PAIR2 is essential for homologous chromosome synapsis in rice meiosis I

Ken-Ichi Nonomura[1,2,*,‡], Mutsuko Nakano[1,*], Mitsugu Eiguchi[1], Tadzunu Suzuki[1] and Nori Kurata[1,2,‡]

[1]Experimental Farm/Plant Genetics Laboratory, National Institute of Genetics, Yata1111, Mishima, Shizuoka 411-8540, Japan
[2]Department of Life Science, Graduate University for Advanced Studies/ Sokendai, Yata1111, Mishima, Shizuoka 411-8540, Japan

*Authors contributed equally to this work
‡Authors for correspondence (e-mail: knonomur@lab.nig.ac.jp; nkurata@lab.nig.ac.jp)

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Summary

The *PAIR2* gene is required for homologous chromosome synapsis at meiosis I in rice (*Oryza sativa* L.) and encodes a HORMA-domain protein that is homologous to *Saccharomyces cerevisiae* HOP1 and *Arabidopsis* ASY1. Immunocytological and electron microscopic analyses indicate that PAIR2 proteins associate with axial elements (AEs) at leptotene and zygotene, and is removed from the AEs of arm regions when homologous chromosomes have been synapsed. Immunocytology against a centromeric histone H3 variant revealed that PAIR2 remains at centromeres until diakinesis, by which time the homologous centromeres had already been synapsed. However, neither precocious segregation of sister chromatids nor kinetochore dysfunction is observed, and AEs are normally assembled in the mutant. In the *pair2*-null mutant, homologous chromosome synapsis is completely eliminated. This study provides the first description of AE-associated protein in monocot plants and indicates that PAIR2 plays an essential role in promoting homologous chromosome synapsis. However, PAIR2 does not play a role in AE formation, sister chromatid cohesion at centromeres or kinetochore assembly in meiosis I of rice.

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Key words: Meiosis, Synaptonemal complex, Homologous chromosome pairing, Gametogenesis, Centromere

Introduction

Meiosis is a crucial event for sexual reproduction of eukaryotes to form haploid spores and gametes. Meiosis is characterized by a single round of pre-meiotic DNA duplication followed by two rounds of chromosome segregation. In this process, homologous chromosomes are aligned and synapsed to prepare for faithful division of each homologous chromosome (homolog) at meiosis I. Although the mechanism of searching for homology among meiotic chromosomes is still unknown, homolog pairing is made solid by an evolutionarily conserved, tripartite, proteinaceous structure called the synaptonemal complex (SC). During early prophase of meiosis I, each chromosome develops a longitudinal axial protein core, called the axial element (AE), to which the chromatin loops are attached (Dobson et al., 1994). At zygotene to pachytene, the cores of each homolog pair become aligned in parallel, and associate with a central element (CE) bridged by transverse filaments (TFs). The AE is called the lateral element (LE) in the mature SCs. Subsequently, the axial cores separate while remaining attached at a few points, called chiasmata, which are presumably the sites of crossovers (von Wettstein et al., 1984).

The components of the SC have been isolated mainly in yeast and mammals. In *Saccharomyces cerevisiae*, the SC components include ZIP1, ZIP2, ZIP3, HOP1, RED1 and MEK1. ZIP1 serves as a putative component of the TF (Sym et al., 1993; Sym and Roeder, 1995). ZIP2 and ZIP3 are present on meiotic chromosomes at discrete foci that correspond to the sites where synapsis initiates, and these proteins are required for the proper assembly of ZIP1 (Smith and Roeder, 1997). HOP1 and RED1 are components of the AE/LE (Hollingsworth et al., 1990; Smith and Roeder, 1997). SCP1 of rat, mouse and human, and the hamster ortholog SYN1, are similar to yeast ZIP1 and a putative TF component (Meuwissen et al., 1992; Meuwissen et al., 1997; Dobson et al., 1994; Sage et al., 1995). The rat SCP2 and SCP3 are components of the AE/LE (Heyting et al., 1987; Heyting et al., 1989; Moens et al., 1987; Offenberg et al., 1998; Dietrich et al., 1992). The hamster SCP3 (COR1) is thought to have a role in the regulation of sister chromatid cohesion, because this protein persists in the chromosome arm until metaphase I, and in the centromeric region until anaphase II (Dobson et al., 1994). Cohesin proteins also constitute part of the AE and are required for the assembly of AEs (Klein et al., 1999; Eijpe et al., 2000; Pelttari et al., 2001).

By contrast, few SC components have been identified in the plant kingdom. ASY1 is the only protein that is homologous to yeast HOP1, and it localizes to the meiotic chromosome axis in *Arabidopsis* and *Brassica* (Caryl et al., 2000; Armstrong et al., 2002). HOP1 and ASY1 include a conserved HORMA (HOP1, REV7, MAD2) domain, which is thought to recognize chromatin that contains DNA adducts or double-stranded breaks, or that fails to attach to the spindle, and acts as an adaptor that recruits other proteins involved in repair (Aravind and Koonin, 1998). Identified genes encoding a HORMA-domain protein are *PAIR2* in rice (*Oryza sativa* L.) (Nonomura et al., 2004b) and *HIM3* in *Caenorhabditis elegans* (Zetka et

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al., 1999; Couteau et al., 2004), and experiments involving mutation or RNA interference of these genes results in the absence of homolog synapsis.

In budding yeast, the meiotic chromosomal proteins RED1, MEK1 and HOP1 are required for the pachytene checkpoint that prevents meiotic nuclear division when completion of homologous recombination and chromosome synapsis fails (Roeder and Bailis, 2000). HOP1 physically interacts with RED1 in vitro (Woltering et al., 2000), and HOP1 localization to meiotic chromosomes depends on prior RED1 localization (Smith and Roeder, 1997). RED1 is phosphorylated by MEK1 kinase and is localized to chromosomes early in meiotic prophase, but is dephosphorylated by GLC7 phosphatase and delocalized from chromosomes around the end of pachytene (Bailis and Roeder, 2000). HOP1, also an in vitro target of MEK1 kinase, dissociates from chromosomes before late pachytene, whereas RED1 remains along the chromosomes (Smith and Roeder, 1997). Taken together, in addition to stoichiometric and mutational analyses, phosphorylated RED1 is thought to signal a defect in meiotic chromosome metabolism to downstream components of the checkpoint pathway (Roeder and Bailis, 2000). However, the details of the function of HOP1 on the chromosome axis and the mechanism of HOP1 localization to, and delocalization from, the axes are still unclear.

