Dynamic localization and functional implications of Aurora-C kinase during male mouse meiosis

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Abstract

Aurora-C was first identified during screening for kinases expressed in mouse sperm and eggs. Herein, we report for the first time the precise subcellular localization of endogenous Aurora-C during male meiotic division. The localization of Aurora-C was analyzed by immunofluorescence staining on chromosome spreads of mouse spermatocytes or in squashed seminiferous tubules. Aurora-C was first detected at clusters of chromocenters in diplotene spermatocytes and was concentrated at centromeres in metaphase I and II. Interestingly, Aurora-C was also found along the chromosome axes, including both the regions of centromeres and the chromosome arms in diakinesis. During the anaphase I/ telophase I and anaphase II/telophase II transitions, Aurora-C was relocalized to the spindle midzone and midbody. A similar distribution pattern was also observed for Aurora-B during male meiotic divisions. Surprisingly, we detected no Aurora-C in mitotic spermatogonia. Furthermore, immunoprecipitation analyses revealed that INCENP associated with Aurora-C in the male testis. We propose that INCENP recruits Aurora-C (or some other factor(s) recruit INCENP and Aurora-C) to meiotic chromosomes, while Aurora-C may either work alone or cooperate with Aurora-B to regulate chromosome segregation during male meiosis.
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Introduction

The Aurora protein kinases play essential roles in many aspects of cell division including the control of centrosome and spindle function, kinetochore–microtubule interactions, and cytokinesis (reviewed by Andrews et al., 2003; Carmena and Earnshaw, 2003; Meraldi et al., 2004; Giet et al., 2005). In mammals, three distinct members of the Aurora kinases (Aurora-A, -B, and -C) have been identified, and all share similar structures at their central catalytic kinase domains. However, their N- and C-terminal sequences are quite different (Tseng et al., 1998). It is interesting to note that Aurora-C and -B share 77.6% amino acid sequence identity in their catalytic domains, while Aurora-C and -A share about 66.5% sequence identity (Tseng et al., 1998), suggesting a functional relationship between Aurora-B and -C.

Aurora-A is present at the spindle poles of mitotic cells and has emerged as a critical regulator of centrosome and spindle

function (Carmena and Earnshaw, 2003). The Aurora-A gene maps to human chromosome 20q13.2, a region that is amplified in a number of cancer cell lines and many forms of cancer (reviewed by Keen and Taylor, 2004; Meraldi et al., 2004). Overexpression of Aurora-A causes centrosome amplification and aneuploidy, which leads to the transformation of mammalian cells (Zhou et al., 1998; Meraldi et al., 2002; Littlepage et al., 2002). Recent studies showed that a Ran signaling pathway mediated by Aurora-A regulates spindle assembly (Tsai et al., 2003). The activated form of Ran (Ran-GTP) stimulates Aurora-A kinase activity by releasing spindle assembly factor TPX2 from the inhibitory binding of importin-α and -β (Tsai et al., 2003). The released TPX2 thus in turn binds to Aurora-A and stimulates its activation through autophosphorylation (Tsai et al., 2003; Eyers et al., 2003).

Aurora-B is a chromosomal passenger protein that localizes at centromeres from the prophase to the metaphase. It then dissociates from the centromeres and relocalizes to the spindle midzone and midbody during the anaphase-to-telophase transition (Andrews et al., 2003). Aurora-B forms a complex with INCENP and survivin, and the reduction of any of these three

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proteins affects the localization of the other two, suggesting their dependence on each other for correct targeting and function during mitosis (Wheatley et al., 2001; Honda et al., 2003). Furthermore, depletion or inactivation of Aurora-B, INCENP, or survivin results in similar defects in chromosome segregation and cell division (Adams et al., 2000; Kaitna et al., 2000; Wheatley et al., 2001). Microinjection of anti-Aurora-B antibodies blocks chromosome segregation and abrogates the spindle-attachment checkpoint (Kallio et al., 2002). Interestingly, Aurora-B phosphorylates the microtubule depolymerase MCAK, and such phosphorylation inactivates the microtubule depolymerization activity of MCAK and targets MCAK to the kinetochores (Lan et al., 2004; Ohi et al., 2004; Andrews et al., 2004). Aurora-B has also been reported to play an essential role in cytokinesis (Murata-Hori and Wang, 2002; Mishima and Glotzer, 2003).

Aurora-C (mouse AIE1/human AIE2) was first identified in our laboratory during screening for kinases expressed in sperm and eggs (Tseng et al., 1998). Unlike Aurora-A and -B, which are ubiquitously expressed in many tissues, particularly in mitotically dividing cells, Aurora-C was found prominently in the testis (Bernard et al., 1998; Tseng et al., 1998; Kimura et al., 1999). RNA in situ hybridization showed that Aurora-C mRNA was mainly restricted to meiotically active germ cells, with the highest levels detected in late pachytene spermatocytes (Hu et al., 2000). Aurora-C was also reported to be overexpressed in a variety of human tumor cell lines (Kimura et al., 1999), however, its presence in both normal mitotic cells and cancer cell lines is still debatable (see Discussion).

Recently, it has been reported that Aurora-C is a novel chromosomal passenger protein that binds to INCENP (Li et al., 2004; Sasai et al., 2004; Chen et al., 2005) and can complement Aurora-B kinase function in mitotic cells (Sasai et al., 2004). Interestingly, overexpression of an Aurora-C kinase-deficient mutant not only inhibits centromere/kinetochore localization of Aurora-B, Bub1, and BubR1, but also disrupts the association of INCENP with Aurora-B (Chen et al., 2005), suggesting that Aurora-C may play similar roles as Aurora-B in mitosis.

The conclusion that Aurora-C is a chromosomal passenger protein is based on results obtained either with ectopically expressed green fluorescent protein (GFP)-tagged constructs or with the immunofluorescence of tag epitopes in somatic cells (Li et al., 2004; Sasai et al., 2004; Chen et al., 2005). Direct immunofluorescence of endogenous Aurora-C has never been detected in somatic cells. The slow progress in the characterization of Aurora-C during the past few years has been due to a lack of specific antibodies and very limited cell sources (such as meiotic germ cells) for such a study. In this study, we have generated both a polyclonal antibody and a monoclonal antibody that specifically recognize mouse Aurora-C. Our biochemical and immunofluorescence analyses showed that the expression of Aurora-C appeared to be restricted mainly to meiotic germ cells and was not found in the examined normal mouse somatic tissues or cell lines. Herein, we report for the first time the precise sublocalization of endogenous Aurora-C during male meiotic division. The putative functions of Aurora-C in meiosis are discussed.

Materials and methods

Antibodies

We previously showed that the three mammalian Aurora kinases revealed high sequence homology in the middle kinase domain (Tseng et al., 1998). However, much-lower homology was found in sequences outside the kinase domain. To generate Aurora-C-specific antibodies, two synthetic peptides derived from the unique sequences located at the N-terminal (N1; residues 1–14) and C-terminal (C1; residues 263–274) regions of mouse Aurora-C were synthesized and coupled to multiple antigenic peptides (MAPs, Genemed Synthesis, South San Francisco, CA). The peptides were combined and used as immunogens to raise antibodies in rabbits (polyclonal antibody). The polyclonal antibodies were then affinity-purified from whole serum using the GST-Aurora-C fusion protein on an affinity matrix. Generation and purification of hybridoma and ascites against GST-Aurora-C were carried out following previously described procedures (Tang et al., 1993). A rabbit anti-INCENP antibody was generated against a mixture of two KLH-conjugated peptides (peptide 1: REPPQSVRRKRSYK; peptide 2: KRNTPLRVDPKEKE) of mouse INCENP and was similarly affinity-purified.

