Condensins I and II are essential for construction of bivalent chromosomes in mouse oocytes

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ABSTRACT **In many eukaryotes, condensins I and II associate with chromosomes in an ordered fashion during mitosis and play nonoverlapping functions in their assembly and segregation. Here we report for the first time the spatiotemporal dynamics and functions of the two condensin complexes during meiotic divisions in mouse oocytes. At the germinal vesicle stage (prophase I), condensin I is present in the cytoplasm, whereas condensin II is localized within the nucleus. After germinal vesicle breakdown, condensin II starts to associate with chromosomes and becomes concentrated onto chromatid axes of bivalent chromosomes by metaphase I. REC8 "glues" chromosome arms along their lengths. In striking contrast to condensin II, condensin I localizes primarily around centromeric regions at metaphase I and starts to associate stably with chromosome arms only after anaphase I. Antibody injection experiments show that condensin functions are required for many aspects of meiotic chromosome dynamics, including chromosome individualization, resolution, and segregation. We propose that the two condensin complexes play distinctive roles in constructing bivalent chromosomes: condensin II might play a primary role in resolving sister chromatid axes, whereas condensin I might contribute to monopolar attachment of sister kinetochores, possibly by assembling a unique centromeric structure underneath.**

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INTRODUCTION

On entry into mitosis, long and entangled chromatin fibers are shortened, resolved, and packaged into mitotic chromosomes, each of which is composed of a pair of sister chromatids. This process, known as chromosome condensation or sister chromatid resolution, is believed to be an essential prerequisite for the rapid yet accurate segregation of chromosomes in anaphase. Accumulating lines of evidence during the last decade or so suggest that a multisubunit protein complex called condensin is a central player in this process (Swedlow and Hirano, 2003; Nasmyth and Haering, 2005).

The condensin complex was originally identified in *Xenopus* egg extracts as a major chromosomal component that contributes to both the assembly and the structural maintenance of metaphase chromosomes (Hirano and Mitchison, 1994; Hirano *et al.*, 1997). A series of concurrent and subsequent genetic studies provided evidence that condensin is essential for chromosome architecture and segregation in many organisms, including fission yeast (Saka *et al.*, 1994; Sutani *et al.*, 1999), budding yeast (Strunnikov *et al.*, 1995; Lavoie *et al.*, 2000; Bhalla *et al.*, 2002), *Drosophila melanogaster* (Bhat *et al.*, 1996; Steffensen *et al.*, 2001), and *Caenorhabditis elegans* (Lieb *et al.*, 1998; Hagstrom *et al.*, 2002). Although it remains under debate to what extent condensin functions might directly be involved in the "compaction" of chromosomes, one of the phenotypes commonly observed in condensin-deficient cells is the formation of anaphase bridges, which argues strongly that condensin plays an essential role in chromosome resolution and segregation.

It is now widely accepted that most eukaryotes contain two different types of condensin complexes (Hirano, 2005). The canonical condensin complex, now known as condensin I, is composed of two structural maintenance of chromosomes (SMC) core subunits (SMC2 and SMC4) and three non-SMC regulatory subunits (CAP-D2, -G, and -H). The second complex, termed condensin II, shares the same

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Abbreviations used: Ana-I, anaphase I; GV, germinal vesicle; GVBD, germinal vesicle breakdown; Meta-I, metaphase I; Meta-II, metaphase II; NEBD, nuclear envelope breakdown; PN, pronucleus; topo IIα, topoisomerase IIα.

pair of the SMC core subunits with condensin I but also contains a distinct set of non-SMC regulatory subunits (CAP-D3, -G2, and -H2; Ono *et al.*, 2003; Yeong *et al.*, 2003). Notably, some species, including budding yeast and fission yeast, possess only condensin I. In HeLa cells, it was shown that the two condensin complexes are differentially regulated during the cell cycle and make distinct contributions to mitotic chromosome assembly (Hirota *et al.*, 2004; Ono *et al.*, 2004). Condensin II is present within the nucleus during interphase and contributes to an early stage of chromosome condensation in prophase before nuclear envelope breakdown (NEBD). In contrast, condensin I is sequestered into the cytoplasm throughout interphase and prophase and gets associated with chromosomes only after NEBD in prometaphase. By metaphase, condensins I and II are concentrated along chromatid axes in an alternate pattern, with a subfraction of condensin II enriched near the inner kinetochore region. Depletion of either one of the condensin I and II subunits causes distinct aberrations in chromosome morphology, indicating that the two complexes have nonoverlapping functions in regulating chromosome architecture (Ono *et al.*, 2003, 2004).

Meiosis is a specialized form of cell divisions that is designed to produce haploid gametes from diploid germ cells (Petronczki *et al.*, 2003). Accordingly, chromosomes undergo two successive rounds of segregation after a single round of duplication. In prophase of meiosis I (prophase I [Pro-I]), homologous chromosomes pair and recombine with their own partners. As a consequence, two homologues are connected with each other along their arm regions distal to chiasmata, leading to the formation of the so-called bivalent chromosomes at metaphase I (Meta-I). The linkage between sister chromatid arms in the bivalent chromosomes is mediated by meiotic cohesin containing REC8 that persists until the onset of anaphase I (Klein *et al.*, 1999; Watanabe and Nurse, 1999; Eijpe *et al.*, 2003; Lee *et al.*, 2003). This behavior of meiotic cohesin is in striking contrast to that of its mitotic counterpart (containing RAD21 rather than REC8), most of which is released from chromosome arms in prophase (Losada *et al.*, 1998; Waizenegger *et al.*, 2000). Thus, in meiosis I, bivalent chromosomes must assemble in the persistent presence of cohesin along arms, which would, in principle, counteract the resolution of sister chromatids. Another unique characteristic of meiosis I is the spatial arrangement of sister kinetochores. Sister kinetochores in Meta-I are arranged into a side-by-side orientation so that they attach to microtubules from the same pole, hence establishing the so-called monopolar attachment (Moore and Orr-Weaver, 1998). This type of microtubule attachment is fundamentally different from bipolar attachment observed in mitosis and meiosis II, in which sister kinetochores are arranged in a back-to-back orientation and bind to opposite poles.

Despite extensive characterization of cohesin functions, we have rather limited information about the potential roles of condensin(s) in meiotic chromosome assembly and segregation (Firooznia *et al.*, 2005). In budding yeast, condensin I (the sole condensin complex in this organism) is required for axial compaction, individualization, and resolution of meiotic chromosomes, as well as proper assembly of the synaptonemal complex (Yu and Koshland, 2003). In *Drosophila*, it was reported that CAP-D3 and -H2 are necessary for the condensation and individualization of chromosomes in prophase I (Hartl *et al.*, 2008). However, the existence of a putative condensin II complex in this organism remains to be established because the gene encoding CAP-G2 is apparently missing in the *Drosophila* genome. Furthermore, mutations in *Drosophila* CAP-G cause a delay in the disassembly of the synaptonemal complex and a defect in retention at Meta-I in female meiosis (Resnick *et al.*, 2009). In *C. elegans*, an organism that has unique holocentric chromosomes, condensin II localizes to the core of sister chromatids after pachytene exit and is required for chromosome reorganization and segregation in both meiosis I and II (Chan *et al.*, 2004). Condensin I, which displays distinct and somewhat peculiar localization patterns, plays an apparently less important role than condensin II in this organism (Csankovszki *et al.*, 2009). Thus it appears that the dynamics and functions of condensins in meiotic chromosome organization and segregation substantially diverge among different species. Very crucially, no systematic characterization of condensins I and II has been performed in mice, one of the most important model organisms for our understanding of meiotic chromosome architecture and dynamics.

In the present study, we investigate for the first time the spatiotemporal dynamics of both condensins I and II during meiotic divisions in mouse oocytes by immunoblot and immunofluorescence analyses. Furthermore, we report antibody injection experiments to address the functions of the two condensin complexes in chromosome assembly and segregation in mammalian meiosis.

RESULTS

Spatial and temporal dynamics of condensins I and II in mouse oocytes

To characterize the dynamics and functions of condensin I and condensin II in mouse meiotic cells, we generated specific antibodies against all putative subunits of the mouse condensin complexes (for details, see *Materials and Methods*). Immunoblot analyses against total extracts of NIH3T3 cells demonstrated that each antibody recognized a specific band of an expected size, whose intensity was reduced by treatment with small interfering RNA (siRNA) designed for the corresponding subunit (Supplemental Figure S1A). We then performed immunoprecipitation experiments, using testis extracts as a starting material, and found that the extracts contain two distinct condensin complexes, namely, condensin I, consisting of SMC2, SMC4, CAP-D2, -G, and -H, and condensin II, consisting of SMC2, SMC4, CAP-D3, -G2, and -H2 (Supplemental Figure S1, B–D), as reported in human somatic cells and *Xenopus* eggs (Ono *et al.*, 2003).

With these specific antibodies in hand, we examined expression patterns of all condensin subunits during meiosis in mouse oocytes. Mouse oocytes were dissected out from the ovary, cultured in vitro, and harvested at several time points to obtain extracts at various meiotic stages, namely, germinal vesicle (GV), Meta-I, and metaphase II (Meta-II) stages. Meta-II oocytes were artificially activated and cultured for another 6 h to obtain eggs at the pronucleus (PN) stage. We found that all of the condensin subunits were expressed throughout these stages as judged by immunoblot analyses (Figure 1A; also see Supplemental Figure S1E). Notably, electrophoretic mobility of some of the subunits (CAP-D2, -G, -H, and -D3) was retarded at M phase (Meta-I and Meta-II) compared with at interphase (GV and PN stages), possibly due to M phase–specific phosphorylation, as reported in *Xenopus* and human condensin subunits (Kimura *et al.*, 2001).