In a previous study, we characterized an insertional mutation of the *PAIR2* gene whose homolog synapsis is completely eliminated in male and female meiocytes (Nonomura et al., 2004b). We demonstrate here that PAIR2 associates with the meiotic chromosome axis, and is required for homolog synapsis in rice. In addition, it is also revealed that the PAIR2 protein that accomplishes its role in SC formation is rapidly removed from the axial cores, but remains at centromeric regions until diakinesis. PAIR2 is a rice ortholog of yeast HOP1 and *Arabidopsis* ASY1, and is the first SC-related protein identified in monocot plants. The *O. sativa* ssp. *japonica* genome is 389 Mb in size (International Rice Genome Sequencing Project, 2005), which is considerably larger than the 12 Mb of *S. cerevisiae* (Goffeau et al., 1996) and 125 Mb of *Arabidopsis thaliana* (Arabidopsis Genome Initiative, 2000). Despite disadvantages in molecular analyses, the larger genome size aids observation of the dynamics of chromosomes and chromosomal proteins in higher resolution. This study is the first description of a relationship between a meiotic HORMA-domain protein and centromeres in a model organism of monocot plants, and we believe that these findings will contribute to add new insights into meiotic protein functions of other eukaryotes.

Results

Meiosis-specific accumulation of the PAIR2 protein

Rice *PAIR2* was previously identified as a meiotic gene strongly expressed in both male and female meiocytes, and insertional mutation of the gene resulted in the complete absence of homolog synapsis (Nonomura et al., 2004b). *PAIR2* encodes a 69 kDa protein with a HORMA domain (DDBJ Accession No. AB109238). To reveal PAIR2 localization in meiocytes, a polyclonal antibody was raised against the entire length of a recombinant PAIR2, which was histidine-tagged at the 5′ terminus and purified using a Ni^{2+}-charged column.

To determine the precise expression profile of the PAIR2 protein, anthers of 0.4-1.1 mm in length were collected at 0.1 mm intervals and used for western analysis. The anti-PAIR2 antiserum clearly recognized both the recombinant protein and proteins extracted from the anthers of *O. sativa* cv. Nipponbare at the predicted 69 kDa position of PAIR2 (Fig. 1). The PAIR2 signal became detectable in 0.5-0.9 mm anthers. The most intense signal was observed in 0.7-0.8 mm anthers. A negative control of pre-immune serum displayed no signal (Fig. 1). The longitudinal elongation of rice anthers correlates well with meiosis progression. The 0.7-0.8 mm anthers, in which a large amount of PAIR2 is accumulated, usually possess pollen mother cells (PMCs) in zygotene or pachytene, in which homolog synapsis has been proceeded (Itoh et al., 2005). This expression profile is consistent with the asynaptic phenotype of the *pair2* mutant shown in a previous report (Nonomura et al., 2004b). Thus, we concluded that the antiserum in this study could specifically recognize the native PAIR2.

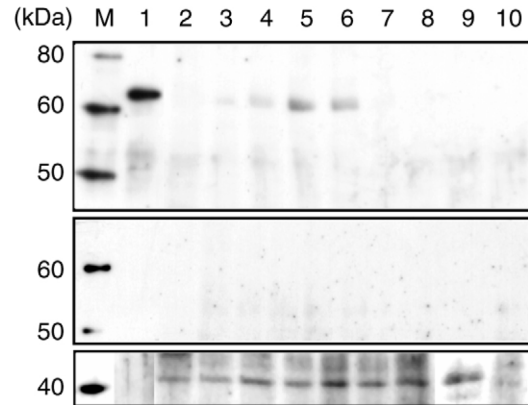

Fig. 1. Western blot analysis using anti-PAIR2 antibody (top), pre-immune serum as a control (middle) and anti-α-tubulin monoclonal antibody (bottom) against recombinant PAIR2 protein and crude extracts from anthers and vegetative tissues. Lanes M and 1 represent a molecular weight marker and 6×His-tagged PAIR2, respectively. Lanes 2-8 show crude extracts from anthers of the following length ranges: 0.4~0.5 mm (2), 0.5~0.6 mm (3), 0.6~0.7 mm (4), 0.7~0.8 mm (5), 0.8~0.9 mm (6), 0.9~1.0 mm (7), 1.0~1.1 mm (8). Lanes 9 and 10 are from seedlings and roots, respectively.

PAIR2 accumulation in the nucleus occurs just after pre-meiotic DNA synthesis

HORMA-domain proteins generally act in the nucleus (Hollingsworth et al., 1990; Armstrong et al., 2002). Western analysis in this study revealed that accumulation of PAIR2 began in young 0.4-0.5 mm anthers (Fig. 1), which frequently included pre-meiotic PMCs (Itoh et al., 2005). Thus, pulsed incorporation of bromodeoxyuridine (BrdU; a thymidine analog) for 4 hours, followed by immunocytological observation, were carried out to investigate whether PAIR2 accumulation in meiotic nuclei is initiated earlier or later than the onset of pre-meiotic DNA synthesis. Pulsed BrdU incorporation into young panicles enables visualization of synchronized de novo DNA synthesis of pre-meiotic PMCs (Nonomura et al., 2004b). The immunostaining pattern of pre-meiotic nuclei was classified into five categories: (1) neither a BrdU nor a PAIR2 signal; (2) faint BrdU but no PAIR2 signal; (3) intense BrdU and faint PAIR2 signals; (4) faint BrdU and intense PAIR2 signals; and (5) no BrdU and a filamentous

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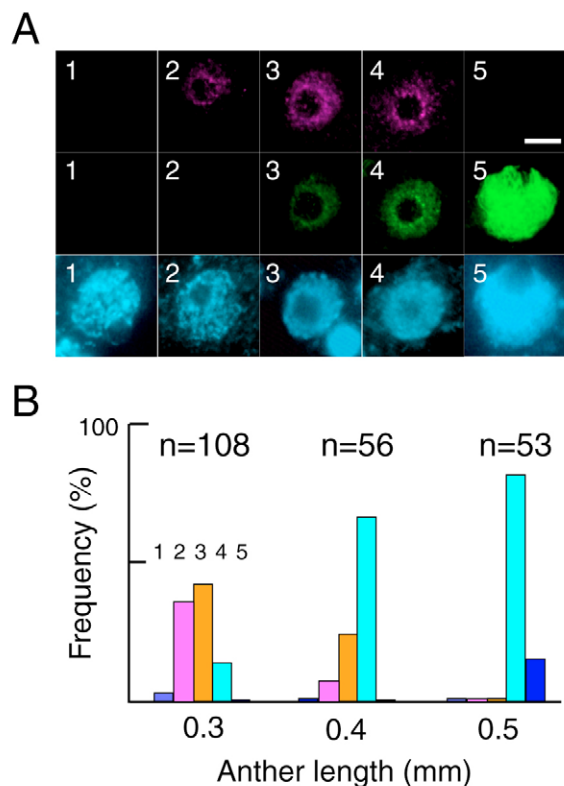