The following antibodies were purchased from the indicated commercial sources: rabbit polyclonal antibodies against SMC3 (Novus Biologicals, Littleton, CO) and ACA (Antibodies Inc., Davis, CA); mouse monoclonal antibodies against Aurora-B (AIM-1; BD Biosciences, San Jose, CA), CENP-H (BD Biosciences), GFP (BD Biosciences), α-tubulin (DM1A; Sigma-Aldrich, St. Louis, MO), Flag (M2, Sigma-Aldrich), and β-actin (AC-74; Sigma-Aldrich); and goat polyclonal antibody against Aurora-A (Santa Cruz Biotechnology, Santa Cruz, CA).

Fractionation and preparation of male germ cells

Spermatogenic germ cells from the testes of 8- to 10-week-old mice were fractionated by sedimentation as previously described (Bellve, 1993) with modifications. Briefly, testes were detunicated and digested with collagenase and trypsin. The resulting cell suspension was layered on top of a discontinuous density gradient prepared with the following Percoll concentrations: 45% and 70% in PBS. After centrifugation, the cellular fraction on top of the 45% layer was collected. The enriched single-cell suspension of germ cells was further purified by sedimentation through a 2%–4% BSA gradient at unit gravity in a STA-PUT chamber as previously described (Bellve, 1993). Fractions were examined for morphology and purity by light microscopy. The DNA content of fractionated male germ cells was analyzed by a flow cytometer FACS Calibur (Fluorescence Activated Cell Sorter, BD Biosciences, San Jose, CA) as previously described (Hung et al., 2004). The present study was approved by the Animal Committee for Care and Use of Laboratory Animals, Institute of Biomedical Sciences, Academia Sinica.

Squashing of mouse spermatocytes

Adult male C57BL/6 mice (>8 weeks) were sacrificed, and their testes were removed and detunicated. The seminiferous tubules were processed using a squashing method as previously described (Page et al., 1998; Parra et al., 2003). Briefly, seminiferous tubules were fixed in freshly prepared 2% formaldehyde in PBS containing 0.1% Triton X-100 for 5–10 min at room temperature. After fixation, several seminiferous tubule fragments were placed in a drop containing fixing solution on a slide coated with poly-L-lysine. The tubule fragments were squashed by placing a coverslip on top of the tubules. After squashing, the tubule-containing slides were quickly frozen in liquid nitrogen, and the coverslip was removed. The slides were washed in PBS and processed for immunofluorescence analysis.

Preparation of chromosome spreads of mouse spermatocytes

Spermatogenic germ cells were fractionated and collected using the discontinuous Percoll gradient method described above. For chromosome spreads of spermatocytes, we followed the drying-down method described by Peters et al. (1997). Briefly, enriched spermatocytes were first placed in a

hypotonic solution containing 30 mM Tris, 50 mM sucrose, 17 mM trisodium citrate, 5 mM EDTA, 0.5 mM DTT, and 0.5 mM PMSF (pH 8.2) for 30 min. The cell suspension was then dispersed on a coverslip that contained the fixation solution (1% paraformaldehyde (pH 9.2) and 0.15% Triton X-100). The coverslip was allowed to slowly dry in a humid chamber for several hours.

Immunofluorescence and confocal microscopy

The spermatogenic cells prepared by the squashing method were fixed with 2% formaldehyde in PBS (Parra et al., 2003). After fixation, cells were permeabilized with 0.1% Triton X-100/PBS for 10 min followed by incubation with a blocking solution containing 10% sheep serum in PBS for 30 min. Cells were then probed with the indicated primary antibodies in blocking solution at room temperature for 60 min. After washing with PBST (0.1% Tween 20 in PBS), cells were incubated with the appropriate secondary antibodies conjugated with either Alexa 488, Alexa 568, or Alexa 647 (Molecular Probes, Eugene, OR) for 60 min at room temperature. DNA was counterstained with DAPI (Sigma-Aldrich). Samples were observed with a laser scanning confocal system (MRC 2100, Bio-Rad Laboratories, Hercules, CA).

Preparation of mouse testis extracts and immunoprecipitation

Nuclear extracts of testes were prepared from 6- to 8-week-old C57BL/6 mice as previously described (Lee et al., 2003) in a modified RIPA buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na deoxycholate, 30 μg DNase, 30 μg RNase, 20 mM β-glycerophosphate, 50 mM NaF, 0.2 mM sodium vanadate, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml chymostatin, 1 μg/ml aprotinin). For immunoprecipitation experiments, the nuclear extracts prepared above were incubated with the indicated antibodies for 2 h at 4°C. After incubation, Protein A or Protein G sepharose beads were added to the mixture and incubated for another 2 h at 4°C. The sepharose beads were then washed 4 times with the RIPA buffer and one time with PBS. The immunoprecipitates were analyzed by SDS-PAGE followed by immunoblotting with anti-Aurora-C or anti-Aurora-B antibodies.

Results

Specificity of Aurora-C antibodies

We generated two antibodies, a polyclonal and a monoclonal one, both of which recognize mouse Aurora-C. To test the specificity of these antibodies, we performed an immunoblot analysis. The expression constructs encoding full-length mouse Aurora-A, -B, and -C tagged at their N-termini or the C-terminus with the Flag epitope were transfected into HeLa cells. Immunoblot analyses showed that the anti-Flag antibody detected all three Flag-tagged proteins (Fig. 1A). However, our monoclonal antibody recognized only Aurora-C, indicating its high specificity (Fig. 1A). The specificity of the affinity-purified polyclonal Aurora-C antibody was also examined and found to have no cross-reactivity with Aurora-A or -B (Fig. 1D).

4C meiotic cells are the major germ cells that express Aurora-C

We previously reported that Aurora-C transcripts appeared to be expressed mainly in testes, with few or no Aurora-C transcripts detected in normal somatic tissues (Tseng et al., 1998). We first examined the expression of endogenous Aurora-C protein in various mouse tissues and cell lines using our newly generated antibodies.

Total cell lysates prepared from extracted tissues or cells were immunoblotted with either a monoclonal (Fig. 1B) or a polyclonal (Fig. 1E) anti-Aurora-C antibody. As shown in Fig. 1B, no Aurora-C signal was detected in the examined mouse tissues (including the liver, lungs, kidneys, brain, and spleen) except the testis. To investigate which cell types in the testis expressed Aurora-C, we partially purified the spermatogenic cells from mouse testes using the STA-PUT chamber (Bellve et al., 1993). The average purities of 4C cells (mostly representing meiotic primary spermatocytes at the prophase), 2C cells (mostly representing mitotic spermatogonia and Sertoli cells), and 1C cells (mostly representing haploid spermatids at different differentiation stages) were ∼90% (fractions 1 to 3), ∼55% (fractions 6 and 7), and ∼80% (fractions 10 and 11), respectively (Fig. 1C).

We next analyzed the lysates prepared from enriched 4C, 2C, and 1C cells by immunoblotting using either a monoclonal (Fig. 1B) or a polyclonal (Fig. 1E) antibody. Fig. 1B shows that endogenous Aurora-C was mainly detected in enriched 4C cells, however, a weaker Aurora-C signal was also observed in fractions containing 2C and 1C cells. The detection of Aurora-C in 2C cells (∼55% purity) could have resulted from contamination of 4C cells during purification (Fig. 1C). However, the detection of Aurora-C in 1C cells was possibly due to incomplete dissociation of Aurora-C from the chromocenters during meiotic II division since our immunofluorescence results showed that Aurora-C was detected within the nuclei of early round spermatids (1C) (Fig. 5D).

Furthermore, we also examined other mouse tissues (heart and muscle) and several mouse cell lines including F0 (a myeloma), TSA (an adenocarcinoma), 3T3 (a fibroblast cell line), Hepa1–6 (a hepatoma), and TM4 (a testis Sertoli cell line) using the Aurora-C monoclonal antibody (Fig. 1B). Again, no detectable Aurora-C signal was found in the examined tissues (unpublished data) or cell lines (Fig. 1B) even after a long exposure. Similar results were also observed using the polyclonal anti-Aurora-C antibody (Fig. 1E and unpublished data). Together, our results indicate that 4C meiotic cells in the testis are the major germ cells expressing Aurora-C.

