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Chromatin-induced Spindle Assembly Plays an Important Role in Metaphase Congression of Silkworm Holocentric Chromosomes

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2   **metaphase congression of silkworm holocentric chromosomes**

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Abstract

The kinetochore plays important roles in cell cycle progression. Interactions between chromosomes and spindle microtubules allow chromosomes to congress to the middle of the cell and to segregate the sister chromatids into daughter cells in mitosis. The chromosome passenger complex (CPC), composed of the Aurora B kinase and its regulatory subunits INCENP, Survivin, and Borealin, plays multiple roles in these chromosomal events. In the genome of the silkworm, *Bombyx mori*, which has holocentric chromosomes, the CPC components and their molecular interactions were highly conserved. In contrast to monocentric species, however, the silkworm CPC co-localized with the chromatin-driven spindles on the upper side of prometaphase chromosomes without forming bipolar mitotic spindles. Depletion of the CPC by RNAi arrested the cell cycle progression at prometaphase and disrupted the microtubule network of the chromatin-driven spindles. Interestingly, depletion of mitotic centromere-associated kinesin (MCAK) recovered formation of the microtubule network but did not overcome the cell cycle arrest at prometaphase. These results suggest that the CPC modulates the chromatin-induced spindle assembly and metaphase congression of silkworm holocentric chromosomes.

1. Introduction

Faithful chromosome segregation during cell division is essential for the genomic integrity of eukaryotic cells and requires the establishment of kinetochores to mediate interactions between chromosomal DNA and spindle microtubules. A large group of kinetochore proteins have been identified and their functions uncovered recently (Przewloka and Glover, 2009). Despite significant variation of centromeric DNA, the kinetochore proteins are relatively conserved among species.

Chromosome architecture can be divided into two different types, monocentric and holocentric. In monocentric chromosomes, which are widely used for the study of kinetochore function (De Wulf et al., 2003; Meraldi et al., 2006; Przewloka and Glover, 2009; Takeuchi and Fukagawa, 2012; Westermann et al., 2003), kinetochores are built at a primary chromosomal constriction. On the other hand, in holocentric chromosomes, which are also reported in a wide range of animal and plant species (Buchwitz et al., 1999; Dernburg, 2001; Maddox et al., 2004; Nagaki, 2005), kinetochores extend along the entire length of each chromatid.

Detailed molecular analysis of holocentric chromosomes performed in *Caenorhabditis elegans* indicates that the core network of kinetochore proteins, especially Cenp-C and the KNL1/Mis12/Ndc80 (KMN) network, is conserved between mono- and holocentric chromosomes (Cheeseman et al., 2006). However, except for Cenp-C, none of the other identified constitutive centromere-associated network (CCAN) proteins found in monocentric chromosomes, including E, I, L, M, N, S, X, T, W, are present in *C. elegans* or *D. melanogaster* (Przewloka et al., 2011; Screpanti et al., 2011).

The silkworm has been reported to possess holocentric chromosomes (Murakami and Imai, 1974). Although cytological observation of holocentric chromosomes and spindles has been carried out in Lepidoptera, resolution by microscopy has been limited (Matsuda and

Yamashiki, 2007; Murakami and Imai, 1974). Genomic information now available enables us to identify and isolate kinetochore genes in silkworm to help define the critical components of its particular holocentric chromosome apparatus. We identified eight putative CCAN proteins (Cenp-E, I, K, L, M, N, S, X) in the silkworm genome whereas Cenp-A and core CCAN factors CENP-C, T and W appear to be lacking. By comparison with *C. elegans*, this suggests that different holocentric species have evolved different molecular strategies for chromosome segregation.

One advantage of a holocentric chromosome is that chromosomal fragments induced by irradiation or endogenous DNA double-strand breaks can be maintained stably through many generations (Fujiwara et al., 2000). In contrast, merotelic attachments, in which a single kinetochore is attached to microtubules from both spindle poles, are likely to be a great disadvantage in holocentric chromosomes. This kind of abnormal kinetochore capture by microtubules is a major source of aneuploidy. To prevent incorrect attachments between microtubules and kinetochores, the spindle assembly checkpoint (SAC) and chromosomal passenger complex (CPC) play important roles to correct improper kinetochore-microtubule attachments.

One of the critical CPC proteins is the Inner Centromere Protein (INCENP), which was originally identified by a monoclonal antibody against mitotic chromosome proteins (Cooke et al., 1987; De Wulf et al., 2003; Meraldi et al., 2006; Przewloka and Glover, 2009; Takeuchi and Fukagawa, 2012; Westermann et al., 2003). INCENP plays a scaffold role and interacts with other components of the CPC, including Aurora-B, Borealin and Survivin. The CPC is diffusely distributed along chromosome arms in prophase of monocentric chromosomes and enriched at the inner centromere in prophase and metaphase. Finally, it moves from the chromosomes to the central spindle in anaphase and concentrates in the midbody in telophase. The dynamic localization of the CPC during mitosis is essential for its

multiple functions (Buchwitz et al., 1999; Dernburg, 2001; Maddox et al., 2004; Nagaki, 2005; Ruchaud et al., 2007).