Fig. 2. PAIR2 accumulation in the meiocyte nucleus is induced following pre-meiotic DNA synthesis. (A) Immunofluorescence of incorporated BrdUs and PAIR2 proteins against pre-meiotic PMCs. Staining pattern of BrdU (magenta) and PAIR2 (green) was divided into five categories: (1) neither a BrdU nor a PAIR2 signal; (2) faint BrdU but no PAIR2 signal; (3) intense BrdU and faint PAIR2 signals; (4) faint BrdU and intense PAIR2 signals; and (5) no BrdU and filamentous PAIR2 signals. Chromosomes were counter-stained with DAPI (blue). Bar, 5 μm. (B) Frequency of each category of meiocyte in anthers 0.3, 0.4 and 0.5 mm long. The numbers above each bar correspond to each category of the meiocytes in A.

PAIR2 signal (Fig. 2A). Category 3, in which PAIR2 signals first appeared, were frequent in 0.3 mm anthers. In meiocytes at categories 3 and 4, PAIR2 signals were observed as punctuate foci (Fig. 2A). The intensity of the PAIR2 signal gradually increased, and that of the BrdU signal decreased, according to the longitudinal elongation of anthers (Fig. 2B). The maximum signal of the incorporated BrdU was linked to the faint signal of PAIR2 accumulation in category 3. This result suggests that pre-meiotic DNA synthesis initiates in the anthers around 0.3 mm and that PAIR2 accumulation into the nucleus begins just after DNA synthesis.

No PAIR2 signal was observed in vegetative tissues (Fig. 1) and mitotic nuclei (nuclei without PAIR2 signals in panel 3 of Fig. 2A), indicating that PAIR2 expression is specific for meiosis.

PAIR2 associates with the axial region of meiotic chromosomes

During pre-meiotic S and G2 phases, the PAIR2 signal appeared as punctuate foci in the nucleus (Fig. 3A). After the PMCs entered into meiosis, PAIR2 foci began to demonstrate

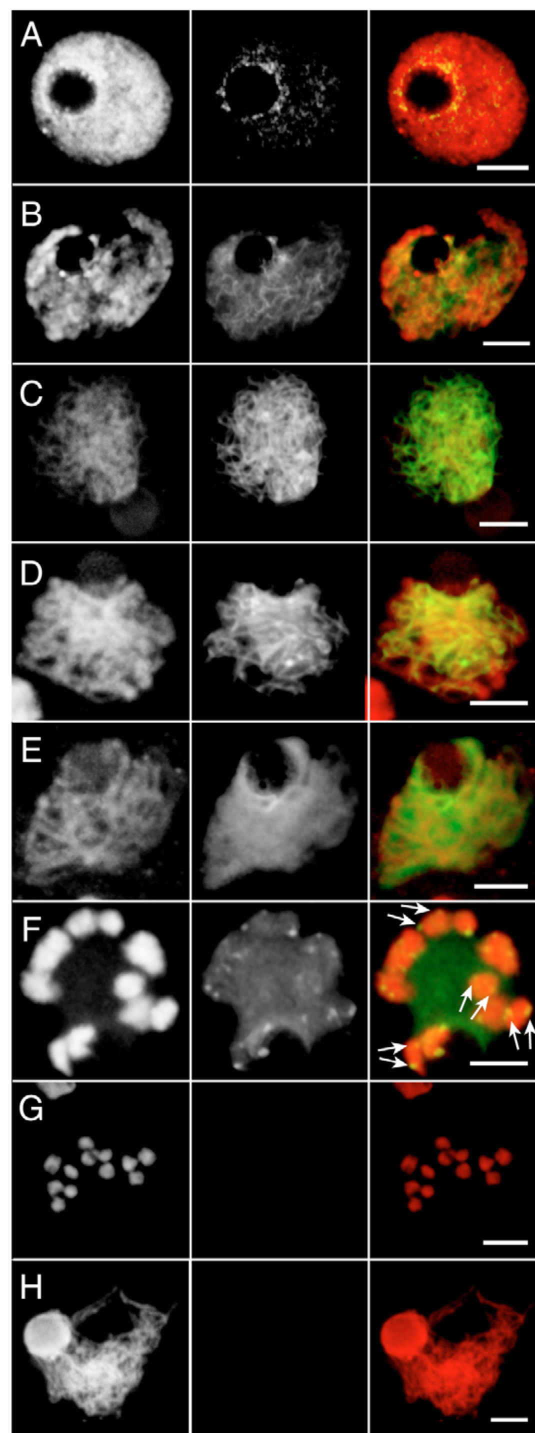

Fig. 3. PAIR2 associates with axial cores of meiotic chromosomes in wild-type (A-G) but not in *pair2*-null mutant PMCs (H). Panels from left to right indicate the localization of histone pan antibody (representing chromatin regions), the localization of PAIR2, and merged image of histone pan antibody (red) and PAIR2 (green). In all images, an unstained circular body within the PAIR2 signals represents the nucleolar region. (A) Pre-meiotic S/G2. (B) Filamentous PAIR2 signals begin to elongate at early leptotene. (C) Late leptotene. (D) Zygotene. (E) Pachytene. (F) Diakinesis. Pairs of arrows indicate pairs of the PAIR2 foci whose bilateral localization on bivalents is clearly detected. (G) Metaphase I. (H) Immunofluorescence against a zygotene PMC of the *pair2* mutant. Bars, 5 μm.