To examine the spatiotemporal dynamics of the two condensin complexes, we sought to immunofluorescently label oocytes at various stages with antibodies against CAP-G (for condensin I), CAP-D3 (for condensin II), and SMC2 (for both condensins I and II). The specificity of these three antibodies in immunofluorescence studies was confirmed in NIH3T3 cells that had been depleted or mock depleted of the corresponding antigens (Supplemental Figure S2). In GV oocytes, CAP-G signals were detected predominantly in the cytoplasm (Figure 1B, a–d). At Meta-I, discrete and focused signals were observed in close proximity to centromeric regions that were

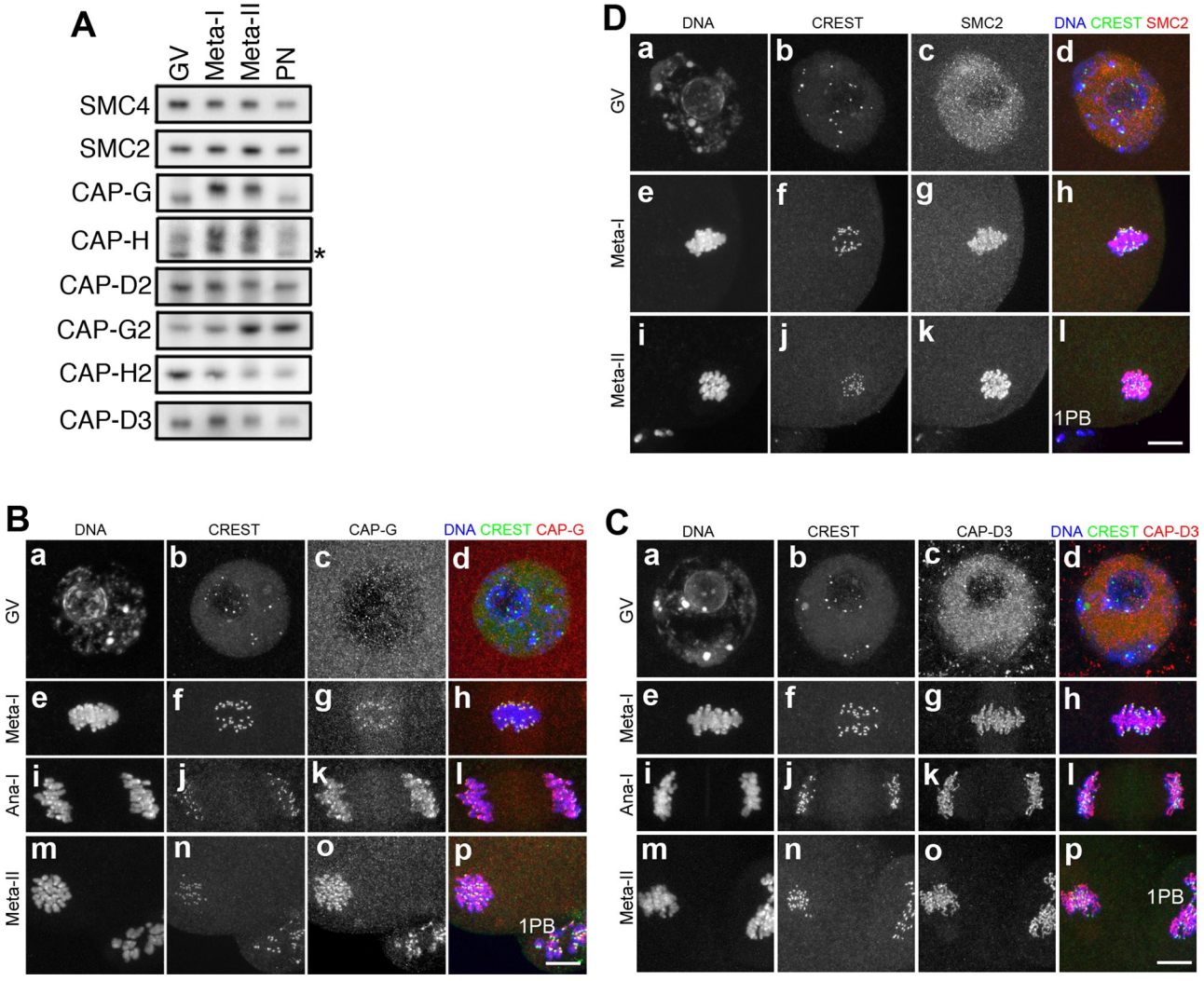

FIGURE 1: Dynamics of condensins I and II during meiosis in mouse oocytes. (A) Mouse oocytes were cultured in vitro and harvested at 0 h for GV, 6 h for Meta-I, and 16 h for Meta-II. Meta-II oocytes were artificially activated and further cultured for 6 h to get eggs at the PN stage. Extracts from the oocytes or the eggs were fractionated by SDS–PAGE and subjected to immunoblot analysis with antibodies against condensin subunits. To detect SMC2, SMC4, CAP-G, and CAP-H, 30 oocytes were used at each stage. To detect CAP-D2, -D3, -G2, and -H2, 100 oocytes were used at each stage. Note that anti–CAP-H antibody detected a pair of bands: the fast-migrating band (indicated by the asterisk) is most likely to be an alternative spliced product or a partially degraded form of full-length CAP-H. (B) Mouse oocytes (n = 38) were immunofluorescently labeled with human CREST (b, f, j, n) and rabbit polyclonal anti–CAP-G (c, g, k, o) antibodies. DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). (C) Mouse oocytes (n = 37) were immunofluorescently labeled with human CREST (b, f, j, n) and rabbit polyclonal anti–CAP-D3 (c, g, k, o) antibodies. DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). (D) Mouse oocytes (n = 31) were immunofluorescently labeled with human CREST (b, f, and j) and rabbit polyclonal anti-SMC2 (c, g, k) antibodies. DNA was counterstained with Hoechst 34580 (a, e, i), and merged images are shown (d, h, l). Projections of stacked images are shown throughout. 1PB, the first polar body. Bars, 10 μm.

labeled with CREST antibody (Figure 1B, e–h). Although very few if any signals were observed along chromosome arms at this stage, CAP-G became detectable along arms at anaphase I (Ana-I; Figure 1B, i–l). The distribution along chromosome arms persisted by Meta-II (Figure 1B, m–p). The dynamics of CAP-D3 was strikingly different from that of CAP-G. At GV stage, unlike CAP-G, CAP-D3 signals were detected within the GV (Figure 1C, a–d). CAP-D3 became detectable along chromatid axes at Meta-I (Figure 1C, e–h), and the axial signals persisted from Ana-I (Figure 1C, i–l) through Meta-II (Figure 1C, m–p). We also noticed that, compared with the CAP-G signals distributing diffusely on chromosome arms from Ana-I to Meta-II, the signals of CAP-D3 were sharper and more confined to the central axis of each chromatid. Consistent with the notion that SMC2 is a common subunit of condensins I and II, the distribution pattern of SMC2 in oocytes at different stages was essentially the sum of those of CAP-G and -D3 (Figure 1D). Our results show that condensins I and II display different spatiotemporal distributions during meiotic divisions in mouse oocytes.

Contribution of condensins and cohesin to bivalent chromosome formation

To investigate the process of bivalent chromosome formation in more detail, we next focused on the dynamics of condensins and cohesin from GV stage to Meta-I. After 45-min culture when the GV

