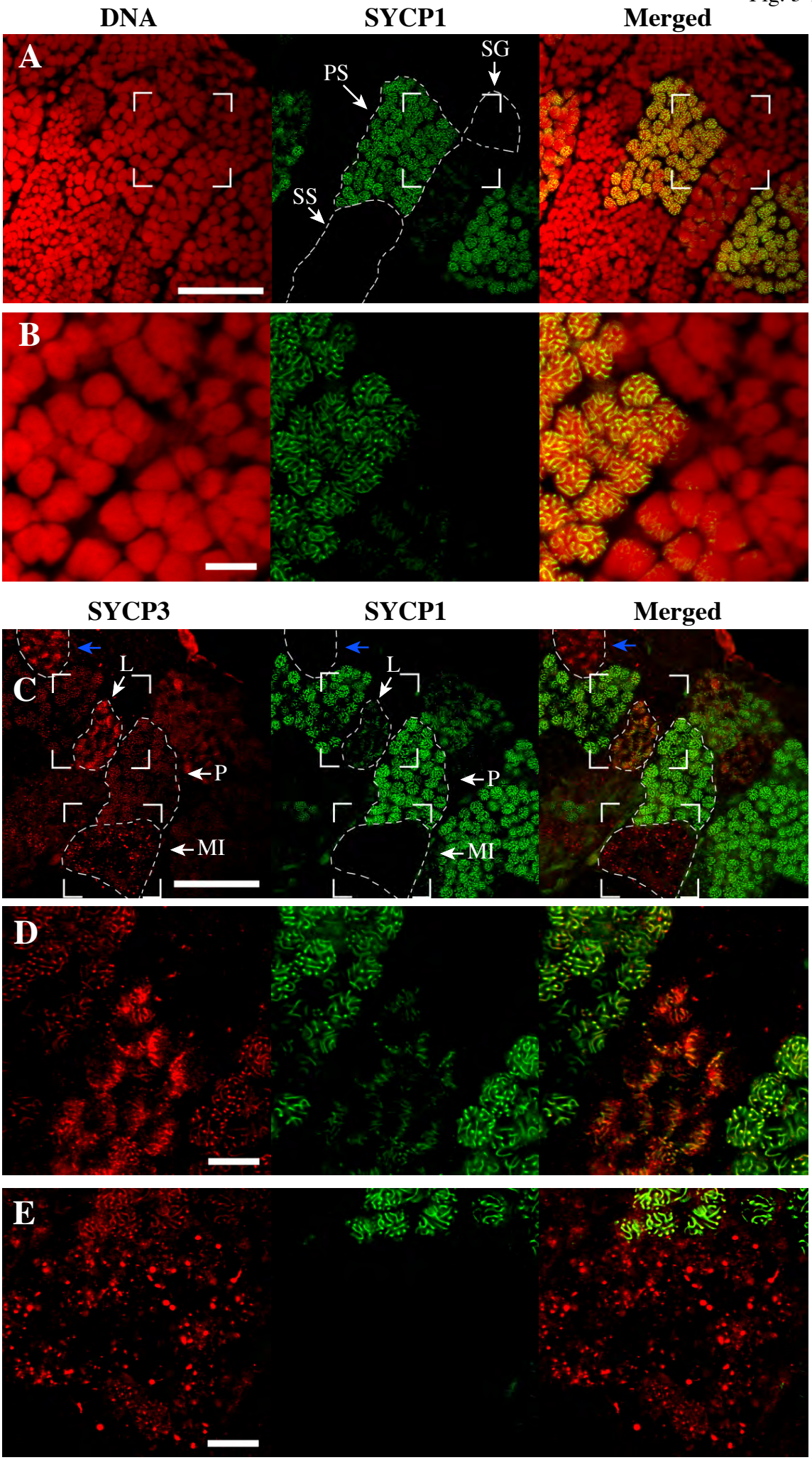
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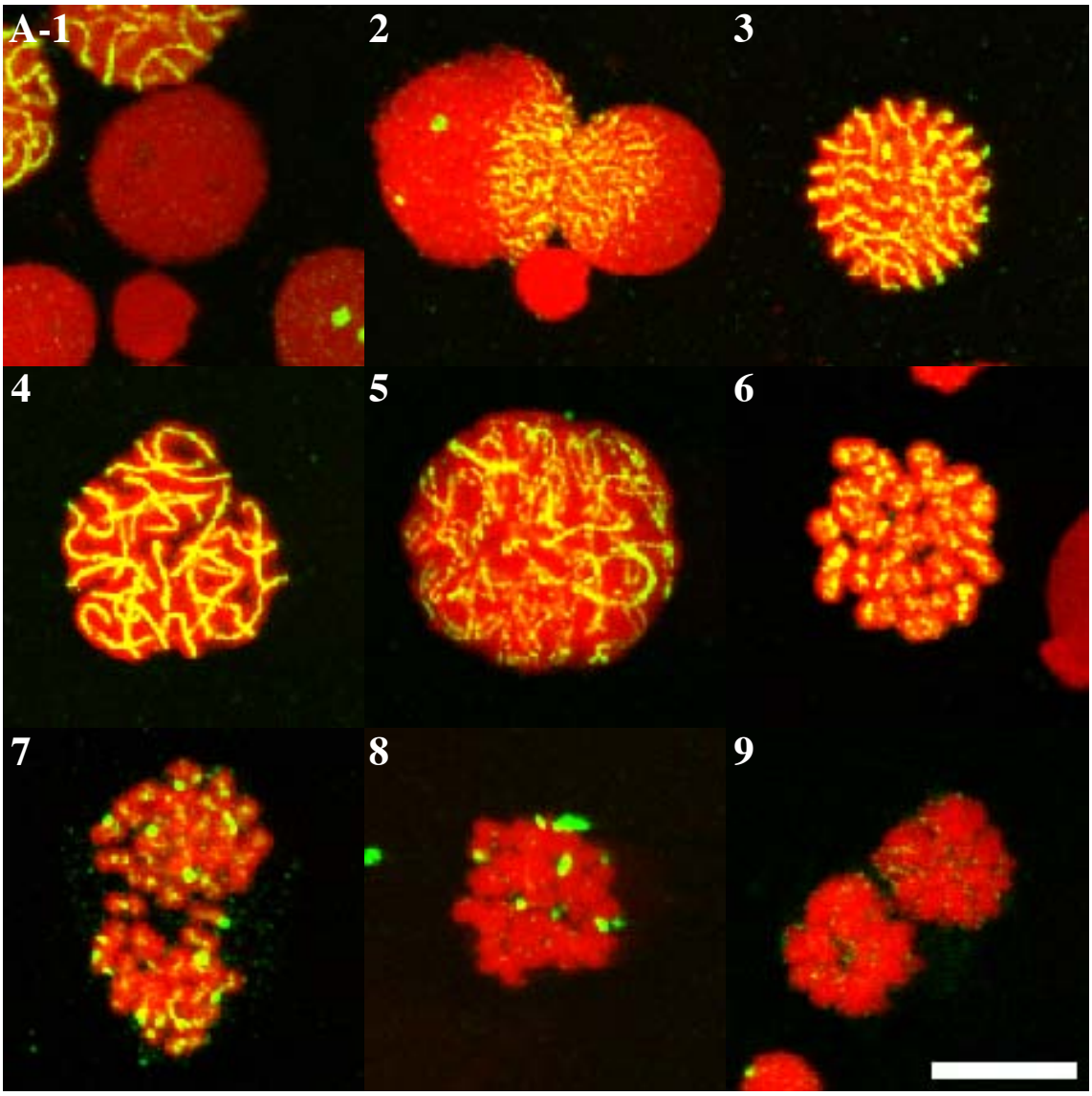
