Meiotic cohesin subunits RAD21L and REC8 are positioned at distinct regions between lateral elements and transverse filaments in the synaptonemal complex of mouse spermatocytes

Mei RONG\textsuperscript{1)}, Atsushi MATSUDA\textsuperscript{2, 3), Yasushi HIRAOKA\textsuperscript{2, 3) and Jibak LEE\textsuperscript{1)}

\textsuperscript{1) Laboratory of Developmental Biotechnology, Graduate School of Agricultural Science, Kobe University, Kobe 657-8501, Japan
\textsuperscript{2) Advanced ICT Research Institute Kobe, National Institute of Information and Communications Technology, Kobe 651-2492, Japan
\textsuperscript{3) Graduate School of Frontier Biosciences, Osaka University, Suita 565-0871, Japan

Abstract. Cohesins containing a meiosis-specific \(\alpha\)-kleisin subunit, RAD21L or REC8, play roles in diverse aspects of meiotic chromosome dynamics including formation of axial elements (AEs), assembly of the synaptonemal complex (SC), recombination of homologous chromosomes (homologs), and cohesion of sister chromatids. However, the exact functions of individual \(\alpha\)-kleisins remain to be elucidated. Here, we examined the localization of RAD21L and REC8 within the SC by super-resolution microscopy, 3D-SIM. We found that both RAD21L and REC8 were localized at the connection sites between lateral elements (LEs) and transverse filaments (TFs) of pachynema with RAD21L locating interior to REC8 sites. RAD21L and REC8 were not symmetrical in terms of synaptic homologs, suggesting that the arrangement of different cohesins is not strictly fixed along all chromosome axes. Intriguingly, some RAD21L signals, but not REC8 signals, were observed between unsynapsed regions of AEs of zygonema as if they formed a bridge between homologs. Furthermore, the signals of recombination intermediates overlapped with those of RAD21L to a greater degree than with those of REC8. These results highlight the different properties of two meiotic \(\alpha\)-kleisins, and strongly support the previous proposition that RAD21L is an atypical cohesin that establishes the association between homologs rather than sister chromatids.

Key words: Cohesin, Meiosis, Recombination, Synapsis, The synaptonemal complex

Meiosis is a special type of cell division that is required for sexual reproduction, in which the chromosome number is halved by two successive meiotic divisions following a single round of DNA replication. Meiosis I differs from mitosis or meiosis II; during meiosis I, homologous chromosomes (homologs) segregate from each other, whereas in meiosis II, sister chromatids segregate. To ensure successful homolog segregation in meiosis I, it is a prerequisite for homologs to establish a connection with their partners. This is achieved by three meiosis-specific events that occur during prophase I, namely pairing, synapsis, and recombination of homologs \cite{1, 2}. These events occur in parallel with the assembly of a tripartite structure called the synaptonemal complex (SC). SC assembly is initiated by the formation of axial elements (AEs) along each chromosome during the leptotene stage. Two AEs of homologous chromosomes begin to synapse through a connection between transverse filaments (TFs) during the zygotene stage, and complete synapsis along the entire length of chromosomes by the pachytene stage. The two AEs connected by TFs are called lateral elements (LEs). Finally, during the diplotene stage, the SC starts to disassemble \cite{2, 3}.

In eukaryotes, meiotic recombination is initiated by the generation of DNA double-strand breaks (DSB), which is catalyzed by SPO11 \cite{4–7}. The generation of DSBs causes a DNA damage response, which is accompanied by the phosphorylation of histone variant H2AX (\(\gamma\)-H2AX) \cite{8}. DSBs are resected to generate 3’ single-stranded overhangs, to which DNA repair proteins such as RAD51 and DMC1 are recruited \cite{9}. Single-stranded DNA then invades the double-stranded DNA of the homolog. As meiotic recombination proceeds, the early recombination intermediates, containing RAD51 and DMC1, are replaced by middle intermediates containing MSH4, and subsequently by late intermediates containing MLH1 in a step-wise manner \cite{10, 11}. During this process, double Holliday junctions are formed and ultimately resolved as either crossover or non-crossover recombinations \cite{12}. After SC disassembly, crossover recombination plays an important role in maintaining the connection between homologs until anaphase I, with the help of sister chromatid cohesion distal to the chiasmata.