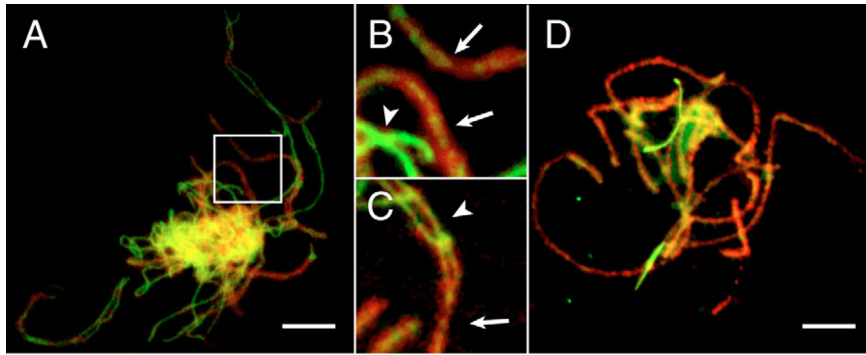

Fig. 4. PAIR2 localizes on the meiotic chromosome axis and delocalizes after synapsis is completed in zygotene and pachytene. (A) Spread chromosomes (red) and PAIR2 localization (green) in the late zygotene meiocyte with destructed nucleus. (B) A magnified image of a box in A. (C) Another magnified image of early pachytene chromosomes. Arrows in B and C indicate synapsed regions of homologous chromosomes, and arrowheads show unsynapsed regions. (D) Spread chromosomes in pachytene. Bars, 5 μm.

a filamentous structure on the chromosome, although their faint signals were also detected in the nucleoplasm (Fig. 3B). At the end of leptotene, the signals extended along the whole length of the chromosome axis, and those in the nucleoplasm were scarcely observed (Fig. 3C). In zygotene, PAIR2 filaments became thicker (Fig. 3D). During zygotene and pachytene, the stages at which the SC formation proceeds and is completed, the PAIR2 signals on the chromosome were diminished; instead, those in the nucleoplasm were increased (Fig. 3D,E). In diakinesis, most of the PAIR2 signal was observed in the nucleoplasm and partly on the restricted regions of all bivalents (Fig. 3F). Regions at which PAIR2 remained are described in the next section. In metaphase I, after the nuclear envelop breakdown, the PAIR2 signals completely disappeared (Fig. 3G). Through all stages described here, no PAIR2 signal was detected at the nucleolar region (Fig. 3).

The same antibody was also applied to *pair2* mutant PMCs in early zygotene, but provided no filamentous PAIR2 signal (Fig. 3H). This result indicates that the *pair2* mutant used here carries a null allele.

For further dissection of PAIR2 localization on the chromosome, localization was observed in spread chromosomes in meiocytes with destructed nuclei. On chromosome spreads in early zygotene, PAIR2 filaments were clearly detected along the entire chromosome axes (Fig. 4A). Although PAIR2 signals were observed on both synapsed and unsynapsed chromosomes, the PAIR2 signal in the central axis of synapsed chromosomes became intermittent and weaker than in unsynapsed regions (Fig. 4B,C). The pachytene meiocytes included synapsed homolog pairs, on which the PAIR2 signal was not clearly observed except for several distinct chromosomal regions (Fig. 4D). These observations correspond to the result of Fig. 3, in which the PAIR2 signals were diminished on chromosomes, and instead increased in the nucleoplasm during zygotene and pachytene. These results suggest that the PAIR2 protein is removed from the chromosome axis after accomplishing its role in SC establishment between homologs.

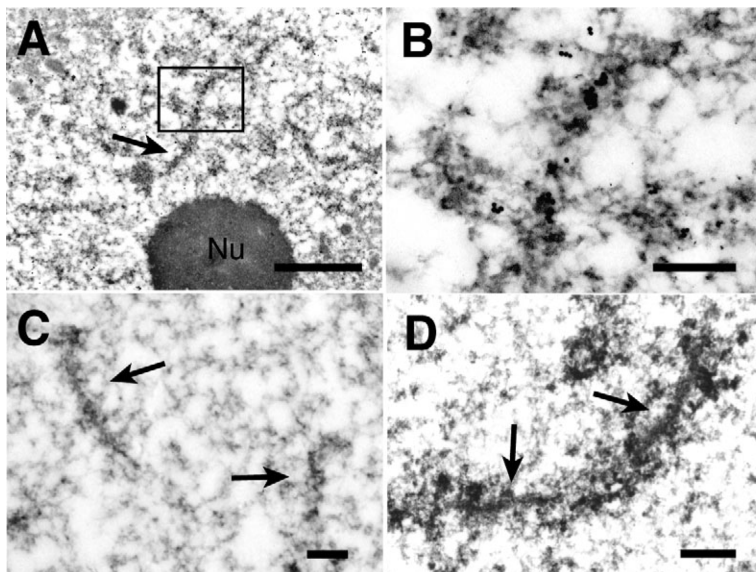

Fig. 5. EM observation of rice meiotic chromosomes. (A) Immunogold localization of anti-PAIR2 antibody in an ultrathin section of a wild-type meiocyte at zygotene. Arrows indicate the electron-dense and filamentous structure observed in the nucleus. Nu, nucleolus. Bar, 1 μm. (B) Magnified view of the box in A. Bar, 0.2 μm. (C) A negative control of A, excluding primary antibody. Bar, 1 μm. (D) EM observation of *pair2* mutant meiocytes. Filamentous and electron-dense chromosome axes (arrows) were observed to be the same as in the wild type (not shown). Bar, 0.2 μm.

An immunogold-labeled antibody was applied to ultrathin sections of meiocytes and observed by an electron microscope (EM). Uranyl/lead-stained filamentous structures, which generally represent AEs (Dobson et al., 1994), were observed at leptotene (Fig. 5A), and immunogold particles were frequently associated with the axis of the electron-dense filaments (Fig. 5B). A negative control excluding primary antibody gave no signal on the filaments (Fig. 5C). This suggests that PAIR2 associates with the axial core of chromosomes at leptotene and zygotene. However, it was not possible to define whether PAIR2 associated with the AEs or with the chromatin regions neighboring the AEs. Even in the *pair2*-null mutant meiocytes, electron-dense AE-like structures were observed in the nucleus at zygotene (Fig. 5D), indicating that PAIR2 is not directly required for constructing AEs in meiosis.