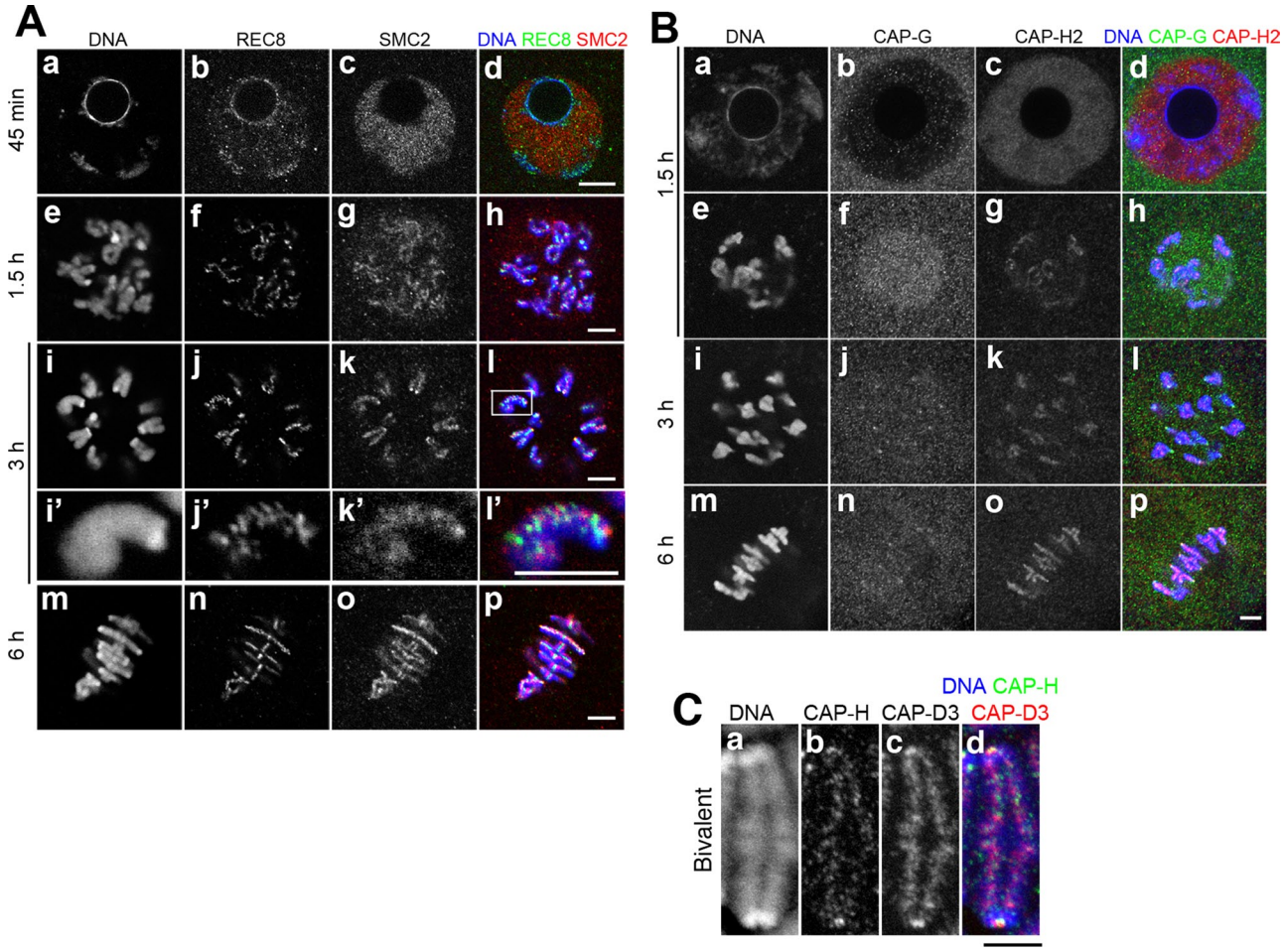

FIGURE 2: Dynamics of condensins and cohesin during the formation of bivalent chromosomes. Mouse oocytes were cultured in vitro and fixed at the indicated time points. (A) The oocytes (n = 37) were immunofluorescently labeled with mouse polyclonal anti-REC8 (b, f, j, j′, n) and rabbit polyclonal anti-SMC2 (c, g, k, k′, o) antibodies. DNA was counterstained with Hoechst 34580 (a, e, i, i′, m), and merged images are shown (d, h, l, l′, p). The region indicated in l is magnified in i′–l′. Bars, 10 μm (d); 5 μm (h, l, l′, p). (B) The oocytes (n = 57) were immunofluorescently labeled with rabbit polyclonal anti–CAP-G antibody (b, f, j, n) and rat polyclonal anti–CAP-H2 antibody (c, g, k, o). DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). Bar, 5 μm. (C) Chromosome spreads from Meta-I oocytes were immunofluorescently labeled with rat polyclonal anti–CAP-H (b) and rabbit polyclonal anti–CAP-D3 (c) antibodies. DNA was counterstained with Hoechst 34580 (a), and merged images are shown (d). Bar, 5 μm.

was still intact, the meiosis-specific cohesin subunit REC8 colocalized with partially condensing chromatin near the periphery of the GV, as well as at the outer surface of the nucleolus (Figure 2A, a, b, and d). At this moment, the population of SMC2 present within the GV was apparently displaced from the chromatin (Figure 2A, a, c, and d). On or immediately after GVBD (1.5 h), both SMC2 and REC8 became detectable on condensing chromosomes (Figure 2A, e–h). At Prometa-I (3 h), alternating layers of SMC2 and REC8 signals were often observed along chromosomes (~67% of the oocytes examined; n = 9), and a subfraction of SMC2 signals was enriched at centromeres (Figure 2A, i–l and i′–l′). By Meta-I (6 h), SMC2 localized to sister chromatid axes, whereas REC8 was confined along the interchromatid regions (Figure 2A, m–p). Thus condensins and REC8-containing cohesin occupies distinctive regions within bivalent chromosomes at Meta-I.

We then examined the spatiotemporal behavior of condensins I and II during the process of bivalent chromosome formation by means of double labeling. Consistent with the results shown in Figure 1, B and C, prior to GVBD, CAP-G was present in the cytoplasm, whereas CAP-H2 was diffusely distributed within the GV (Figure 2B, a–d). On GVBD, a fraction of CAP-G relocated from the cytoplasm to the disassembling GV yet displayed little sign of association with chromatin (Figure 2B, e, f, and h). In contrast, CAP-H2 was apparently concentrated on the condensing masses of chromatin at this stage (Figure 2B, e, g, and h). After 3-h culture, CAP-G was observed primarily at centromeres (Figure 2B, i–l), and CAP-H2 displayed a discrete distribution along chromosome arms. After 6-h culture, CAP-G and -H2 continued to localize at centromeres and along chromosome arms, respectively (Figure 2B, m–p). It should be noted, however, that faint signals of CAP-G were observed along chromosome arms either in some (~20%) of the Prometa-I and Meta-I oocytes or in a very minor population of oocytes that failed to enter Ana-I and stayed at Meta-I even after prolonged (16 h) culture (Supplemental Figure S3). These observations suggest that condensin I might dynamically interact with chromosomes during the formation of bivalent chromosomes. Finally, chromosome spreads were prepared from Meta-I oocytes and double labeled with antibodies against CAP-H (for condensin I) and CAP-D3 (for condensin II). In a

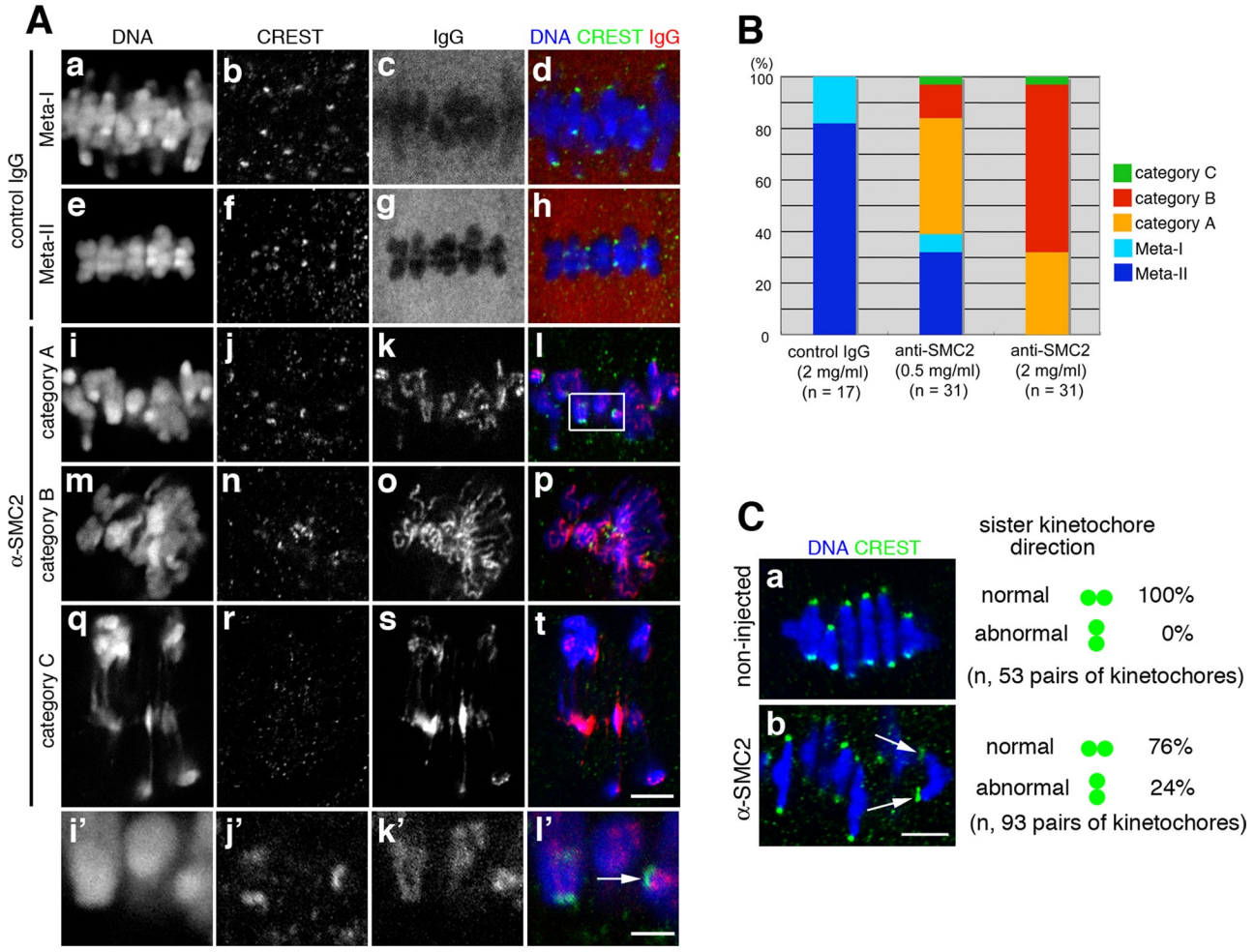

FIGURE 3: SMC2 antibody injection causes severe defects in kinetochore orientation, chromosome condensation, and segregation. (A) Mouse oocytes that had been injected with control rabbit IgG (a–h) or anti-SMC2 antibody (i–t and i′–l′) were cultured for 6 h (a–d) or 16 h (e–t and i ′–l′). After fixation, the oocytes were immunofluorescently labeled with human CREST antibody (b, f, j, n, r, j′). The injected IgG or antibody was also immunofluorescently detected with a secondary antibody alone (c, g, k, o, s, k′). DNA was counterstained with Hoechst 34580 (a, e, i, m, q, i′), and merged images are shown (d, h, l, p, t, l′). The region indicated in l is magnified in i′–l′. Bars, 5 μm (t), 1 μm (l′). (B) Percentages of chromosome morphology observed in oocytes injected with control IgG (2 mg/ml; n = 17) and in oocytes injected with a low (0.5 mg/ml) or high (2 mg/ml) concentration of anti-SMC2 antibody (n = 31 for each). (C) Mouse oocytes were cultured for 4 h in mKSOM culture medium. After adding MG132 into the culture medium, the oocytes were cultured for another 3 h to arrest the cell cycle at Meta-I. They were then injected or uninjected with anti-SMC2 antibody and incubated for 30 min before being fixed and processed for immunofluorescence labeling with human CREST antibody. DNA was counterstained, and merged images are shown. Bar, 5 μm. Also shown are the ratio of sister kinetochore pairs aligned horizontal (normal) or vertical (abnormal) relative to the spindle equator in each group.

representative bivalent chromosome shown in Figure 2C, discrete signals of CAP-H were observed at centromeric regions, which partially overlap with a subfraction of CAP-D3 signals concentrated at the paired sister kinetochores.