Cohesin, a multi-subunit protein complex that is well conserved from yeasts to mammals plays a pivotal role in sister chromatid cohesion \cite{13, 14}. The cohesin complex consists of four different subunits: two structural maintenance of chromosome subunits (SMC1 and SMC3), an \(\alpha\)-kleisin subunit (RAD21), and either SA1/STAG1 or SA2/STAG2 \cite{15–17}, forming a ring-like structure in which...
sister chromatids could be held [18, 19]. Mammals have several meiosis-specific paralogs of cohesin subunits, including SMC1β [20], REC8 [21, 22] or RAD21L [23, 24], and STAG3 [25]. Meiotic cohesins function not only in sister chromatid cohesion but also for formation of AEs, assembly of the SC, and crossover recombination during prophase I [26–33]. Considering that two meiotic α-kleisins, RAD21L and REC8, are conserved among many vertebrate species, it is reasonable to speculate that each of these subunits has its own function in meiotic chromosome dynamics. In fact, there are some phenotypic differences between Rad21L KO and Rec8 KO mice. Rad21L KO mice show sexual dimorphism in fertility [31], whereas both sexes of Rec8 KO mice are infertile [28]. In addition, synopsis occurs between non-homologous chromosomes in Rad21L KO mice [31], whereas synopsis occurs mainly between sister chromatids in Rec8 KO mice [29]. Therefore, it seems that RAD21L or REC8 has specific and distinct important roles in different aspects of chromosomal events. However, their precise roles remain to be elucidated. To investigate their individual functions, further characterization of these two meiotic α-kleisins is needed.

In the present study, we examined the localization of two meiotic α-kleisins in the SC at high resolution by three dimensional structured illumination microscopy (3D-SIM). 3D-SIM has a resolution of 120 nm along the x-y directions, and is advantageous for the localization of molecules of interest with a great robustness in sample preparation [34]. Moreover, immunoreactivity is generally better preserved in the samples fixed with paraformaldehyde and used for 3D-SIM than in those fixed with glutaraldehyde commonly used for electron microscopy [35]. Thus, 3D-SIM is suitable for analyzing the precise localization of proteins in the SC. Here, we showed for the first time that both RAD21L and REC8 are located at the connection sites between AEs and TFs. We also identified the different properties of RAD21L and REC8, and finally proposed potential roles for these individual cohesin subunits.

Materials and Methods

Mice

C56BL/6J mice were used for the analysis of chromosomal localization of meiotic cohesins. This study was approved by the Institutional Animal Care and Committee (Permission number: 25-03-02) and performed according to the Kobe University Animal Experimentation Regulation.

Antibodies

The primary antibodies used in this study were as follows: rabbit and rat polyclonal anti-RAD21L antibodies [24], rabbit polyclonal anti-REC8 [24], mouse polyclonal anti-REC8 antibody [22], mouse polyclonal anti-SYCP3 antiseraum [22], rabbit polyclonal anti-MSH4 antibody (ab58666, Abcam, Cambridge, MA, USA), rabbit polyclonal anti-SYCP1 antibody (ab15087, Abcam), and rabbit polyclonal anti-RAD51 antibody (sc-8349, Santa Cruz Biotechnology, Dallas, TX, USA). The antigen-antibody complexes were detected by appropriate secondary antibodies conjugated with Alexa Fluor 488 or 568 (Molecular Probes, Eugene, OR, USA).