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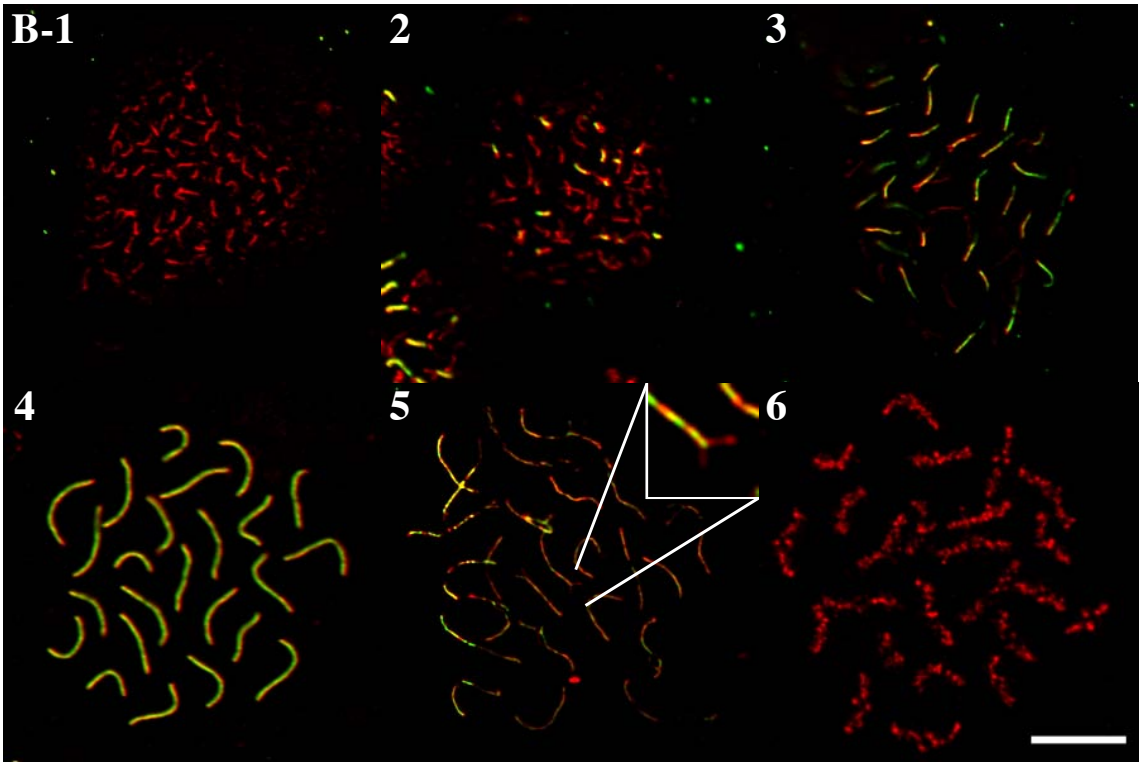
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