Injection of anti-SMC antibody causes severe defects in kinetochore orientation, chromosome condensation, and segregation

To address the role of condensins in meiotic chromosome assembly and segregation, we attempted to disturb their functions by injecting oocytes with an anti-SMC2 antibody. Most of the control oocytes that had been injected with rabbit immunoglobulin (IgG) progressed to Meta-I at 6 h and Meta-II at 16 h after culture (Figure 3A, a–h), a time course indistinguishable from that observed in noninjected oocytes. Moreover, no apparent defect in chromosome assembly or segregation was observed in these oocytes. In brief, at Meta-I, sister kinetochores were placed in a "side-by-side" manner and cooriented toward one of the spindle poles (Figure 3A, a–d). At Meta-II, sister kinetochores were rearranged into a "back-to-back" manner and directed toward the opposite spindle poles (Figure 3A, e–h). In striking contrast to these control oocytes, most of the oocytes injected with the antibody against SMC2 failed to properly segregate their chromosomes (Figure 3, A, i–t, and B). Multiple defective phenotypes were observed, but they could largely be classified into three groups, which we here refer to as categories A, B, and C. In category A, the morphology of chromosomes as judged by Hoechst staining was seemingly normal, but some pairs of sister kinetochores failed to be oriented to the same spindle pole (Figure 3A, i′–l′, arrow). In category B, chromosome architecture was heavily impaired: the chromosomes observed in this class of oocytes

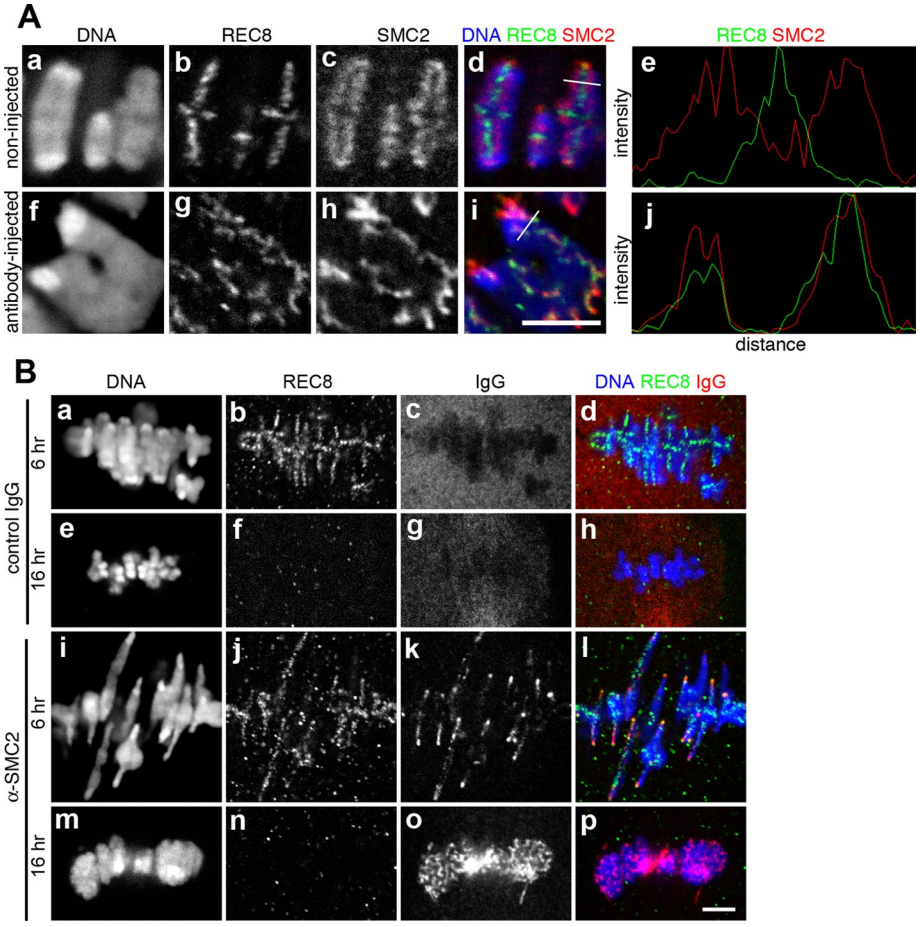

FIGURE 4: SMC2 antibody injection impairs sister chromatid resolution but not cohesin removal in anaphase I. (A) Mouse oocytes were uninjected or injected with anti-SMC2 antibody, cultured for 6 h, and immunofluorescently labeled with mouse polyclonal anti-REC8 (b, g). For the uninjected samples, SMC2 was labeled with rabbit polyclonal anti-SMC2 antibody (c). For the injected samples, the injected antibody was visualized with a secondary antibody alone (h). DNA was counterstained with Hoechst 34580 (a, f), and merged images are shown (d, i). Fluorescence intensities of REC8 and SMC2 signals were measured along the white lines indicated in d and i and are plotted in e and j, respectively. Bar, 5 μm. (B) Mouse oocytes were injected with control IgG (a–h) or anti-SMC2 antibody (i–p), cultured for 6 h (a–d, i–l) or 16 h (e–h, m–p), and then immunofluorescently labeled with mouse polyclonal anti-REC8 antibody (b, f, j, n). The injected IgG or antibody was immunofluorescently detected with a secondary antibody alone (c, g, k, o). DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). Bar, 5 μm.

were poorly condensed, looked fragile, and failed to be individualized (Figure 3A, m–p). It was often difficult to judge how each pair of sister kinetochores was oriented relative to the spindle poles. In category C, which was very rare, the oocytes apparently entered anaphase and attempted to segregate chromosomes without success, leaving massive amounts of chromatin bridges (Figure 3A, q–t). As shown in Figure 3B, these defective phenotypes caused by antibody injection was dose dependent. We observed a similar set of defective phenotypes in oocytes injected with an antibody against SMC4, another core subunit common to both condensins I and II (unpublished data).

To test the impact of antibody injection on kinetochore orientation more directly, oocytes were cultured in the presence of the proteasome inhibitor MG132 to arrest the cell cycle at Meta-I and were then injected or uninjected with the antibody against SMC2. In the control oocytes, 100% of sister kinetochores were placed in the normal, monopolar orientation (Figure 3C, a). On the other hand, 30 min after injection of the anti-SMC2 antibody, ~24% of sister kinetochores became misoriented, apparently being pulled to the opposite poles rather than to the same pole (Figure 3C, b, arrows). Under this condition, no apparent defect in chromosome condensation was observed. These results clearly demonstrate that proper function of condensins is essential not only for the establishment but also for the maintenance of monopolar attachment of sister kinetochores in meiosis I.

Injection of anti-SMC2 antibody impairs sister chromatid resolution but not cohesin removal in anaphase I

We then wanted to understand how REC8 might behave under the condition where condensin functions were impaired by means of antibody injection. At Meta-I in the control oocytes, SMC2 localized along sister chromatid axes, whereas REC8 was concentrated in interchromatid regions (Figure 4A, a–d; also see Figure 2A, m–p). Thus the distributions of SMC2 and REC8 were clearly segregated from each other in bivalent chromosomes at Meta-I (Figure 4A, e). On the other hand, in Meta-I–like chromosomes in the anti-SMC2 antibody–injected oocytes (classified as category B in Figure 3), both structures labeled with anti-REC8 and -SMC2 antibodies were highly distorted, displaying zigzag and less continuous appearances. Notably, the REC8 and SMC2 signals often overlapped, meaning that individual sister chromatid axes and interchromatid regions were no longer clearly distinguishable from each other (Figure 4A, f–j). These results suggest that condensin functions are required for sister chromatid resolution during bivalent chromosome assembly.

In the control oocytes injected with control IgG (Figure 4B, a–h), REC8 localized along interchromatid regions at Meta-I (6 h) but was hardly detectable at Meta-II (16 h), as reported previously (Lee *et al.*, 2006). Likewise, in all of the oocytes injected with anti-SMC2 antibody, REC8 signals were readily detectable in the distorted chromosomes at 6 h (Figure 4B, i–l). By 16 h, however, the REC8 signals became undetectable on the chromosomes (Figure 4B, m–p). These results suggest that cohesin removal from chromosome arms in anaphase I occurs normally in these oocytes injected with anti-SMC2 antibody. Thus the failure of chromosome segregation under this condition is most likely caused by defects in sister chromatid resolution at the preceding stage.