PAIR2 remains on centromeres until diakinesis, but does not affect centromere functions

Even after almost all PAIR2 signal had been removed from chromosomes at diakinesis, bilateral

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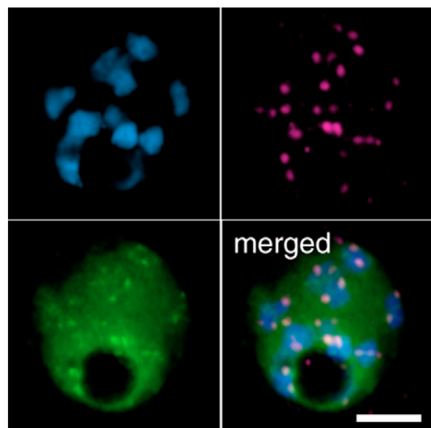

Fig. 6. A pair of PAIR2 spots remains until diakinesis on centromeric regions of each homologous pair. Chromosomes were counter-stained with anti-histone pan antibody (blue). The centromeric chromatin and PAIR2 were stained with anti-OsCenH3 (magenta) and anti-PAIR2 antibodies (green), respectively. Bar, 5 μm.

and symmetrical PAIR2 foci were clearly detected on all bivalents (Fig. 3F). Thus, we performed double staining with antibodies against PAIR2 and the centromere-specific histone H3 variant (OsCenH3). The anti-OsCenH3 antibody recognizes functional centromeric regions in somatic and meiotic rice cells (Fig. S1, supplementary material). Double staining revealed that the PAIR2 dots on every homolog pair completely overlapped the OsCenH3 foci at diakinesis in all 25 cells observed (an example is shown in Fig. 6). The same experiment was also carried out in pachytene. In the meiocytes with destructed nucleii, only faint PAIR2 signals were observed on restricted regions of the spread chromosomes, and those regions always covered the OsCenH3 signals (Fig. S2A, supplementary material). Islands of faint PAIR2 signals were occasionally observed at centromeric and peri-centromeric regions in all five cells observed (an example is shown in Fig. S2B, supplementary material). These observations indicate that, even after almost all PAIR2 proteins are removed from arm regions at pachytene, they continue to stay at centromeric and peri-centromeric regions until diakinesis.

Despite its specific localization on centromeres during pachytene and diakinesis, the PAIR2 protein did not contribute to any centromere functions. In anaphase I of the *pair2*-null mutant, a diploid chromosome number ($2n$=24) was detected, as in the wild type (Fig. 7A,B), indicating that *pair2* mutation affects neither precocious sister chromatid separation nor chromosome fragmentation in rice mieosis I. From the result of immunostaining, two sets of 12 univalents were captured by bipolar spindle microtubules at centromeres, and divided equally and synchronously into both poles in wild-type anaphase I (Fig. 7C). In ten PMCs of the *pair2*-null mutant observed, all univalents, even lagging ones at midzone, were captured by spindle microtubules at centromeric regions at anaphase I (Fig. 7D,E). These results indicate that the PAIR2 function is not required for the sister chromatid cohesion at centromeres or for kinetochore assembly in rice meiosis I. Interestingly, in mid anaphase I, the almost-lagging univalents at midzone attached to microtubules from both poles (Fig.

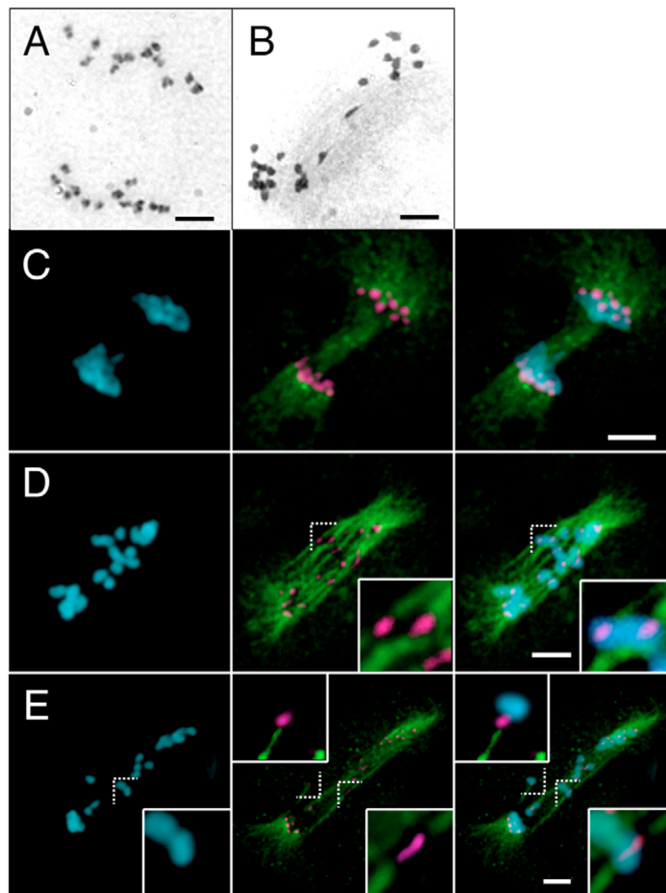

Fig. 7. PAIR2 function is not required for sister chromatid cohesion and kinetochore assembly in rice meiosis I. (A,B) Spread and Giemsa-stained meiotic chromosomes of the wild-type (A) and *pair2*-null mutant (B) at anaphase I. In both meiocytes, a diploid number ($2n$=24) of univalents was detected. (C-E) Spindle microtubules and chromosomes were stained with anti-α-tubulin antibody (green) and anti-histone pan antibody (red), respectively, in the wild-type (C) and *pair2* mutant (D,E) meiocytes. Even lagging univalents at midzone were captured by spindle microtubules at mid anaphase I (two univalents showing bipolar attachment are magnified in the bottom right of D). In E, at late anaphase I, a single OsCenH3 focus attached to bipolar microtubules is elongated towards both poles (magnified in the bottom right), and a univalent shows monopolar microtubule attachment (magnified in the top left). Bars, 5 μm.

7D). Most of the univalents shifted to monopolar attachment until late anaphase I; however, several univalents still exhibited bipolar attachment, in which a centromeric OsCenH3 focus was elongated polewards (Fig. 7E). The results shown here suggest that a single kinetochore assembled on a univalent enables bipolar attachment to spindle microtubules.