Localization of Aurora-C in the meiotic prophase

The meiotic prophase in germ cells consists of five sequential stages: leptonema, zygonema, pachynema, diplonema, and diakinesis. In meiotic prophase (prophase I), homologous chromosomes pair and associate by a zipper-like structure, the synaptonemal complex (SC). Synapsis is a process to pair homologous chromosomes intimately and is mediated by the SCs. Synapsis begins in zygonema and is complete throughout pachynema. Homologous recombination takes place between the paired chromosomes. At meiosis I, homologous chromosomes disjoin, while, at meiosis II, the sister chromatids separate, which finally brings the reduction of DNA content from diploid to haploid (Petronczki et al., 2003).

We previously showed that the expression of Aurora-C transcripts was mainly restricted to meiotically active germ
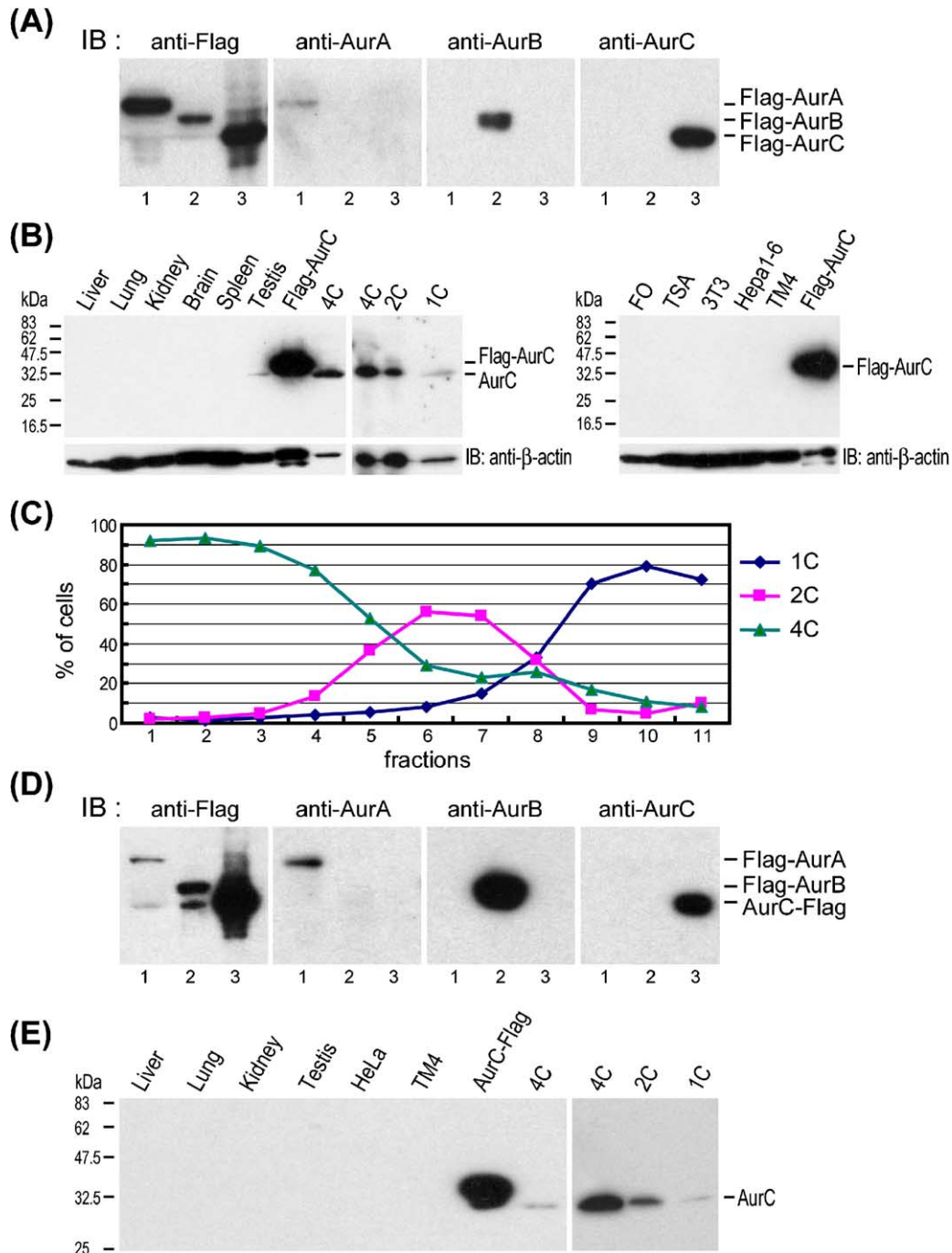
Fig. 1. Expression of Aurora-C in meiotic cells. (A) Specificity test for Aurora-C monoclonal antibody. Flag-tagged Aurora-A (lane 1), -B (lane 2), or -C (lane 3) constructs were transfected into HeLa cells. Twenty-four hours after transfection, cell lysates (~10 μg) were immunoblotted with specific antibodies against Flag, Aurora-A, -B, or -C (monoclonal antibody). (B) Immunoblot analyses with a monoclonal antibody against Aurora-C. Cell lysates prepared from various mouse tissues (~150 μg), fractionated 4C, 2C, and 1C spermatogenic cells (5 × 10^5 cells in each lane), or various mouse cell lines (~150 μg) were analyzed by immunoblotting. The cell lysate prepared from Flag-tagged Aurora-C-transfected cells was used as a positive control. β-Actin was used as an internal control for protein loading. (C) Isolation and fractionation of spermatogenic cells. Spermatogenic cells were fractionated by sedimentation through a 2%–4% BSA gradient. Each cell fraction was collected and analyzed by FACS Calibur. (D) Specificity test for Aurora-C polyclonal antibody. (E) Immunoblot analyses of cell lysates prepared from various mouse tissues and fractionated spermatogenic cells with a polyclonal antibody against Aurora-C.