Injection of anti–CAP-H antibody causes disorganization of centromeric regions and misalignment of chromosomes

Because SMC2 is a common subunit of condensins I and II, the phenotypes observed in the anti-SMC2 antibody–injected oocytes could, in principle, be caused by inhibition or functional disturbance of both condensin complexes. To investigate the specific role of condensin I, if any, in meiotic chromosome assembly and segregation, mouse

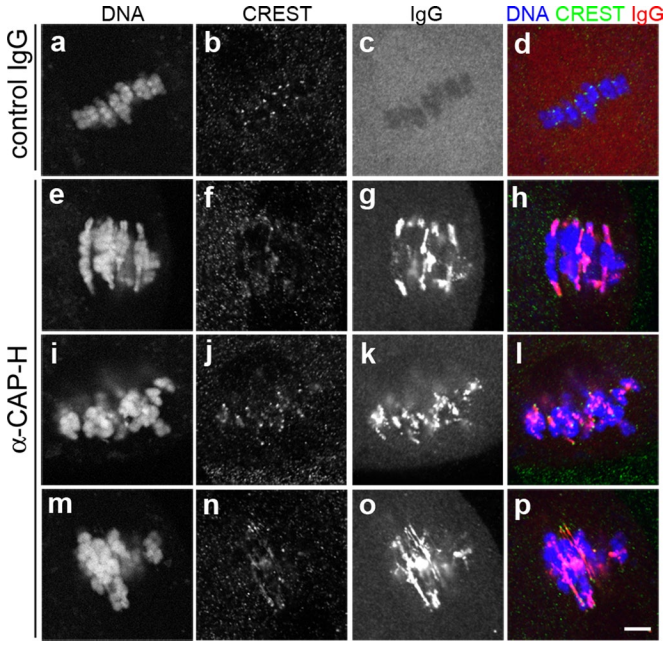

FIGURE 5: CAP-H antibody injection causes disorganization of centromeric regions and misalignment of chromosomes. (A) Mouse oocytes were injected with control rabbit IgG (a–d) or anti–CAP-H antibody (e–p) and cultured for 16 h. After fixation, the oocytes were immunofluorescently labeled with human CREST antibody (b, f, j, n). The injected IgG or antibody was also immunofluorescently detected with a secondary antibody alone (c, g, k, o). DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). Bar, 5 μm.

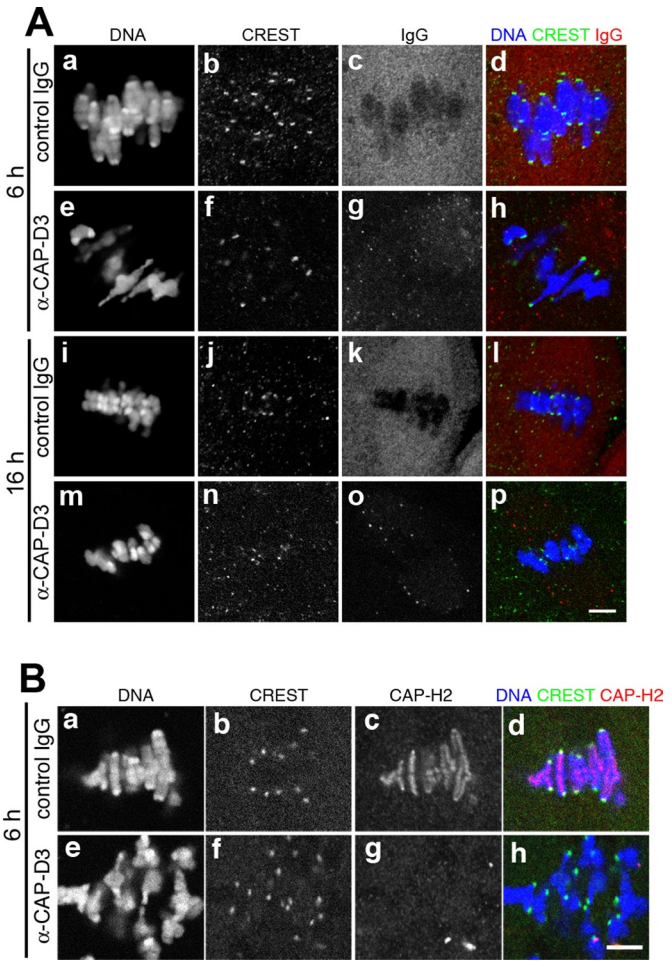

FIGURE 6: CAP-D3 antibody blocks loading of condensin II and causes defects in sister chromatid architecture and resolution. (A) Mouse oocytes were injected with control IgG (a–d, i–l) or anti–CAP-D3 antibody (e–h, m–p), and cultured for 6 h (a–h) or 16 h (i–p). After fixation, the oocytes were immunofluorescently labeled with human CREST antibody (b, f, j, n). The injected IgG or antibody was immunofluorescently detected with a secondary antibody alone (c, g, k, o). DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). Bar, 5 μm. (B) Mouse oocytes were injected with control IgG (a–d) or anti–CAP-D3 antibody (e–h) and cultured for 6 h and immunofluorescently labeled with human CREST (b, f) and rat polyclonal anti–CAP-H2 (c, g) antibodies. DNA was counterstained with Hoechst 34580 (a, e), and merged images are shown (d, h). Bar, 5 μm.

oocytes were injected with an antibody against the condensin I–specific subunit CAP-H. We found that the morphology of chromosomes in these oocytes was substantially impaired, but their defects were apparently milder than those observed in the oocytes injected with the anti-SMC2 antibody (Figure 5, e–p). Chromosomes looked compact but exhibited a bumpy surface. Some of the oocytes showed chromosome alignment like Meta-I (4 of 14; Figure 5, e–h) or Meta-II (7 of 14; Figure 5, i–l), whereas others (3 of 14) showed coalescence of pericentromeric heterochromatin regions, which appeared to be unusually extended (Figure 5, m–p). In all cases, chromosomes failed to align properly and failed to segregate from each other.

We also noticed unexpectedly that the anti–CAP-H antibody injected was heavily concentrated around centromeric regions (Figure 5, e–p). Such antibody-positive regions were often highly extended (also see the antibody signals indicated by an arrow in Figure 7B, l), and the shape of CREST signals was also extremely disorganized. We speculate that the CAP-H antibody modulates the on–off rate of its antigen and thereby induces accumulation of an unusual amount of CAP-H at centromeric regions. These results argue in favor of our idea that condensin I might help to assemble a unique centromeric structure that contributes to the establishment and maintenance of monopolar attachment of sister kinetochores in meiosis I (see *Discussion*).

Injection of anti–CAP-D3 antibody blocks loading of condensin II and causes defects in sister chromatid architecture and resolution

To investigate the specific role of condensin II, we then injected mouse oocytes with an antibody against the condensin II–specific subunit CAP-D3. Unlike the injection of antibodies against SMC2 or CAP-H described earlier, we found unexpectedly that the anti–CAP-D3 antibody injected was undetectable on chromosomes (Figure 6A, e–h and m–p). When a lower concentration of the antibody was used, fainter signals of CAP-D3 were observed on chromosomes (Supplemental Figure S4), suggesting that the injected antibody blocked chromosomal loading of CAP-D3 in a dose-dependent manner. At 6 h after the antibody injection, the oocytes were apparently in a Meta-I–like stage, and their chromosomes failed to be individualized or resolved (Figure 6A, e–h). After 16-h culture, most (19 of 25) of the oocytes progressed to a Meta-II–like stage. However, chromosomes were not aligned well on the metaphase plate, and neither sister kinetochores nor pericentromeric heterochromatin dissolution was discernible (Figure 6A, m–p). CAP-H2, another condensin II–specific subunit, was readily detectable on chromosomes in the control oocytes (Figure 6B, a–d) but was hardly