Discussion

PAIR2 is a good marker to discriminate early meiotic events in plants

In this study, the HORMA-domain protein PAIR2 of rice is revealed to associate with meiotic chromosome axes. This is the third meiotic HORMA protein reported in the plant

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kingdom, following *Arabidopsis* ASY1 and *Brassica* BoASY1 (Armstrong et al., 2002). An advantage of using an anti-PAIR2 antibody in cytological analyses is to facilitate discrimination between the stages of pre-meiosis and early meiosis I in rice. Pre-meiotic S phase is linked closely to several important meiotic events such as reductional chromosome segregation, and homolog pairing and recombination (Martinez-Perez et al., 1999; Watanabe et al., 2001). In addition, stages in early meiosis I are also important for homology recognition. This study revealed that the PAIR2 protein begins to accumulate in the nucleus of meiocytes following the onset of pre-meiotic DNA synthesis (Fig. 2). This means that a single staining with PAIR2 antibody, without BrdU incorporation and immunostaining, is enough to determine the approximate timing of pre-meiotic S initiation in rice meiosis. Furthermore, leptotene can be separated into early and late stages according to the degree of elongation of the PAIR2 filamentous structure. These findings will contribute to dissecting the molecular mechanism of homology recognition and to characterize meiotic mutants of rice.

Function of PAIR2 protein

From the results of immunofluorescent and immunogold analyses, we conclude that PAIR2 associates with unpaired chromosome axes at early meiosis I (Figs 4, 5). This conclusion is supported by the observation that the PAIR2 filamentous structure is extended during leptotene (Fig. 3B), which is the stage at which AEs develop along unsynapsed sister chromatid pairs, and that full association of PAIR2 with the entire chromosomal axis is established at the end of leptotene (Fig. 3C). During zygotene, the stage at which synapsis proceeds by establishing TFs and CEs between homologous AE pairs, filamentous PAIR2 signals observed between synapsed homolog pairs became weaker and less continuous than those on unsynapsed chromosomes (Fig. 4B,C). One possible explanation for the diminished PAIR2 intensity at synapsed regions is that accessibility of the PAIR2 antibody to the chromatin declines as a result of progression of chromosome condensation and/or SC establishment. Another explanation is that an as-yet-unknown signaling pathway degrades the PAIR2 proteins during SC completion. However, during the same stages, the amount of the PAIR2 signal gradually increases in the nucleoplasm instead of its gradual decrease on the chromosome axis (Fig. 3C-E). Thus, the most likely story is that the PAIR2 proteins are removed quickly from the chromosomal axes when SC formation is completed. This speculation corresponds well with earlier observations in yeast that HOP1 dissociates from chromosomes before late pachytene (Smith and Roeder, 1997). Taken together, PAIR2 function is thought to promote synapsis by recruiting TFs, CEs, or both, between homologous AEs. Localization of HOP1 to chromosomal axes depends on RED1 localization to the AEs in *S. cerevisiae* (Smith and Roeder, 1997). Although a RED1 homolog has not yet been identified in rice, similar behavior of HORMA proteins in yeast and rice strongly suggests that a functional ortholog of RED1 also joins AEs, and controls the association and dissociation of PAIR2 with AEs in rice meiosis. However, for further analyses, we must wait for new markers to be able to detect central components of plant SCs.

Punctuate PAIR2 foci were detected in pre-meiotic S/G2 cells (Fig. 2A, Fig. 3A). In *Arabidopsis* and *Brassica*, punctuate ASY1 foci are also detected at meiotic interphase (Armstrong et al., 2002). In *Brassica* cells, punctuate signals mix with some stretches of more-continuous signal during leptotene. Thus, the authors speculate that punctuate foci show initial points of ASY1 elongation, or that meiotic chromosome condensation causes fusion of the punctuate foci during leptotene (Armstrong et al., 2002). This study suggests that the PAIR2 kinetics in pre-meiosis and early prophase I is almost the same as that of ASY1.

PAIR2 function is essential for synapsis but not for AE formation because the *pair2*-null mutant also assembles AEs (Fig. 5D). It is also supported by the appearance of normally condensed and unsynapsed chromosomes in *pair2* mutant meiocytes (Nonomura et al., 2004b). This observation is consistent with the observation that the *S. cerevisiae hop1* mutant also displays extensive AE development (Hollingsworth and Byers, 1989; Loidl et al., 1994).

PAIR2 protein is not required for centromere functions

PAIR2 proteins remained at centromeres in pachytene and diakinesis; by contrast, few PAIR2 signals have been observed at arm regions (Figs 6, 7). A unique aspect of PAIR2 behavior on the centromere is the first case reported for a meiotic HORMA-domain protein among eukaryotes. HOP1 of *S. cerevisiae* dissociates completely from the chromosomes before late pachytene (Smith and Roeder, 1997). The smallest 125 bp centromere unit of *S. cerevisiae* (Cottarel et al., 1989) might interfere with detection of the details of HOP1 kinetics. In meiocytes of *Arabidopsis* and *Brassica*, the behavior of ASY1 and BoASY1 is almost the same as that of rice PAIR2; however, no ASY1 signal is detectable at diakinesis (Armstrong et al., 2002). Thus, even in the plant kingdom, the dynamics of meiotic HORMA-domain proteins might be different among species. By contrast, HIM3 of *C. elegans* remains at the chromosome core after desynapsis (Zetka et al., 1999). It is difficult to compare the case in *C. elegans* to other model organisms because the chromosomes of *C. elegans* are holocentric. According to the conclusion of this study, HIM3 might continue to associate with the chromosome axis of dispersed centromeric regions after desynapsis. However, the HIM3 signal is observed only on the axial core, where homologous chromosomes face each other during diakinesis of *C. elegans* oocytes (Zetka et al., 1999), whereas pairs of PAIR2 foci exhibit bilateral symmetry and are apart from each other on all bivalents (Fig. 3F). Furthermore, the association with spindle microtubules is restricted to one end of each pair of sister chromatids in *C. elegans* meiosis (Albertson and Thomson, 1993), indicating monocentric behavior of meiotic chromosomes. Thus, the system of retaining the meiotic HORMA-domain proteins on the chromosome axis after desynapsis might be different between *C. elegans* and rice.