cells (Hu et al., 2000). However, the precise subcellular localization of the Aurora-C protein in germ cells is not clear. To examine the localization of Aurora-C in spermatogenic cells, we compared the distribution pattern of Aurora-C with those of several well-studied proteins located either at the centromere/kinetochore [Aurora-B (Parra et al., 2003), INCENP (Parra et al., 2003), CENP-H (Fukagawa et al., 2001), and ACA (Page et al., 1998)], at the lateral element of synaptonemal complex [SMC3 (Eijpe et al., 2003)] on chromosome spreads of mouse spermatocytes (Figs. 2 and 4; Fig. S1) or in squashed seminiferous tubules (Figs. 3, 5, and 6).
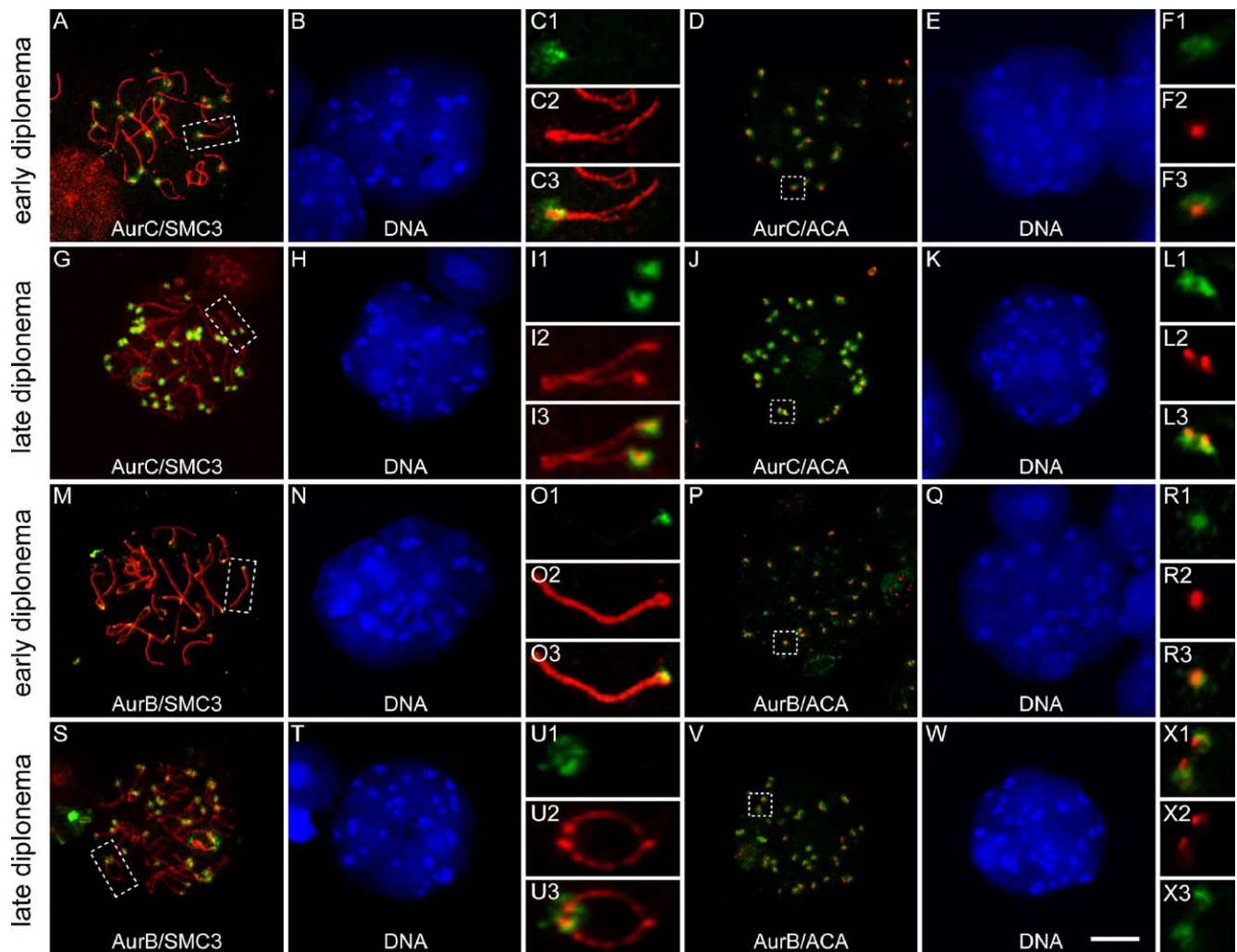
Fig. 2. Localization of Aurora-C at diplotene spermatocytes. Chromosome spreads of mouse spermatocytes at the early (A–F, M–R) or late (G–L, S–X) diplotene stages were double-immunolabeled with anti-Aurora-C (green) and anti-SMC3 (red) antibodies (A–C, G–I), with anti-Aurora-C (green) and ACA (red) antibodies (D–F, J–L), with anti-Aurora-B (green) and anti-SMC3 (red) antibodies (M–O, S–U), or with anti-Aurora-B (green) and ACA (red) antibodies (P–R, V–X). DNA was counterstained with DAPI (blue). Images were collected as Z-stacks using confocal microscopy, converted to maximum-intensity projections, and printed with identical settings. (C1–C3) An enlarged view of the boxed region derived from A; (F1–F3) that derived from D; (I1–I3) that derived from G; (L1–L3) that derived from J; (O1–O3) that derived from M; (R1–R3) that derived from P; (U1–U3) that derived from S; and (X1–X3) that derived from V. Scale bar = 10 μM.

We first examined the temporal expressions of Aurora-C and -B during the meiotic prophase. No Aurora-C or -B signals were detected at the leptotene (unpublished data), zygotene (see Figs. S1A–D, E–H), or pachytene stages (see Figs. S1I–L, M–P). When germ cells progressed to the early diplotene stage, Aurora-C was detected at clusters of chromocenters (Figs. 2A, C1–C3) and appeared to have accumulated at the centromeric regions as evidenced by ACA staining (Figs. 2D, F1–F3). No or a very weak Aurora-C signal was detected along SMC3-labeled synaptonemal complexes (SCs) (Figs. 2C1–C3). At the end of the diplotene stage, Aurora-C was seen as very bright dots in the centromeric regions (Fig. 2G). At this stage, most centromeres of desynapsed chromosomes had separated into two spots (Figs. 2I1–I3) as evidenced by ACA staining (Figs. 2J, L1–L3). A similar distribution pattern was also observed for Aurora-B kinase during the early (Figs. 2M, O1–O3; P, R1–R3) and late (Figs. 2S, U1–U3; V, X1–X3) diplotene stages. Furthermore, the signals detected at the

centromeric regions in diplotene spermatocytes using both Aurora-C and -B antibodies were not non-specific since these centromeric stainings could be competed out by co-incubating the antibody with an excess of antigens (Aurora-C: Figs. S1S–T; Aurora-B: Figs. S1W–X).

Aurora-C shows dynamic localization during male meiosis I

Since chromosome spreads are not convenient for tracing the localization of Aurora-C during various meiotic stages, the squashing immunofluorescence method (Parra et al., 2003) was performed, which allowed observation of spermatogenic cells at different developmental stages in the same preparations. Centromere/kinetochore proteins such as INCENP, Aurora-B (Parra et al., 2003), and CENP-H (Fukagawa et al., 2001) were used as immunofluorescent markers for tracing the distribution of Aurora-C during various meiotic division stages.
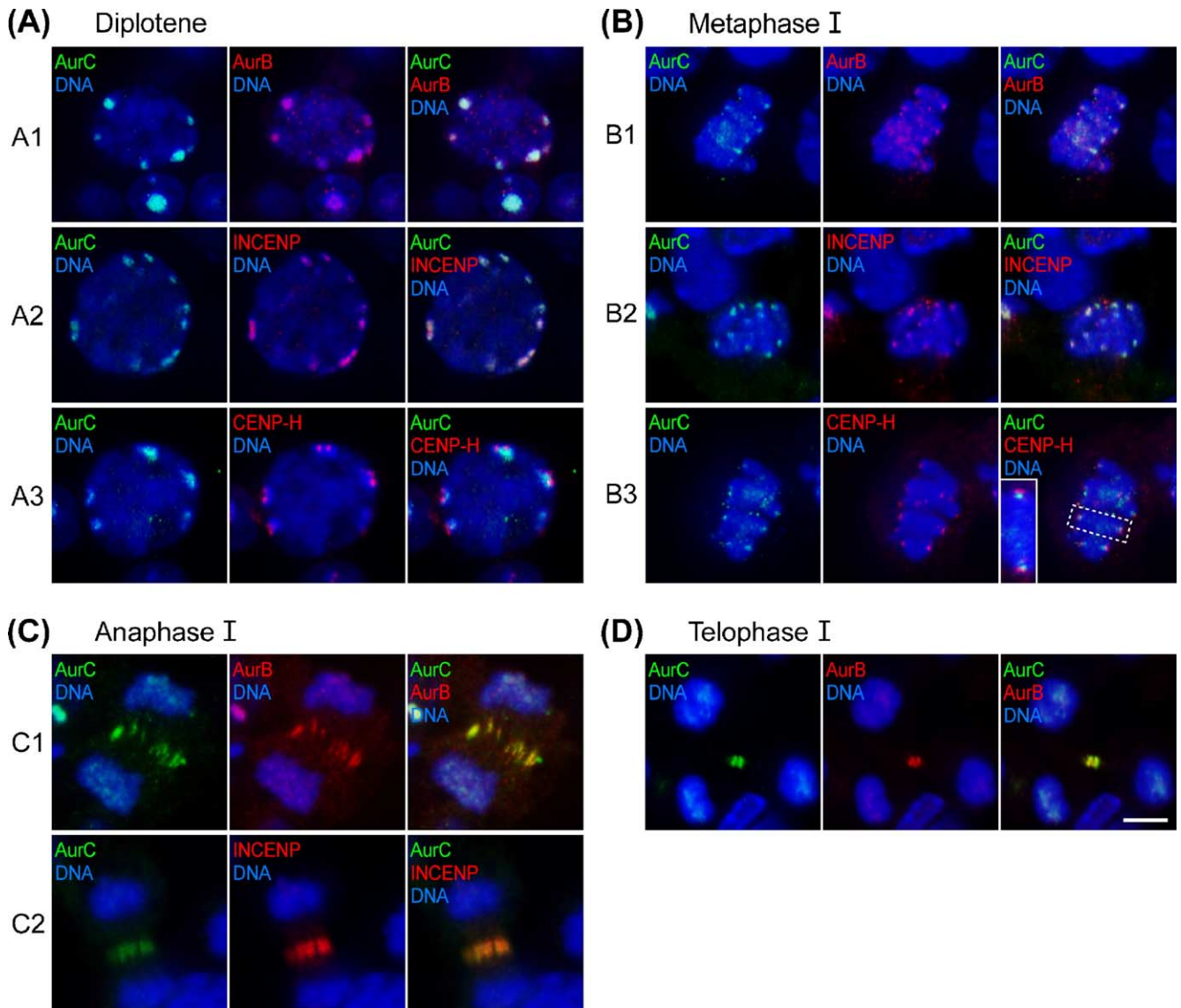
Fig. 3. Dynamic localization of Aurora-C during male meiosis I. Aurora-C expression patterns in mouse spermatocytes at the diplotene (A), metaphase I (B), anaphase I (C), and telophase I (D) stages are shown. Spermatogenic cells were prepared by the squashing method and analyzed by confocal fluorescence microscopy. Aurora-C colocalized with Aurora-B (A1) and INCENP (A2) at the chromocenters, which represent clustered centromeric regions of chromosomes in diplotene spermatocytes. (A3) Aurora-C signals partially overlapped with CENP-H (a known kinetochore marker) signals at the chromocenters. Aurora-C colocalized with Aurora-B (B1) and INCENP (B2) in metaphase I spermatocytes. (B3) Aurora-C was located beneath kinetochores labeled with the CENP-H signals. The inset shows the magnification of a region of B3 marked by a white rectangle. Aurora-C colocalized with Aurora-B and INCENP at the early anaphase I (C1), late anaphase I (C2), and telophase I (D) stages. Scale bar = 5 μm.