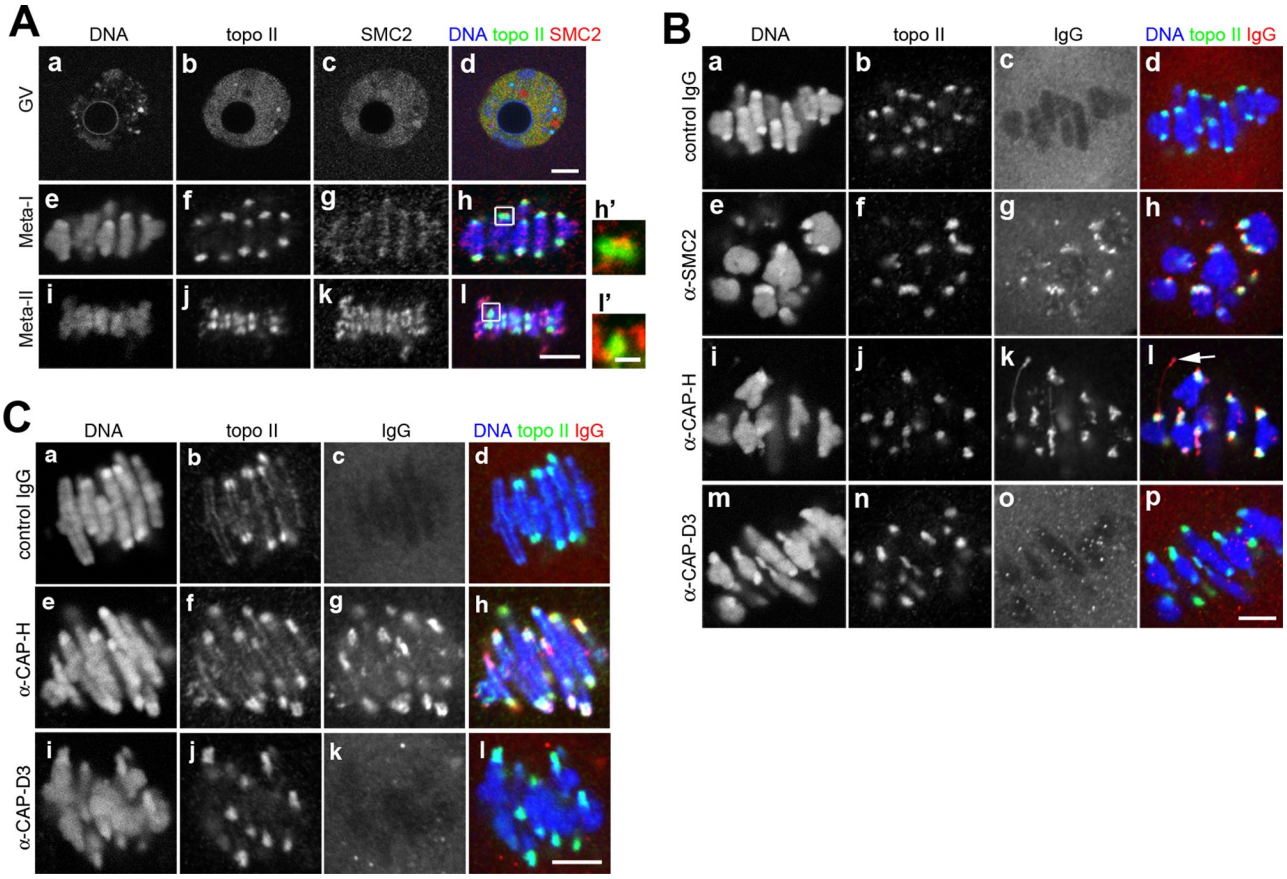

FIGURE 7: Injection of condensin antibodies barely affects pericentromeric localization of topoisomerase IIα. (A) GV (a–d), Meta-I (e–h), and Meta-II (i–l) oocytes were immunofluorescently labeled with rat polyclonal anti–topo IIα (b, f, j) and rabbit polyclonal anti-SMC2 (c, g, k) antibodies. DNA was counterstained with Hoechst 34580 (a, e, i), and merged images are shown (d, h, l). The regions indicated in h and l are magnified in h′ and l′, respectively. Bars, 10 μm (d); 5 μm (l); 1 μm (l′). (B) Mouse oocytes were injected with control IgG (a–d) or antibodies against SMC2 (e–h), CAP-H (i–l), and CAP-D3 (m–p), cultured for 6 h, and immunofluorescently labeled with rat polyclonal anti–topo II α antibody (b, f, j, n). The injected IgG or antibody was immunofluorescently detected with a secondary antibody alone (c, g, k, o). DNA was counterstained with Hoechst 34580 (a, e, i, m), and merged images are shown (d, h, l, p). The arrow indicates an extremely extended centromere. Bar, 5 μm. (C) Mouse oocytes were injected with control IgG (a–d) or antibodies against CAP-H (e–h) and CAP-D3 (i–l), cultured for 16 h, and immunofluorescently labeled with rat polyclonal anti–topo IIα antibody (b, f, j). The injected IgG or antibody was also immunofluorescently detected with a secondary antibody alone (c, g, k). DNA was counterstained with Hoechst 34580 (a, e, i), and merged images are shown (d, h, l). Bar, 5 μm.

observed on chromosomes in the oocytes injected with the anti–CAP-D3 antibody (Figure 6B, e–h), suggesting that the anti–CAP-D3 antibody inhibited chromosomal loading of the whole condensin II complex rather than the CAP-D3 subunit alone. In the antibody-injected oocytes, condensin I (CAP-G) was detected on chromosomes as usual (unpublished data). Taken together, our results demonstrate that condensin II is essential for individualization and resolution of meiotic chromosomes.

Injection of condensin antibodies barely affects pericentromeric localization of topoisomerase IIα

Besides condensins, topoisomerase IIα (topo IIα) plays an important role in chromosome assembly and segregation (Cuvier and Hirano, 2003; Maeshima and Laemmli, 2003). The localization and dynamics of topo IIα during mammalian meiosis had not thoroughly been characterized, although one report had detected topo IIα on chromosomes in mouse Meta-II oocytes (St Pierre *et al.*, 2002). For this reason, we first looked at the distribution of topo IIα in the oocytes at different stages. At the GV stage, topo IIα was diffusely present within the whole nucleus, yet its subfraction was concentrated on pericentromeric heterochromatin that was also heavily stained with Hoechst (Figure 7A, a–d). After GVBD, topo IIα was detected predominantly at the pericentromeric heterochromatin from Meta-I through Meta-II: topo IIα signals along chromatid axes were only faintly observed at Meta-II (Figure 7A, e–l). It was important to note that the localization of topo IIα and SMC2 was distinct at the proximity of centromeric regions. Although topo IIα was detected in the whole region of pericentromeric heterochromatin (stained intensely with Hoechst and colabeled with heterochromatin protein 1β; unpublished data), the signals of SMC2 were confined to a more focused region of centromeres (Figure 7A, h′ and l′).

We then tested how the injections of antibodies against condensin subunits might affect the distribution of topo IIα in oocytes. In the oocytes injected with control IgG, topo IIα localized normally to pericentromeric heterochromatin (Figure 7B, a–d). In the oocytes injected with antibodies against SMC2 (Figure 7B, e–h), CAP-H

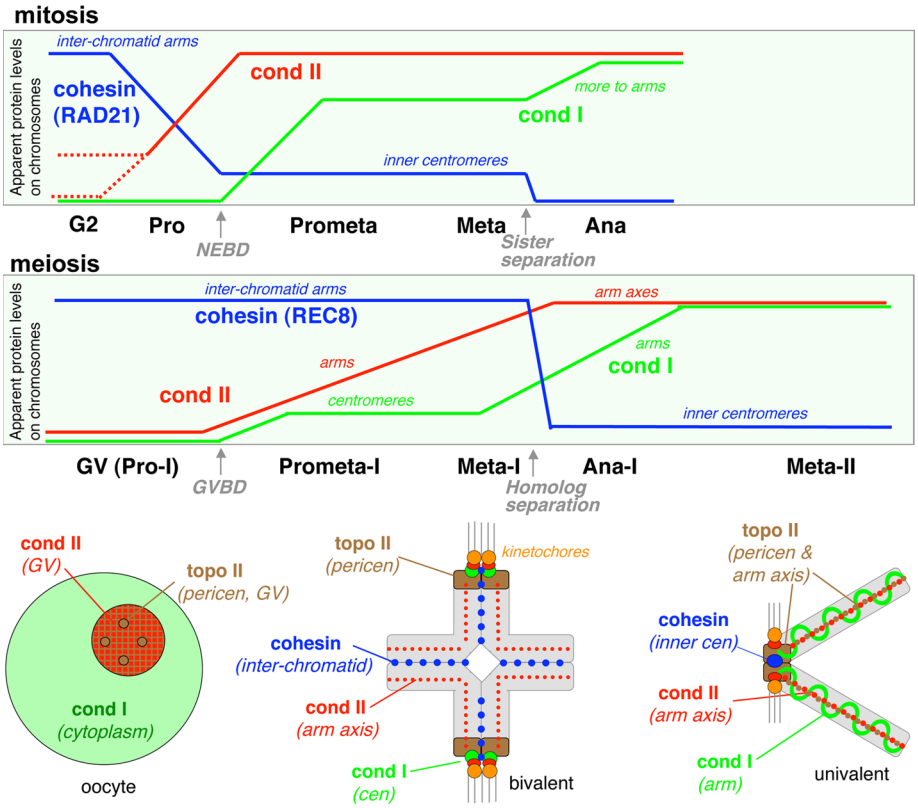

FIGURE 8: Spatiotemporal dynamics of condensins and cohesin in mitosis and meiosis. In mitotic prophase, most cohesin is released from chromosome arms, and condensin II becomes concentrated on chromatid axes. On NEBD in prometaphase, condensin I starts to associate with chromosomes, resulting in the formation of metaphase chromosomes. In anaphase, when the residual population of cohesin primarily concentrated at inner centromeres is released, more condensin I appears to get loaded onto separating chromatid arms. In meiosis, meiotic cohesin (REC8) remains associated with chromosome arms to keep connection between homologous chromosomes by Meta-I. In this situation, the timing of chromosomal association of both condensins I and II is substantially delayed. Condensin II becomes concentrated onto chromatid axes around or immediately after GVBD, whereas condensin I localizes primarily at centromeres, being hardly detectable along chromosome arms by Meta-I. Stable association of condensin I with chromosome arms starts only after Ana-I. Despite these apparent differences, the order of chromosomal association of condensins I and II (i. e., condensin II first, condensin I later) is conserved between mitosis and meiosis. Mutually exclusive association of cohesin and condensin I with chromosome arms is another common feature between mitosis and meiosis.

(Figure 7B, i–l), or CAP-D3 (Figure 7B, m–p), the distribution of topo IIα was also restricted to the same region even though the overall morphology of their chromosomes was heavily distorted. Thus the injection of antibodies against condensin subunits does not have a big impact on the pericentromeric localization of topo IIα.