During transition from metaphase I to anaphase I, sister chromatid cohesion is generally lost at chromosome arms but maintained at centromeres to allow proper reductional segregation of homolog pairs (Suja et al., 1992; Miyazaki and Orr-Weaver, 1994; Petronczki et al., 2003). In the *pair2* mutant meiocytes, no precocious sister chromatid separation is observed (Fig. 7B). In addition, kinetochore function seems normal in the mutant, because all homolog pairs are captured by spindle microtubules at centromeric regions and divided into either of both poles (Fig. 7D,E), although non-disjunction

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events are frequently observed (Fig. 7B,D,E). This suggests that PAIR2 is not required for centromeric cohesion or kinetochore assembly in rice meiosis. Thus, we conclude that, as suggested in *Arabidopsis* and *Brassica*, the meiotic HORMA-domain proteins of higher plants temporally associate with the meiotic chromosome axis and possess distinct functions from the integral AE components. This is also supported by the finding that the *pair2* mutant meiocyte assembles normal AEs (Fig. 5D). In *C. elegans*, Zetka et al. (Zetka et al., 1999) propose that HIM3 is an integral component of the chromosome core and might act to promote arm cohesion within bivalents to stabilize chiasmata and establish a tension at the metaphase I plate. Meiotic HORMA-domain proteins certainly conserve an essential role in homolog synapsis of eukaryotic meiosis; however, their roles after desynapsis are unclear and are difficult to be ruled out among model organisms.

This study clearly demonstrated that lagging univalents carrying a single OsCenH3 focus connected with bipolar microtubules at anaphase I in *pair2* mutant meiocytes (Fig. 8D). Formation of the normal metaphase plate is seldom observed in the *pair2* mutant (data not shown); by contrast, in rare cases, the rice *pair1* mutant, which also lacks homolog synapsis, forms the metaphase I plate (Nonomura et al., 2004a). Both rice mutants can form abnormal but bipolar spindles (Fig. 8D,E) (Nonomura et al., 2004a). These results support the idea in maize that homolog pairing and bilaterally symmetrical kinetochores are not required for bipolar spindle formation and chromosome alignment at the spindle midzone of plant meiosis I (Chan and Cande, 1998). In this study, bipolar attachment of univalents at early and mid metaphase I gradually shifted to monopolar attachment at late anaphase I (Fig. 8E). It has been proposed that proper attachment and alignment at the metaphase plate is followed by tension from pulling forces towards opposite poles and that tension is monitored by the checkpoint mechanism (Li and Nicklas, 1995). Thus, it is suggested in the *pair2* mutant that a single kinetochore captured by bipolar microtubule bundles is pulled towards opposite poles, and that the breakage of either of the bipolar microtubules results in monopolar attachment. Bipolar attachment of a single kinetochore might contribute to a path through the metaphase checkpoint in the *pair2* mutant.

A possible explanation for PAIR2 remaining on centromeres is that the components necessary to delocalize PAIR2 have difficulty accessing the chromosome axes as a result of the centromeric higher structure. Centromeres are composed of centromeric DNA that is packaged with histones and other proteins to form a specialized type of chromatin (reviewed by Amor et al., 2004). As for other eukaryotes, the rice centromere is composed of many reiterated sequences commonly involved in all chromosomes (Dong et al., 1998; Nonomura and Kurata, 2001; Cheng et al., 2002; Nagaki et al., 2004; Wu et al., 2004; Zhang et al., 2004). OsCenH3-binding regions have abundant dimethylation of histone H3 at Lys9 and are frequently linked to gene inactivation in rice centromeres (Nagaki et al., 2004). These findings suggest that the centromeric heterochromatin of rice also forms a complex structure as in other eukaryotes. In *S. cerevisiae*, HOP1 localization to meiotic chromosomes depends on prior RED1 localization (Smith and Roeder, 1997), and RED1 is dephosphorylated by GLC7 phosphatase and delocalized from chromosomes around the end of pachytene (Bailis and Roeder, 2000). If dephosphorylation of rice RED1 is insufficient as a result of the centromere structure being inaccessible to GLC7, then PAIR2 might fail to dissociate from the pericentromeric heterochromatin.

The meiotic centromere undergoes unique chromatin modification and sister chromatid cohesion. The pattern of histone H3 modification differs between plants and animals at meiosis (Manzanero et al., 2000). The unique behavior of rice PAIR2 on the centromere observed in this study could be related to a unique histone modification of plants. Further dissection of plant meiosis by molecular-based analyses will help understanding of the mechanism of faithful synapsis and sorting of homologous chromosomes in eukaryotes.

Materials and Methods

Plant material and genetics

Anthers of *O. sativa* cv. Nipponbare (2*n*=24) were used for western blot and immunocytological analyses. For immunocytology, a *pair2* mutant from the fifth generation after regeneration, which was derived from a *Tos17*-tagged line NC0122 (Nonomura et al., 2004b), was also used. All materials were grown in a field in the city of Mishima, Shizuoka, Japan, or in a greenhouse at 30°C during the day and 24°C at night.

Antibody production

The entire coding region of *PAIR2* cDNA (DDBJ Accession No. AB109238) (Nonomura et al., 2004b) was amplified by PCR using primers P609 (5′-CACCATGGTGATGGCTCAGAAGACGAAG-3′) and P610 (5′-TCACTGAA-CTTGAACTTGAACTTGGGAC-3′). The PCR product was cloned into the pENTR-TOPO plasmid and re-inserted into pDEST17 with a 6× histidine (6×His) repeat at the 5′-end of the multiple cloning site using the Gateway system (Invitrogen). After transformation of *Escherichia coli* strain BL21-AI (Invitrogen) with the plasmid, expression of the fusion protein was induced by adding L-arabinose to a final concentration of 0.2% (w/v) in LB liquid culture.

The clone E30313, carrying the OsCenH3 cDNA in pBluescript II SK+, was kindly provided by T. Sasaki, Ministry of Agriculture, Forestry and Fisheries (MAFF) DNA Bank, Tsukubu, Japan. This clone is identical to the CenH3 cDNA (GenBank accession No. AY438639) used by Nagaki et al. (Nagaki et al., 2004). The sequence encoding the 41 N-terminal amino acids of OsCenH3 (AEPKKKLQFERSPRPSKAQRAGGGTGTSATTRSAAGTSASG) was used to generate a GST-fusion peptide. The fragment was amplified by PCR using primers, Tz1 (5′-GGAATTCCCGCGGAGCCCAAGAAGAAGC-3′) and Tz2 (5′-GAGTCGACCCTGAAGCCGATGTTCCAG-3′), then digested with *Eco*RI and *Sal*I, and ligated between the *Eco*RI and *Sal*I sites of the GST-tagged protein expression vector pGEX-6P-2 (Amersham Biosciences). Expression of the fusion peptide was induced in *E. coli* BL21(DE3) by addition of 0.1 mM IPTG to the culture medium.