Consistent with observations of chromosome spreads (Fig. 2), we detected no or very weak signals of Aurora-C and -B in pachytene spermatocytes using the squashing method (unpublished data). However, Aurora-C was strongly detected in diplotene spermatocytes (Fig. 3A) as it was in chromosome spreads (Figs. 2A, G). As shown in Fig. 3A, Aurora-C signals appeared as large bright nuclear staining corresponding to the heterochromatic chromocenters commonly found at the nuclear periphery. These chromocenters represented clustered centromere heterochromatic regions of chromosomes. Fig. 3A also shows that Aurora-C was colocalized with Aurora-B (Fig. 3A1) and INCENP (Fig. 3A2) at the chromocenters in diplotene spermatocytes. Interestingly, the CENP-H antibody recognized sister kinetochores, which appeared as pairs of dots located on top of the Aurora-C signals (Fig. 3A3). The appearance of Aurora-B and INCENP in diplotene spermatocytes agrees with a previous report (Parra et al., 2003).

During metaphase I, Aurora-C was colocalized with Aurora-B (Fig. 3B1) and INCENP (Fig. 3B2) mainly in the centromeric regions. Interestingly, most of the Aurora-C labeling was detected beneath the kinetochore CENP-H signals, although some degree of overlap was observed (Fig. 3B3). Thus, Aurora-C is most likely located between CENP-H and the heterochromatin. At the onset of anaphase, Aurora-C, like Aurora-B, was transferred from the centromeres (early anaphase I, Fig. 3C1) to the spindle midzone (late anaphase I, Fig. 3C2) and was eventually concentrated at the midbody (telophase I, Fig. 3D).

To examine the localization of Aurora-C during the diakinesis-to-metaphase I transition in more detail, immuno-fluorescence staining of chromosome spreads of meiotic cells was performed. Surprisingly, a considerable amount of Aurora-C signal was detected along the chromosomal axes, which covered both the regions of the centromere and the chromosome arms during diakinesis (Figs. 4A, D1–D4). Intense Aurora-C signals were frequently observed in the arm regions proximal to the centromeres (Figs. 4D1–D4). At the MI stage, however, most Aurora-C signals were detected at the centromeres (Figs. 4E–G, H1–H4). Similar results were also observed for Aurora-B kinase (Figs. 4M–O, P1–P4).

By comparing Aurora-C signals between the diakinesis (Fig. 4D1) and MI stages (Fig. 4H1), it is reasonable to speculate that Aurora-C gradually dissociates from the arms and accumulates at the centromeres during the diakinesis-to-MI transition. Since very few cells are present at this transition stage during normal meiotic division, we treated pachytene spermatocytes with okadaic acid (OA), a protein phosphatase inhibitor. It has been reported that OA can induce a rapid and premature G2/M transition which is accompanied with the disassembly of SCs (Wiltshire et al., 1995). After OA treatment, discontinuous signals of Aurora-C dotted along the chromosome arms were clearly visible in some (~10%) OA-
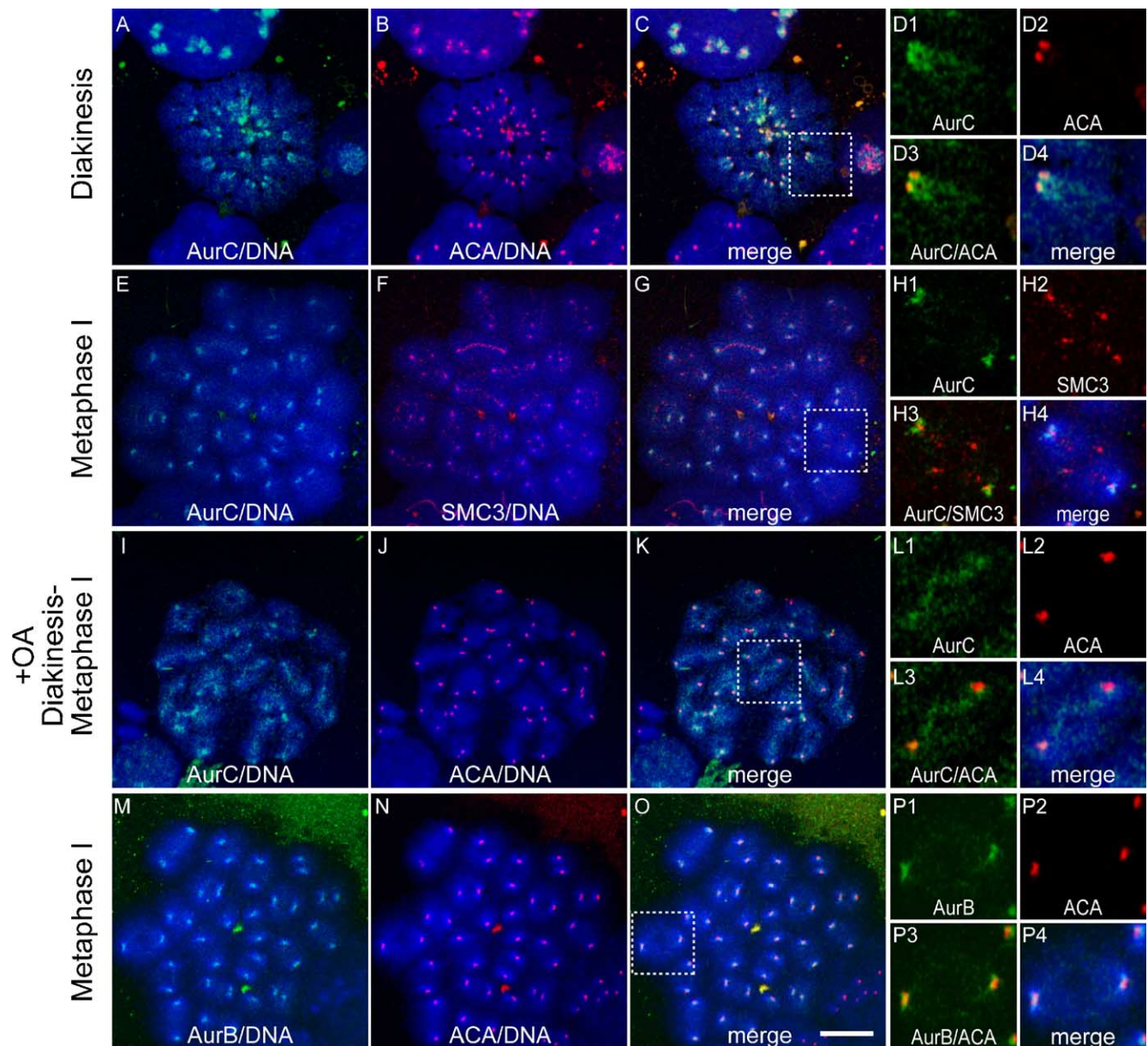