We noticed that, when cultured in vitro, ~10% of oocytes failed to enter Ana-I and stayed at Meta-I even after prolonged culture (16 h). When we looked at such Meta-I–arrested oocytes, topo IIα signals became detectable along chromatid axes, as well as pericentromeric heterochromatin (unpublished data). Likewise, topo IIα signals were observed along chromatid axes in such Meta-I–arrested oocytes injected with control IgG (Figure 7C, a–d) or anti–CAP-H antibody (Figure 7C, e–h). Remarkably, however, such axial distribution of topo IIα was hardly observed in the Meta-I–arrested oocytes injected with anti–CAP-D3 antibody (Figure 7C, i–l). These results suggest that condensin II, but not condensin I, might help to load topo IIα to chromatid axes at Meta-I, although such function becomes fully recognizable only under the Meta-I–arrested condition.

DISCUSSION

Although emerging studies in invertebrate model organisms have begun to uncover essential functions of condensins in meiotic chromosome structure and function, only fragmentary and limited information is available from studies in vertebrates such as *Xenopus laevis* (Watrin *et al.*, 2003), pig (Liskova *et al.*, 2010), and mouse (Viera *et al.*, 2007). The present report is the first comprehensive study to investigate the expression, chromosomal localization, and potential functions of the two condensin complexes in mouse oocytes. Our results reveal differential, spatiotemporal distributions of condensins I and II and their involvement in multiple aspects of meiotic chromosome dynamics.

Spatiotemporal dynamics of condensins I and II during meiosis in mouse oocytes

Our understanding of fundamental aspects of chromosome dynamics would be deepened by comparing and contrasting the spatiotemporal dynamics of condensins I and II between mitosis and meiosis (Figure 8). In mitosis, condensin II associates with chromosomes in prophase prior to NEBD, whereas condensin I does so only after NEBD (Hirota *et al.*, 2004; Ono *et al.*, 2004). By contrast, in mouse oocytes, condensin II appears to associate with chromosome arms around or immediately after GVBD (equivalent to NEBD in mitosis). Condensin I is hardly detected on chromosome arms from GVBD through Meta-I (Figures 2, B and C, and 2B) and becomes detectable reproducibly along arms only after Ana-I (Figure 1B). Thus, in meiosis, there is a substantial delay in loading of condensins I and II onto chromosomes relative to the disassembly of the nuclear envelope/germinal vesicle. What factor might cause the difference between mitosis and meiosis? Although cohesin and condensins are largely incompatible with each other on chromosomes in both mitosis and meiosis, a crucial difference is that a large amount of meiotic cohesin remains between sister chromatid arms from Pro-I through Meta-I in meiosis. We suggest that the robust mechanical linkage between the arms mediated by meiotic cohesin would counteract resolving forces supported by condensin II, thereby slowing down its loading and action. Consistent with this idea, we showed recently that chromosomal loading of condensin II is greatly suppressed in *Xenopus* egg extracts depleted of Wapl, a protein required for cohesin release in mitotic prophase (unpublished data). As for condensin I, its stable association with chromosomes is delayed in meiosis even more drastically than in mitosis. In fact, we failed to detect condensin I on the arms of bivalent chromosomes in the majority of Meta-I oocytes, as judged by immunofluorescence labeling following the standard fixation. However, very faint signals on arms are occasionally detectable in a minor population of Prometa-I and Meta-I oocytes (Supplemental

Figure S3) and in chromosome spreads of bivalents (Figure 2C), implying that condensin I may interact with the bivalent chromosomes in a highly dynamic manner.

Despite the apparent differences in condensin dynamics between mitosis and meiosis, some similarities are also noticeable. For instance, the order of chromosomal association of the condensin complexes (i.e., condensin II first, condensin I later) is common between mitosis and meiosis. This order of action would be a natural consequence of the fact that condensin II, but not condensin I, is already within the nucleus (or the germinal vesicle) during interphase in both mitosis and meiosis. It is also reasonable to assume that cohesin and condensin II are at least partially compatible with each other, whereas cohesin and condensin I do not coexist on chromosome arms in unperturbed mitosis or meiosis (Figure 8).

Roles of condensins I and II in constructing bivalent chromosomes

During bivalent chromosome assembly in meiosis I, chromosome individualization, compaction, and resolution must proceed in the presence of meiotic cohesin containing REC8, which maintains the linkage between homologous chromosome arms until the onset of Ana-I. At the cytological level, a jumbled set of signals of condensins and cohesin observed on chromosomes at Prometa-I (Figure 2A, i′–l′) is gradually reorganized and sorted out, eventually being converted into a pair of sister chromatid axes positive for SMC2 that are "glued" by a structure positive for REC8 by Meta-I (Figure 2A, m–p; Figure 4A, a–e). It is of great interest to understand how this intricate series of large-scale conformational changes of chromosomes might occur.

When oocytes were injected with an antibody against SMC2 or CAP-H, the antibody not only bound to the corresponding antigen, but it also apparently promoted its accumulation on chromatid axes (and centromeric regions). In contrast, injection of an antibody against CAP-D3 depleted the corresponding antigen (as well as another condensin II subunit CAP-H2) from chromosomes. Although the underlying mechanisms of inhibition of condensin(s) are likely to be different, poorly individualized, fuzzy chromosomes are commonly observed in these antibody-injected oocytes. In fact, the signals of condensins and cohesin partially overlapped and failed to be sorted out in the oocytes injected with the anti-SMC2 antibody (Figure 4A, f–j), implying that condensins plays a crucial role in resolving sister chromatid axes to make the "tripartite" structure along the bivalent chromosome arms.

Might condensins I and II play differential roles in the formation of bivalent chromosome arms? Although the technical limitation inherently associated with the antibody injection experiments preclude us from making a strong conclusion, we can speculate about putative functions of the two condensin complexes based on their differential localizations and dynamics. In Meta-I, condensin II, but hardly condensin I, is detected along chromatid axes. The chromosomes depleted of condensin II apparently lose compactness and stiffness to some extent, as judged by their extended appearance pulled by spindle microtubules (Figure 6A, e–h). Equally important, topo IIα fails to be loaded onto the axes of condensin II–depleted chromosomes (Figure 7C, i–l). Thus condensin II is most likely to play a major role in assembling the chromatid axes during bivalent chromosome construction. In striking contrast, condensin I primarily localizes to near centromeres at this stage. Nonetheless, injection of oocytes with the antibody against CAP-H produces chromosomes with a bumpy surface (Figure 5), implying that dynamic association of condensin I with chromosome arms may also contribute to establishing and/or maintaining their shapes.

Moreover, we suspect that a large amount of meiotic cohesin accumulated in the interchromatid regions might also provide stiffness to linked sister chromatid arms as a structural component. In this sense, it is interesting to find that condensin I becomes detectable on chromatid arms at the same time that bulk cohesin is removed from them in Ana-I. Stable association of condensin I with chromosome arms at this stage would help to reinforce their rigidity to resist the tension acting on separating chromatid arms, as proposed for anaphase chromosome segregation in mitosis (Gerlich *et al.*, 2006).

Possible contribution of condensins to monopolar attachment of sister kinetochores in meiosis I

The molecular mechanism that ensures monopolar attachment of sister kinetochores in meiosis I has been investigated primarily in fission and budding yeasts but barely in other species. In fission yeast, it was shown that meiotic cohesin containing Rec8 plays a key role in this process (Yokobayashi *et al.*, 2003). In addition, Moa1, a meiosis I–specific kinetochore protein, is believed to facilitate the localization of Rec8-containing cohesin to the core centromere, thereby contributing to monopolar attachment (Yokobayashi and Watanabe, 2005). In budding yeast, monopolar attachment of sister kinetochores in meiosis I depends on a four-subunit complex, known as monopolin, that is composed of Csm1, Lrs4, Hrr25 (a casein kinase), and Mam1 (Toth *et al.*, 2000; Rabitsch *et al.*, 2003; Petronczki *et al.*, 2006). Among them, Mam1 is the sole subunit whose expression is meiosis specific. Unlike fission yeast's Moa1, the monopolin complex is sufficient to induce sister kinetochore coorientation in a manner independent of meiotic cohesin (Monje-Casas *et al.*, 2007). It is unknown whether vertebrates might possess proteins that have equivalent functions to Moa1 or monopolin.

In the present study, we provide evidence that condensins may contribute to monopolar attachment of sister kinetochores in mouse oocytes: microinjection of oocytes with an antibody against SMC2 causes severe defects in chromosome alignment in Meta-I, resulting in massive formation of bipolar or merotelic attachment of sister kinetochores (Figure 3, A, i–l, and C, b). Of interest, similar defects are observed even when the same antibody is microinjected into oocytes arrested at Meta-I, implying that condensin functions are required not only for establishment but also for maintenance of monopolar attachment. Then, how might condensins contribute to these processes? As judged by CREST signals, the distance between sister kinetochores is not increased in the antibody-injected oocytes. Thus condensins are unlikely to be involved in the process of linking sister kinetochores, a function expected for meiotic cohesin with Moa1 (in fission yeast) or the monopolin complex (in budding yeast). It is possible that kinetochore structure itself is impaired, at least partially, in the absence of proper functions of condensins (Ono *et al.*, 2004; Bernad *et al.*, 2011). An alternative and more likely possibility is that condensins contribute to assembling a specialized structure of centromeric chromatin that helps direct cooriented sister kinetochores toward a single pole in Meta-I. Although it remains to be determined which condensin complex (or whether both) might play a major role in establishing and maintaining monopolar attachment, it is remarkable to find that condensin I is concentrated at the vicinity of cooriented kinetochores in Meta-I. Such an enrichment of condensin I at centromeric regions is not observed in mitosis or meiosis II and is highly characteristic of meiosis I, which suggests that condensin I may have a specific contribution to monopolar attachment at this stage.