Immunofluorescence analysis

A testicular cell suspension from adult mice was prepared according to the method described by Heyting and Dietrich [36]. The cells were placed on poly-L-lysine-coated coverslips and fixed with 2% paraformaldehyde in buffer H [120 mM HEPES-KOH (pH 7.6), 30 mM HEPES-NaOH, 2 mM EDTA-3Na, 0.5 mM EGTA, 0.5 mM spermidine 3HCl, 0.2 mM spermine 4HCl, 15 mM 2-mercaptoethanol] for 10 min [37]. Testicular cells on coverslips were permeabilized with 0.2% triton X-100 in PBS for 5 min, and washed in PBS three times for 10 min each. The cells were incubated with primary antibodies at appropriate dilutions in blocking buffer at 4°C overnight. Cells were then washed in PBS for 10 min, in detergent solution for 10 min, and in PBS for 10 min. Cells were then incubated with secondary antibodies at appropriate dilutions in blocking buffer. DNA was counterstained with DAPI (Wako, Osaka, Japan). Samples were mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). Images were acquired with a DeltaVision OMX microscope (GE Healthcare, Buckinghamshire, UK) with a 100 × UPlanSAp2 NA1.40 oil immersion objective lens (Olympus, Tokyo, Japan) for 3D-SIM or with a confocal laser scanning microscope (LSM; Olympus, Lake Success, NY, USA) with a Plan Apochromat 10 × and 100 × /1.46 oil DIC objective lens. All Images were processed with DeltaVision SoftWorx software (GE Healthcare). Some of the images shown were projected views of z-stacks.

Image analysis

For every measurement, images were acquired as z-sections encompassing the entire nuclei. To measure the distance between two signals on the SC, 3D images of synapsed chromosomes were linearized (Fig. 1H). For linearization, binary images were first made by automatic iterative thresholding [37]. Next, individual SCs in binary 3D images were identified by our program using functions from the “ndimage” module of the Scipy package (http://www.scipy.org). Subsequently, the lines of the SC in binary, single-color images were traced with the “Trace” function in Priism suite (http://msg.ucsf.edu/IVE/). Finally, using the “Trace” output function as the model file, the original multicolor 3D images were linearized using the “Straight” function of Priism suite. The distance between two fluorescence peaks on the synaptic LEs was measured in at least five areas. The statistical significance between different samples was tested using a Student’s t-test.

Co-localization (overlap) was analyzed in individual nuclei of 3D-SIM images. We used automatic thresholding (mentioned previously) with the same value for the “delta” coefficient for automatic thresholding to keep the threshold visually constant for all images [37]. Then, using the threshold, Mandars co-localization coefficients [38] were measured. The ratios of the α-kleisin-recombination intermediates-double-positive areas (overlapping areas) to the recombination intermediates-positive areas were calculated and are shown in Fig. 4. We corrected chromatic shifts using our adaptive image registration algorithm [37]. The statistical significance between different samples was tested using a Student’s t-test.
Results

The arrangement of SYCP3 and SYCP1 in the pachytene stage of mouse spermatocytes visualized by 3D-SIM

To identify the framework structure of SCs at the resolution of our imaging system using 3D-SIM, and to validate the experimental workflow, we first examined the localization of SYCP3 and SYCP1, which are well-defined components of AEs/LEs and TFs, respectively, of the SC [39–41]. At the pachytene stage, when homologs were completely synapsed, SYCP3 and SYCP1 signals were observed as a single line on each bivalent using a confocal laser-scanning-microscope (LSM) (Fig. 1A and C). In contrast, SYCP3 signals
were observed as two distinct lines representing LEs by 3D-SIM (Fig. 1B), as observed in previous 3D-SIM analyses in animal and plant meiocytes [42, 43]. SYCP1 signals were also observed as two lines on each bivalent (Fig. 1D), which was unexpected as SYCP1 is localized along the TFs formed between LEs. Because SYCP1 forms a homodimer through its N-terminus and since the anti-SYCP1 antibody used was raised against a C-terminal peptide, the two separate lines indicated that we were able to resolve the C-terminal ends of the SYCP1 homodimer. Thus, the spatial resolution in our study was high enough to distinguish two ends of a single SYCP1 homodimer. From 3D images, we could observe that such fine structures of SYCP1 and SYCP3 were oriented in various directions with curving and twisting in the nucleus of pachytene spermatocytes (Fig. 1E–G).