Fig. 4. Aurora-C association with chromosome arms and centromeres during the diakinesis/MI transition. Chromosome spreads of mouse spermatocytes at diakinesis (A–D), MI (E–H, M–P), or the OA-induced diakinesis/MI transition (I–L) stages were double-immunolabeled with anti-Aurora-C (green) and ACA (red) antibodies (A–D, I–L), with anti-Aurora-C (green) and anti-SMC3 (red) antibodies (E–H), or with anti-Aurora-B (green) and ACA (red) antibodies (M–P). DNA was counterstained with DAPI (blue). (I–L) Chromosome spreads of mouse spermatocytes treated with 0.5 μM okadaic acid (OA) for 6 h to induce a premature G2/M transition. (D1–D4) An enlarged view of the boxed region derived from C; (H1–H4) that derived from G; (L1–L4) that derived from K; and (P1–P4) that derived from O. Scale bar = 10 μM.

treated cells, most likely representing the diakinesis-to-MI transition (Figs. 4I–K, L1–L4). While in others (∼90%), Aurora-C signals were prominently found at the centromeres of MI spermatocytes (a pattern similar to Fig. 4E). Together, our results suggest that Aurora-C is localized along the chromosome arms and centromere regions, while its arm association is gradually lost during the diakinesis-to-meiosis I transition.

Distribution pattern of Aurora-C during meiosis II

In metaphase II spermatocytes, Aurora-C was also colocalized with Aurora-B as detected in squashed seminiferous tubules. They were detected not only at the centromeres, but also in interbridge areas between sister kinetochores (Fig. 5A, upper panel). This pattern of localization is similar to that of INCENP in metaphase II spermatocytes (Parra et al., 2003). Again, partial colocalization of Aurora-C with CENP-H was also observed, and Aurora-C signals were frequently found in the region between sister kinetochores in metaphase II spermatocytes (Fig. 5A, lower panel). Aurora-C then relocated from the centromere to the spindle midzone during anaphase II (Fig. 5B) and finally concentrated at the midbody at telophase II (Fig. 5C). In early round spermatids, Aurora-C appeared to be colocalized with chromocenters within the nuclear interior as revealed by CENP-H and DAPI staining (Fig. 5D). Interestingly, Aurora-C did not completely dissociate from the chromocenters during the transition either from anaphase I to telophase I (Figs. 3C, D) or from anaphase II to telophase II (Figs. 5B, C). The latter event may explain why Aurora-C was commonly detected within the nuclei of early round spermatids.

The absence of Aurora-C from mitotic spermatogonia

Unlike Aurora-B and -C found in male meiotic germ cells, Aurora-C signals were not detected in spermatogonia during various mitotic stages in squashed seminiferous tubules (Fig. 6A). In contrast, Aurora-B was detected at centromeres in mitotic spermatogonia at the metaphase and was later found in the midzone and midbody at the anaphase and telophase, respectively (Fig. 6A). Our results showed that Aurora-C revealed a similar distribution pattern to that of many chromosome passenger proteins during male meiotic divisions. Since we detected no Aurora-C protein expression in mitotic spermatogonia (Fig. 6A) or in the examined somatic cells or tissues (Fig. 1C), Aurora-C may represent a new meiotic chromosomal passenger protein.

Association of Aurora-C with INCENP in male testis

The colocalization of Aurora-C with INCENP in male meiotic cells (Fig. 3) suggests that Aurora-C may interact with INCENP in vivo. To investigate this possibility, we immunoprecipitated testis nuclear extracts with antibodies against several chromosomal passenger proteins and analyzed the immunoprecipitates by immunoblotting. As shown in Fig. 6B

(left panel), anti-INCENP immunoprecipitate contained Aurora-C, suggesting that the protein complex exists in vivo that contains both Aurora-C and INCENP. In contrast, no Aurora-C was detected in the immunoprecipitate using the control antibody. Furthermore, anti-INCENP immunoprecipitate contained both Aurora-B and INCENP (Fig. 6B, right panel). Together, these results suggest that INCENP can form a complex with either Aurora-C or -B in the testis.

Interestingly, the anti-Aurora-B immunoprecipitate appears to contain a little amount of Aurora-C (Fig. 6B, left panel) and that the reverse experiment using the anti-Aurora-C antibody confirms that result (Fig. 6B, right panel). The shifted Aurora-B signal in the anti-Aurora-C immunoprecipitate may suggest that the Aurora-B kinase present together with Aurora-C in the complex is modified (possibly by phosphorylation). Together, we concluded that two major but distinct complexes (Aurora-B/INCENP and Aurora-C/INCENP) are present in the testis. However, the possible presence of a minor complex that contains both Aurora-B and -C kinases cannot be excluded.

Discussion

Dynamic localization of Aurora-C during male meiotic division

In this study, we have analyzed the distribution and subcellular localization of Aurora-C during male mouse meiosis. We unexpectedly found that Aurora-C is colocalized with Aurora-B and INCENP during male meiotic division. A summary of the subcellular localization of Aurora-C during male meiotic division is given in Fig. 7. The Aurora-C signal was first detected in centromeric regions in early diplotene spermatocytes (Fig. 2A) and then gradually increased in amount and was concentrated at the centromeres in the late diplotene stage (Fig. 2G). At diakinesis, Aurora-C was localized along the chromosomal axes between sister chromatids, including the centromeres and arm regions. During the transition from diakinesis to MI, Aurora-C gradually dissociated from the chromosome arms and became concentrated at the centromeres near the kinetochores. It then relocalized to the spindle midzone and midbody at anaphase I and telophase I, respectively. Aurora-C also showed a dynamic change in localization from chromosomes to the central spindle during meiosis II (Fig. 5).