Intriguingly, emerging lines of evidence implicate a possible connection between condensin I and monopolin components in budding yeast. For instance, Csm1 and Lrs4, two subunits of the

monopolin complex that are expressed also during the mitotic cell cycle, actively recruit condensin I to the replication fork barrier site within rDNA through physical interactions (Johzuka and Horiuchi, 2009). When the function of condensin I is compromised in meiosis I, association of Mam1 with kinetochores is weakened, and kinetochore orientations are impaired (Brito *et al.*, 2010). It is therefore possible that condensin I and the monopolin complex directly interact with each other and collaborate to establish monopolar attachment, albeit through distinct mechanisms in budding yeast. We suggest that condensin I might help assemble or reinforce a centromeric platform on which the side-by-side arrangement of sister kinetochores is firmly ensured. In this way, a pair of sister kinetochores would be recognized by the meiotic spindle as if they were a single kinetochore. It is tempting to speculate that such a structural role of condensin I in assembling centromeres of bivalent chromosomes may widely be conserved among eukaryotes.

Conclusions and perspectives

The present study investigated the spatiotemporal dynamics and function of condensins I and II in mammalian meiosis and provided evidence that they play crucial roles in multiple aspects of chromosome dynamics, including monopolar attachment of sister kinetochores, chromosome individualization, compaction, (sister chromatid) resolution, and segregation. Our data also argue that condensins I and II are most likely to play distinctive roles in these processes. Because the present study used fully grown oocytes and focused on the role of condensins after diplotene, future studies will be required for clarifying their functions at early stages of meiosis prior to diplotene, as revealed in budding yeast (Yu and Koshland, 2003) and *C. elegans* (Mets and Meyer, 2009).

MATERIALS AND METHODS

Animal study compliance

All animal experiment protocols used in the present study were approved by the Director of RIKEN Wako Institute, following a review by the Wako Animal Experiment Committee.

Antibodies

To produce specific antisera, recombinant fragments of mouse condensin subunits were expressed in *Escherichia coli*, purified, and used as antigens to immunize rabbits or rats. The following fragments were used as antigens: SMC2 (amino acids [aa] 817–1191), SMC4 (aa 845–1286), CAP–D2 (aa 858–1079), CAP-G (aa 637–1004), CAP-H (aa 1–233), CAP-D3 (aa 1160–1506), CAP-G2 (aa 789–1138), CAP-H2 (aa 1–394), and CAP-H2 (aa 267–607). The accession numbers of the mouse condensin subunit sequences are as follows: SMC2 (NM_008017), SMC4 (NM_133786), CAP-D2 (NM_146171), CAP-G (XM_485604), CAP-H (AK028919), CAP-D3 (AK139081), CAP-G2 (BC076631), and CAP-H2 (AK146642). Synthetic peptides corresponding to the C-terminal 14 amino acids of SMC2 (KIPKEAKSRGKEPN) and 16 amino acids of CAP-G (QKSKLNLAEFLNEDTS) were also used to immunize rabbits. In addition, a fragment of mouse topoisomerase IIα (aa 1162–1528) expressed in *E. coli* was also used to immunize rabbits or rats. Other antibodies used in this study included mouse polyclonal anti-REC8 antibody (Lee *et al.*, 2003) and CREST antibody (a gift from Y. Muro, Nagoya University, Nagoya, Japan). The specificity of anti-REC8 antibody was tested previously (Lee *et al.*, 2003).

Collection and culture of mouse oocytes

Three-week-old female ICR or BDF1 mice were injected with 5 IU of pregnant mare serum gonadotropin (ASKA Pharmaceutical, Tokyo, Japan). After 45 h, fully grown GV oocytes were collected and cultured for oocyte maturation and activation as described previously (Lee *et al.*, 2006). Briefly, for oocyte maturation, denuded GV oocytes were cultured for up to 16 h in mKSOM culture medium (95 mM NaCl, 2.5 mM KCl, 0.37 mM KH_2PO_4, 0.2 mM $MgSO_4$, 0.14 mM kanamycin, 2.2 mM glucose, 0.2 mM Na pyruvate, 1.7 mM $CaCl_2$, 1 mM glutamine, 25 mM $NaHCO_3$, 7.8 mM Na lactate, and 3 mg/ml bovine serum albumin [BSA]). For oocyte activation, Meta-II–arrested oocytes were cultured in mTBM culture medium (20 mM Tris, 113 mM NaCl, 3 mM KCl, 11 mM glucose, 5 mM Na pyruvate, and 2 mg/ml BSA) containing 7.5 mM strontium chloride for 6 h. To obtain Meta-I–arrested oocytes, the GV oocytes were cultured for 4 h in mKSOM culture medium and then cultured for another 3 h in the medium supplemented with MG132 (Merck Calbiochem, Darmstadt, Germany) at a final concentration of 1 µg/ml. All of the cultures were conducted under mineral oil in an atmosphere of 5% CO_2 in air at 37°C.

Antibody injection

Immature GV oocytes or maturing oocytes arrested at Meta-I with MG132 were microinjected with 6 pl of an antibody solution (antibodies against condensin subunits or control mouse IgG at a concentration of 0.5 or 2 mg/ml in PBS), using a micromanipulator with a pressure injector (Narishige, Tokyo, Japan).

Immunoblot analysis

The GV, Meta-I, and Meta-II oocytes were harvested at 0, 6, and 16 h after maturation culture, respectively. The eggs at the PN stage were harvested at 6 h after oocyte activation. The oocytes were boiled in sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, 1% [vol/vol] saturated bromophenol blue, and 50 mM dithiothreitol) at 100°C for 3 min. Thirty or 100 oocytes were used for each lane, depending on the detection limit of each antibody. The samples were separated by 7.5% SDS–PAGE and blotted onto hydrophobic polyvinylidene fluoride membranes (Immobilon-P; Millipore, Billerica, MA). The membranes were blocked with 1% BSA in TBST (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween 20) for 1 h and then incubated primary antibodies in the same buffer. After washing three times with TBST, the membranes were incubated with horseradish peroxidase–conjugated anti–rabbit IgG in TBST containing 5% skim milk. After washing with TBST, antigen signals on the membranes were visualized with a Super Signal West Femto detection system (Pierce, Thermo Fisher Scientific, Rockford, IL).

Fixation and immunofluorescence analysis

Cultured oocytes were fixed and processed for immunofluorescence labeling as described previously (Lee *et al.*, 2008). In brief, the oocytes were fixed with 2% paraformaldehyde in KB (20 mM Tris-HCl, pH 7.5, and 150 mM NaCl) for 30 min. After 15-min treatment with 0.2% Triton X-100 in KB-BSA (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% BSA), the oocytes were washed with KB-BSA twice and stored for 1 or 2 d at 4°C. The fixed oocytes were then incubated with primary antibodies at appropriate dilutions. After washing with KB-BSA three times, Alexa-labeled secondary antibodies (Invitrogen) were used for the detection of signals. DNA was counterstained with Hoechst 34580. The samples were mounted with Vectashield Mounting Medium (Vector Laboratories, Burlingame, CA) and observed under a Zeiss (Jena, Germany) LSM5 DUO or LSM710 confocal microscope with a Plan-Apochromat 100×/1.46 oil differential interference contrast objective lens at room temperature. Most of the images, unless annotated, were obtained by scanning a single section of samples using ZEN2008 software (Zeiss),

whereas the images shown in Figures 1, B–D, and 2C were obtained by a projection of serially scanned sections. All images were imported into Photoshop (Adobe, San Jose, CA), and then gamma adjustment was performed for each of the RGB channels. ImageJ software (National Institutes of Health, Bethesda MD) was used to measure signal intensities of REC8 and SMC2 on the images shown in Figure 4A.

Chromosome spreads from oocytes

Chromosome spreads were prepared from mouse oocytes for immunofluorescence analysis according to the method described previously (Hodges and Hunt, 2002).

Culture of NIH3T3 cells and siRNA treatment

NIH3T3 cells were cultured in DMEM culture medium (Gibco, Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum, 100 IU/ml penicillin (Wako Pure Chemical Industries, Osaka, Japan), and 100 μg/ml streptomycin (Wako). For siRNA treatment, the cells were transfected with Stealth siRNAs using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Briefly, the cells were treated with Opti-MEM1 supplemented with 200 nM siRNAs and 0.25% (vol/vol) Lipofectamine 2000 for 6 h, cultured in the culture medium for 42 h, and then fixed with 2% paraformaldehyde in KB for immunofluorescence analysis or extracted with sample buffer for immunoblot analysis. The Stealth siRNAs used in the present study are listed in Supplemental Table S1.

Immunoprecipitation

For immunoprecipitation analysis, a whole extract of testes of 8- to 10-wk-old ICR mice was prepared as follows. Five pairs of testis were removed, decapsulated surgically, and homogenized with a Dounce homogenizer in 10 ml of extraction buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid–KOH, pH 8.0, 100 mM KCl, 2 mM $MgCl_2$, 0.2 mM EDTA, and 10% glycerol) supplemented with a Protease Inhibitor Cocktail tablet (Roche Diagnostics, Indianapolis, IN). The homogenized solution was sonicated and centrifuged at 200,000 × *g* at 4°C for 30 min, and the supernatant was collected and used as a testis extract. Aliquots of the extract were incubated with affinity-purified antibodies against condensin subunits at 4°C for 1.5 h with rotor agitation. Protein A–Sepharose (GE Healthcare, Piscataway, NJ) beads were then added to the mixtures and further incubated for 1 h. The beads were washed five times with the extraction buffer, and the immunoprecipitates recovered were analyzed by SDS–PAGE followed by immunoblotting.

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