The recombinant peptides expressed were extracted 4 or 6 hours after induction using the BugBuster protein extraction reagent (Novagen). The 6×His- and GST-tagged peptides in the soluble fraction were purified using a HisTrap kit and glutathione Sepharose 4B (both from Amersham Biosciences), respectively, according to the manufacturer's instructions. 250 μg of PAIR2 and 750 μg of OsCenH3 recombinant peptides were injected into a rabbit and guinea pig every two weeks, respectively. Immune sera were extracted 52 days after the first injection.

Western blotting

Proteins from plant tissues were extracted in 2% SDS, 6% β-mercaptoethanol, 10% glycerol and 50 mM Tris-HCl (pH 6.8), and insoluble materials were removed by centrifugation. Protein samples were separated by SDS-PAGE on a 7.5% polyacrylamide gel and electroblotted onto Hybond-P PVDF membrane (Amersham). Western blots were incubated with anti-PAIR2 antiserum diluted 1/5000 followed by anti-rabbit IgG antibodies conjugated to horseradish peroxidase (Amersham) diluted 1/25,000. Signals were detected by the ECL Plus detection system (Amersham). An anti-rat α-tubulin monoclonal antibody OBT0614S (Oxford Biotechnology) was used as a positive control.

Indirect immunofluorescence

Young panicles containing PMCs entering into meiosis were fixed with 4% (w/v) paraformaldehyde (PFA) in PMEG buffer (25 mM PIPES, 5 mM EGTA, 2.5 mM MgSO4, 4% glycerol, and 0.2% DMSO, pH 6.8) for 3 hours, washed six times with PMEG for 20 minutes each, and stored at 4°C. Using a single anther of a floret, the meiotic stage of the PMCs was determined by an acetic-carmine squashing method. The remaining five anthers in appropriate meiotic stages were incubated in a 20:0.75

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mixture of an enzyme cocktail with 100 mg/ml cytohelicase (Sigma) for 20 minutes at 37°C, and another 5 minutes at 4°C. The enzyme cocktail contained 2% cellulase Onozuka-RS (Yakult Honsha, Japan), 0.3% pectolyase Y-23 (Kikkoman) and 0.5% Macerozyme-R10 (Yakult Honsha) in PMEG (pH 6.9). The anthers were washed five times with PMEG on a poly-L-lysine-coated glass slide, and squashed in PMEG by a needle. After the cell debris was removed, the cell suspension was covered by a cover slip. The slip was removed on dry ice, and the samples were air-dried. The slide was washed three times with PMEG for 5 minutes each, blocked with 3% BSA (Sigma) in PMEG for 30 minutes followed by a PMEG wash for 5 minutes, and used for antibody staining.

The slide was incubated at 4°C overnight with rabbit anti-PAIR2 antibody and guinea pig anti-OsCenH3 antisera, diluted 1/3000 and 1/2000, respectively, with 3% BSA/PMEG. After three washes with PMEG for 5 minutes each, the slide was incubated in a dark chamber for 3 hours at room temperature with Alexa Fluor 488-conjugated anti-rabbit IgG and Alexa Fluor 568-conjugated anti-guinea pig IgG (both from Molecular Probes) diluted 1/200 with 3% BSA/PMEG, followed by three washes with PMEG for 5 minutes each. Then, 40 μg/ml of propidium iodide (Sigma) or 4′,6-diamidino-2-phenylindole (DAPI) (Sigma) in Vectashield solution (Vector) was applied to counter-stain the chromatin or, alternatively, the chromatin was immunologically stained with mouse anti-histone pan antibody (1/1000 dilution; Roche) and anti-mouse IgG-Cy5 conjugate (1/200 dilution; Amersham). The signals were observed using a Fluoview FV300 CLSM system (Olympus). Captured images were enhanced and pseudo-colored by Photoshop 7.0 software (Adobe).

To stain spindle microtubules, monoclonal antibody against rat-α-tubulin subunit (OBT0614S; Oxford Biotechnology) was used. A condition to stain the spindle has been described previously (Nonomura et al., 2004a).

BrdU incorporation and detection

Fresh young panicles of 3-6 cm in length were cut from stems and placed in 100 μM BrdU solution in the dark for 4 hours. The panicles were fixed with 4% PFA/PMEG as described above. Anthers 0.3-0.5 mm long were isolated from flowers 1.5-2.0 mm long, which often included meiocytes from pre-meiotic S phase to early meiosis (Nonomura et al., 2004b), and then digested with the enzyme mixture used for PMC preparation on a poly-L-lysine-coated glass slide as described above. Mouse anti-BrdU monoclonal antibody (Becton Dickinson) was diluted 1/3000 and used to detect incorporated BrdU in the PMCs.

Electron microscope observation and immunogold localization

For transmission EM, a flower including the PMCs at zygotene was fixed with 2.5% glutaraldehyde/PBS at 4°C for 2 hours. After five washes with PBS at 4°C for 1 hour each, samples were incubated at 4°C overnight to remove the glutaraldehyde completely. Flowers were briefly washed three times with PBS, fixed with 1% osmium tetroxide/PBS at room temperature for 1 hour, dehydrated in an ethanol series, and embedded in Epon resin. For immunogold localization, a flower was fixed with 0.1% glutaraldehyde/4% PFA/0.1 M phosphate buffer for 3 hours, then washed three times with 0.14 M sucrose/0.1 M phosphate buffer. Samples were incubated at 4°C overnight, then dehydrated through an ethanol series and embedded in LR White acrylic medium (Polysciences). After standard procedures to make ultrathin sections for EM observation, the sections were mounted on nickel mesh grids.

For immunogold localization, sections were washed with PBS for 10 seconds and with 1%BSA/PBS for 30 minutes, and incubated in 1/100 diluted anti-PAIR antiserum/1%BSA/PBS at 4°C overnight. After three washes with PBS for 30 minutes each, sections were incubated in a 1/10 dilution of the goat anti-rabbit IgG, and conjugated with a 20 nm gold colloidal particle (EY laboratories) in 1% BSA/PBS at 4°C for 2 hours. After three washes with PBS for 30 minutes each, sections were fixed with 1% glutaraldehyde/PBS for 10 minutes, washed with distilled water five times for 5 minutes each and air-dried. Samples were stained with saturated uranyl acetate for 2 minutes followed by lead citrate for 5 minutes, as described by Dobson et al. (Dobson et al., 1994). Micrographs were obtained with a JEM100S EM (JOEL).

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