Aurora-C expression is mainly restricted to meiotic germ cells

There are conflicting reports on the expression of Aurora-C in human cancer cell lines. According to a very recent study, Aurora-C does not seem to be overexpressed in tumor cells nor does its expression correlate with Aurora-A or -B expression (Keen and Taylor, 2004). Using both polyclonal and monoclonal antibodies that specifically recognize mouse Aurora-C, we detected very little or no expression of Aurora-C protein in normal mouse somatic tissues, including the liver, lungs, kidneys, spleen, muscle, brain, and heart (Fig. 1B and unpublished data), or in several mouse cell lines including TM4 (a Sertoli cell line), TSA (an adenocarcinoma), Hepa1–6
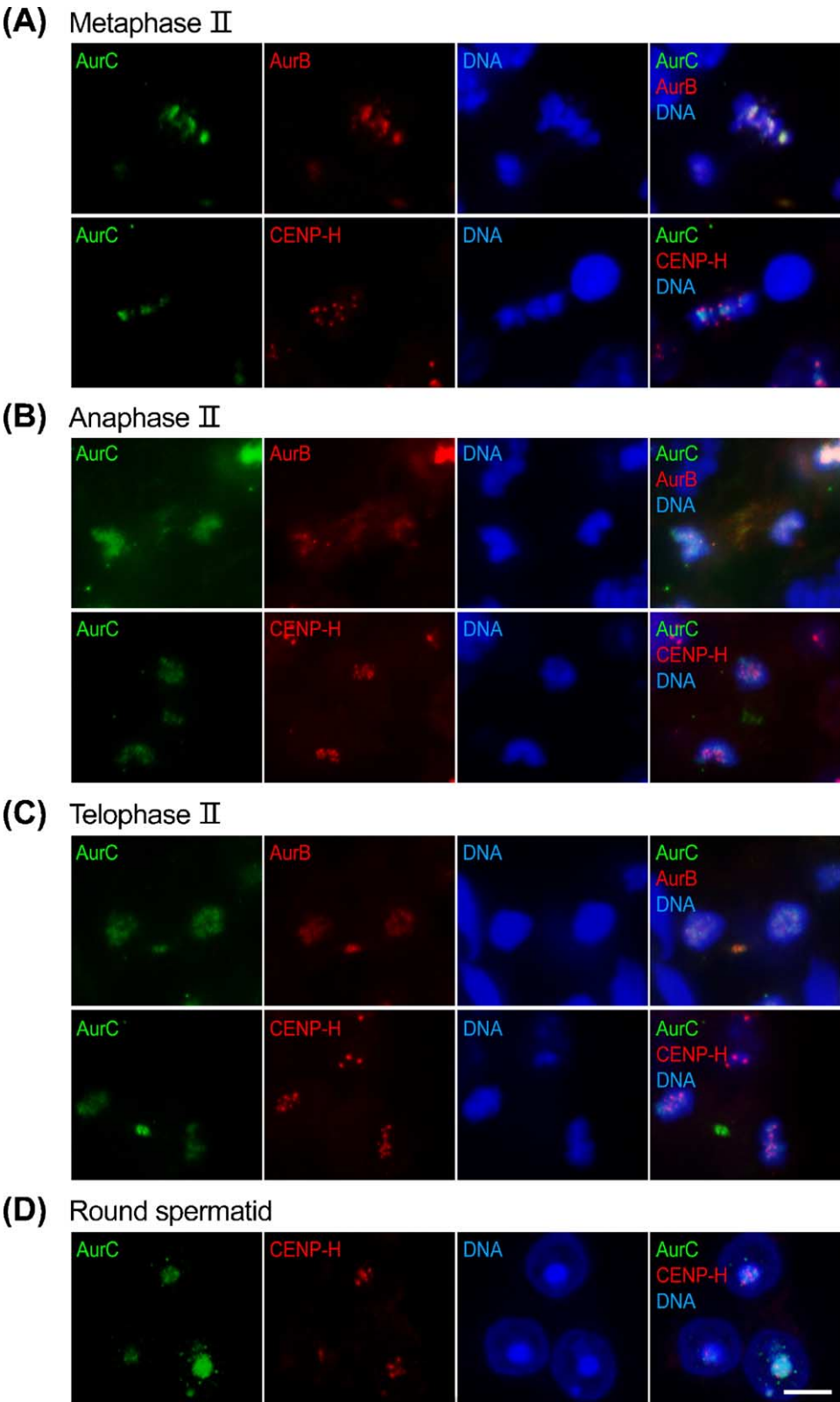
Fig. 5. Localization of Aurora-C during male meiosis II. Aurora-C expression in spermatocytes at metaphase II (A), anaphase II (B), telophase II (C), and the round spermatid stage (D). Spermatogenic cells were prepared by the squashing method and analyzed by confocal fluorescence microscopy using the indicated antibodies. Scale bar = 5 μM.

(a hepatoma), F0 (a myeloma), and 3T3 (Fig. 1B and unpublished data), or even in mitotic spermatogonia (Fig. 6A). At the present stage, we cannot exclude the possibility that Aurora-C is expressed in other normal tissues or cell lines in which we did not examine in this report. On the basis of the current results, we concluded that Aurora-C may be regarded as
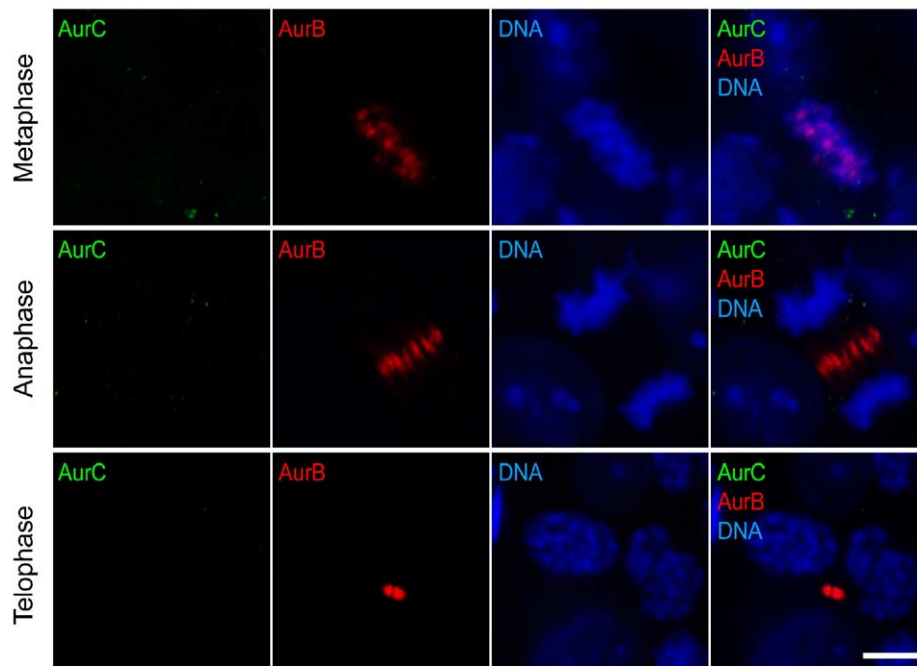
(A) Spermatogonia at mitosis

(B)

Fig. 6. (A) The absence of Aurora-C from mitotic spermatogonia. Spermatogenic cells were prepared by the squashing method and analyzed by confocal fluorescence microscopy using antibodies against Aurora-C (green) and -B (red). DNA was counterstained with DAPI (blue). The distribution of Aurora-B in mitotic spermatogonia was similar to that observed in cultured somatic cells; however, no Aurora-C was detected in spermatogonia during the entire mitotic cycle. Scale bar = 5 μM. (B) Direct association of INCENP with Aurora-C or -B in the testis. Nuclear cell extracts prepared from spermatogenic cells were immunoprecipitated (IP) with anti-Aurora-C, anti-Aurora-B, anti-INCENP, or control (normal rabbit IgG) antibodies. The precipitated protein complexes were then analyzed by immunoblotting (IB) with an anti-Aurora-C (left panel) or anti-Aurora-B antibody (right panel).

a meiotic chromosomal passenger protein, which appears to mainly be expressed in and restricted to meiotic cells. The inability of our antibodies to recognize human Aurora-C prevented us from examining Aurora-C in human samples. Future generations of a human-specific Aurora-C antibody may help to resolve this discrepancy.

Possible interaction between INCENP and Aurora-C during meiosis

In this study, we found that Aurora-C was colocalized with Aurora-B and INCENP during male meiotic division. Similar results were also observed in somatic cancer cell lines using ectopically expressed tagged Aurora-C constructs (Li et al., 2004; Sasai et al., 2004; Chen et al., 2005). INCENP appears in meiotic cells prior to Aurora-B and -C. INCENP was first detected at the central element (CE) of the SC from the zygotene to the late pachytene stage. It then relocalized to heterochromatic chromocenters during the diplotene stage (Parra et al., 2003). As shown in Fig. 2, both Aurora-B and -C were first detected in the early diplotene stage at chromo centers that were already labeled by INCENP. After the

diplotene stage, INCENP appeared to colocalize with Aurora-B and -C throughout the entire meiotic division. Thus, it is possible that INCENP may recruit both Aurora-B and -C to appropriate sites within cells during meiotic division. Alternatively, some other protein(s) may recruit both INCENP and Aurora-C to meiotic chromosomes to perform their functions during meiosis.