To analyze the structure of SCs in more detail, 3D images were computationally linearized (Fig. 1H and I). This facilitated the measurement of spacing between two linear signals of a protein of interest and could be applicable to a quantitative analysis (Fig. 1H and J). In this way, we measured the spacing between synapsed LEs (two linear SYCP3 signals) and between both ends of the TFs (two linear SYCP1 signals) and compared these values. Since the meiotic chromosome axes were twisting, the spacing between signals on paired homologs was measured at the widest regions of the helices (indicated by scale marks in Fig. 1H and J). As expected, the spacing of SYCP1 signals (mean value ± SD = 0.157 ± 0.004 µm) was significantly shorter than that of SYCP3 signals (0.249 ± 0.008 µm) (n = 98 homologs, P < 0.01) (Fig. 1I). Because differences in fluorescent emission wavelengths might influence the measurement, we repeated the experiments after swapping fluorophores on secondary antibodies. Regardless of the wavelengths tested, similar results were obtained: the spacing of SYCP3 was 0.245 ± 0.004 µm and the spacing of SYCP1 was 0.154 ± 0.005 µm (n = 72 homologs, P < 0.01) (Fig. 1J and K). This proved that our measurements were not affected by the specific fluorophores and that the results were reproducible within approximately 5 nm.

**RAD21L is interior to REC8 in the SC**

It has been shown that all cohesin subunits examined so far are localized along the AEs/LEs during mammalian meiosis [20–25, 44, 45]. However, their precise localization in the SC remains elusive. Therefore, we examined the precise positioning of RAD21L and REC8 in the SC. Mouse spermatocytes were immunofluorescently labeled with antibodies against α-kleisins (RAD21L or REC8) and recombination intermediate molecules (RAD51 or MSH4), and were analyzed by 3D-SIM. During the zygotene stage, some MSH4 and RAD51 signals were detected between synaptic AEs, and thus appeared to overlap with RAD21L or REC8 signals (Fig. 4A–D). To further investigate this localization, we calculated the ratio of overlapping areas (α-kleisins and recombination intermediates-double-positive areas) to recombination intermediates-positive areas. We discovered that the ratio of overlapping areas of MSH4-RAD21L (mean value ± SD = 0.440 ± 0.067) was significantly greater than that of MSH4-REC8 (mean value ± SD = 0.217 ± 0.041, n = 80 homologs, P < 0.01) (Fig. 4E). The ratio of overlapping areas of RAD51-RAD21L (mean value ± SD = 0.226 ± 0.075) was greater than that of RAD51-REC8 although this was not significant (mean value ± SD = 0.127 ± 0.051, n = 80 homologs) (Fig. 4F). These results suggest that RAD21L locates more closely to recombination intermediates than REC8.

**Discussion**

**The assembly of the SC with its constituent components and cohesins in mammals**

It is well known that the SC is assembled in two steps. First, AEs/LEs composed of SYCP2/SYCP3 heterodimers are formed for each homolog [39, 41, 46]. Then, TFs composed of SYCP1 molecules form a bridge between two AEs/LEs of paired homologs [47–49]. However, it remains elusive as to how AEs/LEs are connected by TFs because a direct interaction between LE and TF components has never been reported. Although previous studies using KO mice have suggested that RAD21L and REC8 function redundantly in the formation of cohesin cores that are essential for AE formation [32], little is known about how cohesins might function in SC assembly after AE formation. In the present study using 3D-SIM, we have shown for the first time the geometry of the meiotic α-kleisin subunits of cohesin, RAD21L and REC8, relative to AEs/LEs (SYCP3) and...
TFs (SYCP1) in the SC in mouse spermatocytes. Our study provides new evidence that both meiotic α-kleisins are localized to connection sites (interspace) between LEs and TFs with RAD21L localizing more centrally in the SC. The spacing between respective molecular axes in the SC is summarized in Fig. 5. RAD21L and REC8 are located at the innermost sides of two LEs of the pachynema, in agreement with a previous electron microscopy-based analysis [33]. Meiotic α-kleisins have been co-immunoprecipitated with SYCP3 and/or...
SYCP1 [22, 24, 33]. Therefore, when considering previous findings, our results suggest that cohesin cores are positioned at the connection sites and mediate associations between AEs and TFs during SC assembly. A recent study utilizing 3D-SIM in barley has revealed two different SC configurations that contain one or two axes of the TF component ZYP1 for each homolog [42]. However, in the present study, such different configurations of TFs were not observed. This discrepancy might be attributed to differences in TF components or in TF arrangements between these two species.