Recently, INCENP was reported to be a substrate and a positive regulator for Aurora-B kinase in somatic cells. INCENP contains both a conserved carboxy-terminal IN-box that binds Aurora-B (Honda et al., 2003) and a non-conserved amino-terminal region that is essential for its targeting to centromeres (Ainsztein et al., 1998). Phosphorylation of the carboxyl terminus of INCENP by Aurora-B enhances the activity of the kinase (Bishop and Schumacher, 2002; Honda et al., 2003). Thus, as it occurs in somatic cells, INCENP might also bind and activate Aurora-C kinase in meiotic germ cells in a similar fashion. Indeed, our observation that Aurora-C co-immunoprecipitates with INCENP (Fig. 6B) and the finding that INCENP binds (Li et al., 2004; Chen et al., 2005) and activates Aurora-C in transfected somatic cells (Li et al., 2004; unpublished data) are consistent with this hypothesis.
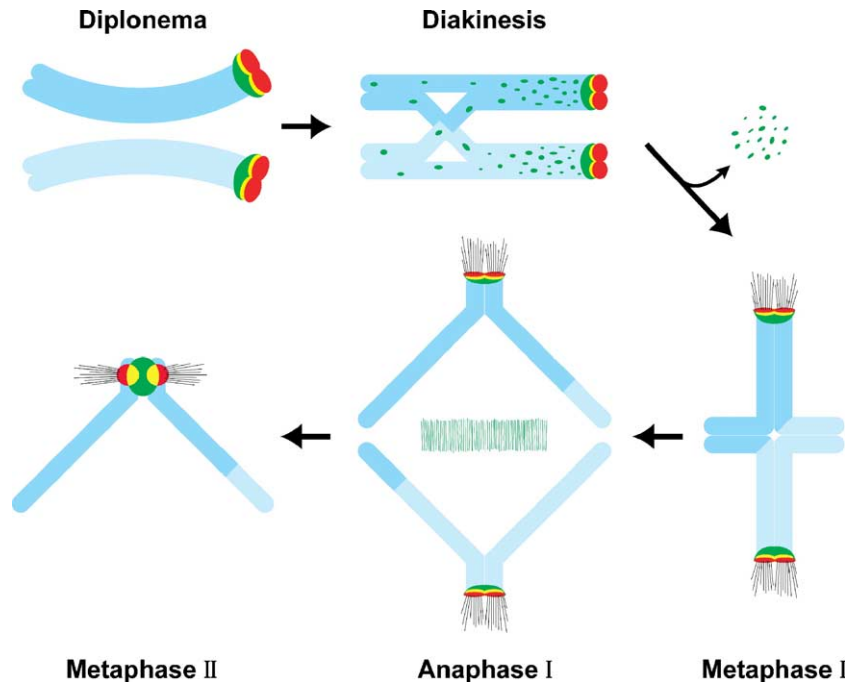
Fig. 7. Schematic representation of the distribution of Aurora-C during male meiotic division. Aurora-C is labeled green, and centromeres/kinetochores are labeled red. Chromosomes are labeled blue. Regions of overlap are yellow. Aurora-C signals were gradually lost from the chromosome arms and accumulated at the centromeres during the diakinesis-to-MI transition.

Aurora-C in meiosis: functional implications

There are five major processes that are unique to chromosome segregation and cytokinesis during mammalian meiotic division: (1) reciprocal recombination and formation of chiasmata between homologous chromosomes, (2) cosegregation of sister kinetochores at meiosis I, (3) protection of centromeric sister chromatid cohesion, (4) no DNA replication between the two meiotic divisions (reviewed by Petronczki et al., 2003), and (5) asymmetric cell division during meiosis I and II in eggs. Failures in chromosome segregation at meiosis result in aneuploidy, which is a major cause of miscarriages and birth defects in humans. Currently, we know very little about the molecular mechanisms underlying these processes in mammals. The dynamic localization of Aurora-C during male meiosis suggests that Aurora-C plays important roles in chromosome segregation and cytokinesis during meiotic division.

First, it was reported that most cohesion complexes dissociate from the chromosome arms during the mitotic prophase due to their phosphorylation (Waizenegger et al., 2000; Warren et al., 2000; Losada et al., 2002). Aurora-B and the Polo-like kinases were reported to participate in this mitotic event (Losada et al., 2002; Sumara et al., 2002). Interestingly, in meiosis, some SC components (e.g., SCP2 and SCP3) and cohesion subunits (e.g., SMC1b and SMC3, but not REC8) are gradually released from the chromosome arms and accumulate around the centromeres during the transition from the prophase I to the MI stage (Eijpe et al., 2003; Lee et al., 2003). The observation that a significant portion of Aurora-C was dissociated from the chromosome arms and was concentrated at the centromeres during the diakinesis–MI transition (Fig. 4)

raises the possibility that Aurora-C may regulate the release of cohesion and SC components from the chromosome arms during meiosis I.

Recently, it has been reported that the Aurora-B kinase, AIR-2, functions in the release of chromosome cohesion (Rogers et al., 2002) and is required for separation of homologous chromosomes in *Caenorhabditis elegans* meiosis (Kaitna et al., 2002). They proposed that Aurora-B promotes the release of chromosome cohesion possibly by phosphorylation of REC 8 at specific chromosomal regions (Rogers et al., 2002). Interestingly, our unpublished data showed that REC 8 can also be phosphorylated by Aurora-C in vitro. Presently, it is not clear whether Aurora-C in mammals can perform a similar function as Aurora-B in *C. elegans*. It will be interesting to test this possibility in the future.

Second, it has been proposed that, in mitosis, Aurora-B promotes amphitelic kinetochore attachment to microtubules by destabilizing syntelic attachment of sister chromatids (Tanaka et al., 2002; Carmena and Earnshaw, 2003; Hauf et al., 2003). However, a different mechanism must be applied to meiosis I since meiosis sister kinetochores attach to microtubules in a syntelic manner, which ensures that maternal and paternal chromosomes segregate to opposite poles during meiosis I. Interestingly, Aurora-C shares a high sequence identity with Aurora-B (77.6%) in its kinase catalytic domain (Tseng et al., 1998), and both Aurora-B and -C colocalized at the centromeres of MI chromosomes, suggesting that Aurora-C may possess a similar but different function to that of Aurora-B. Here, we propose that Aurora-C works by itself or coordinates with Aurora-B's function, particularly in regulating kinetochore–microtubule interactions during meiotic chromosome separation.

Finally, during mitosis, Aurora-B was shown to play critical roles in cytokinesis (Carmena and Earnshaw, 2003). Over-expression of a catalytically inactive Aurora-B disrupts cleavage furrow formation and prevents cytokinesis (Terada et al., 1998). The results showing depletion of Aurora-B by RNAi experiments also confirm these findings (Adams et al., 2001; Giet and Glover, 2001). Our immunofluorescence studies showed that a major portion of Aurora-C was dissociated from the centromeres and relocated to the spindle midzone and midbody during the transition from anaphase I to telophase I (Figs. 4C, D), suggesting an essential role for Aurora-C in meiotic interkinesis (a process similar to cytokinesis in mitosis). Interestingly, microinjection of antibodies against either Aurora-C or -B or their dominant negative mutants into mouse oocytes caused interkinetic failure and inhibited asymmetric egg division (unpublished data) consistent with this hypothesis.

There are three Aurora kinases (Aurora-A, -B, and -C) in mammals, two in toads, *Drosophila*, and *C. elegans* (Aurora-A and -B), and only one in yeast. It is unclear why mammals need three Aurora kinases. In lower species such as yeast, a single Aurora kinase likely suffices for both mitosis and meiosis. However, in higher species, more than one kinase may be necessary. Since chromosome dynamics in mammals during meiosis are more complicated than those in lower species, they may require a more-specialized Aurora kinase such as Aurora-C, which either works by itself or is a functional complement with Aurora-B, to regulate accurate chromosome segregation and interkinesis during meiosis. Obviously, more studies are required to assess the meiotic functions of Aurora-C in mammals.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2005.11.036.

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