**The distinct roles of RAD21L and REC8 in meiotic chromosome dynamics**

The discovery of the second meiotic α-kleisin RAD21L, in addition to REC8, suggested that the two types of meiotic cohesins might play distinctive roles in specific aspects of meiotic chromosome dynamics. In this regard, at least after the diplotene stage, REC8 but not RAD21L should be responsible for sister chromatid cohesion because RAD21L is expressed only in meiotic prophase I until mid pachytene, whereas REC8 is present throughout meiosis up to metaphase II [22, 24]. In fact, using mice carrying TEV protease-cleavable REC8 or RAD21, it was demonstrated that REC8 is essential for maintaining sister chromatid cohesion at both centromeres and arm regions during meiosis [50]. In contrast, from leptotene to mid-pachytene, both subunits are expressed and localized to the SC; hence, it is not easy to distinguish their respective functions. In the present study, we found several differences between RAD21L and REC8, providing evidence to address this issue. RAD21L and REC8 were observed at different positions in the longitudinal axes of chromosomes,
and these homologs did not form a mirror image, suggesting that the chromosomal positions were flexible rather than fixed. If this is true, it argues against a symmetric distribution of two types of α-kleisin along chromosomal axes that contributes to the recognition of homologs for pairing and synapsis [23]. However, the sparseness of antibody labeling using fixed samples is often a major limitation for 3D-SIM in visualizing the entire fraction of molecules present in cells. Although it is difficult to label all molecules without affecting the physiology of the cell, these results need to be clarified. We also observed bridge-like signals for RAD21L, but rarely for REC8, between unsynapsed AEs spaced ~400 nm apart (Fig. 3; Supplementary movies S1, S2, and S3); these structures are reminiscent of inter-axis bridges corresponding to sites of DSB-mediated interhomolog interactions that are formed when homologs are co-aligned in various organisms [2]. Thus, it is probable that RAD21L is localized to the DSB-mediated interhomolog bridges of zygonema. In support of this notion, RAD21L was observed closer to recombination intermediates than REC8 (Fig. 4). It seems that RAD21L-containing cohesin might localize close to the recombination intermediates either through direct or indirect association with the recombination intermediate molecules or by recognizing the configuration of the DNA (chromatin) during the process of recombination from zygotene to mid pachytene. Alternatively, but not mutually exclusively, RAD21L might have an intrinsic feature allowing it to make and/or maintain the connection between non-sister chromatids rather than between sister chromatids. In support of the latter case, recent studies have suggested that homolog recognition and pairing occur in a DSB-independent manner before AE formation [33, 51]. In addition, this early recognition and pairing are mainly dependent on the function of RAD21L but only partly on REC8 since homolog co-alignment was observed in Rec8/Spo11 double KO but not Rad21L/Spo11 double KO spermatocytes [33]. Our observation that RAD21L locates to a position interior to the site of REC8 in the SC (Fig. 2) might reflect the fact that RAD21L but not REC8 can couple the DNA of non-sister chromatids from homologs irrespective of recombination position (Fig. 5). A recent work using 3D-SIM in fission yeast meiosis also suggests that Rec8, the only meiotic α-kleisin in this species, plays an essential role in building a platform to support the chromosome architecture necessary for the spatial alignment of homologs [52]. Therefore, it seems that the role of meiosis-specific cohesin subunits in establishing the linkage between homologs is conserved among eukaryotes.

In summary, the present study highlights the different properties of two meiotic α-kleisins, strongly supporting the view that RAD21L is an atypical cohesin that establishes an association between homologs rather than between sister chromatids [24, 33].

Acknowledgments

We are grateful to the members of our laboratory for their valuable support and discussions. This work was supported in part by JSPS KAKENHI (Grant Numbers 26292169 to JL, 15K14500 and 16H01440 to AM, and 26251037 to YH).

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