MCAK, which is a substrate of Aurora-B, belongs to the kinesin-13 family and depolymerize improperly attached microtubules to ensure accurate chromosome segregation, and its activity is regulated by CPC (Ems-McClung and Walczak, 2010; Lan et al., 2004; Paul D Andrews et al., 2004). In CHO cells, depletion of MCAK results in lagging chromosomes in anaphase, but no significant defects during prometaphase and metaphase (Maney et al., 1998). In kangaroo rat (PtK2) cells, overexpression of a dominant negative form of MCAK induces mitotic arrest at prometaphase and perturbs chromosome congression (Kline-Smith et al., 2004).

In the present study, we performed molecular analysis of the CPC using cultured silkworm BmN4 cells. During prometaphase, BmINCENP is localized along the sister chromatids of mitotic chromosomes but not between the sister chromatids, the so-called inner centromere. In addition, we used RNAi to analyze the effects of loss of CPC proteins and demonstrated that they are essential for spindle assembly near chromosomes, chromosome segregation and cytokinesis in silkworm. These results provide the first insights into understanding the detailed structure and function of the holocentric kinetochore in Lepidoptera.

1 **2. Materials and methods**

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3 *2.1. Cloning of silkworm CPC*

4   The *BmINCENP* gene was cloned by 5' rapid amplification of cDNA ends using a

5 SMART RACE cDNA Amplification kit (Clontech, Shiga, Japan). cDNA was prepared from

6 the testes of *B. mori*, strain p50 larvae on day 3 of the fifth instar. Amplified PCR products

7 were subcloned into the *Eco*RV site of plasmid pZErO-2 (Invitrogen, Carlsbad, CA). After

8 the identification of potential start and stop codons, the full length of *BmINCENP* (Genbank

9 accession number AB777644) was cloned into pENTR11 (Invitrogen), generating pENTR-

10 BmINCENP. The cDNAs of putative *BmAurora-B*, *BmBorealin* and *BmSurvivin* were

11 determined using the SilkBase and KAIKOBLAST databases

12 (http://kaikoblast.dna.affrc.go.jp/). The cDNA fragments encoding *BmAurora-B* (AB777868),

13 *BmBorealin* (AB777869), *BmSurvivin* (ADM32525) were amplified by PCR using a cDNA

14 mixture transcribed from the total RNA isolated from the testes of *B. mori*, strain p50 larvae

15 on day 3 of the fifth instar. The amplified cDNAs were digested with *Xho*I and subcloned

16 into the *Nco*I/blunt-*Xho*I site of pENTR11 (Invitrogen), and their nucleotide sequences were

17 determined via dye-terminator cycle sequencing using a DNA sequencer (Applied

18 Biosystems). The ORFs of *BmAurora-B*, *BmBorealin*, *BmSurvivin* and *BmHP1α* (Mitsunobu

19 et al., 2012) were transferred to the HA-tagged destination vector (Yamashita et al., 2007) by

20 using LR Clonase II enzyme mix (Invitrogen). The expression vector of Flag-tagged

21 BmINCENP was generated by the Gateway LR reaction between pie2FW (Tatsuke et al.,

22 2009) and pENTR11-BmINCENP. All PCR reactions were performed using KOD-Plus-neo

23 DNA polymerase (TOYOBO, Japan).

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1   *2.2. RNAi*

2   Double-stranded RNA was made by in vitro transcription using T7 RNA polymerase.

3   The fragments of the genes *BmINCENP*, *BmAurora-B*, *BmBorealin*, *BmSurvivin*, *BmMCAK*

4   and *BmDgt6* were amplified by PCR. The following primer sets were used: *BmINCENP* (5'-

5   GAATTCTTCAGCTAAAAATGATAAACAG-3'   and   5'-

6   CTCGAGTCCGTTTAGGTCTGCCCTCAAC-3');   *BmAurora-B*   (5'-

7   ACAATGAAGAGCGAAGTGCTCGAACTTGAAAC-3'   and   5'-

8   CTATACTGTCTGAAATTGTTGGACC-3');   *BmBorealin*   (5'-

9   GTAATGTTGAATTGGCCTTCAAGATTC-3'   and   5'-

10   CACCTTAGGCGTGATAGCAGGGTAGTAC-3');   *BmSurvivin*   (5'-

11   GAGAACGAGAGCTCTTTACTATTTCTGG-3'   and   5'-

12   TTATTTCCTTGCTAAGCATTTTCTTTG-3'); *BmMCAK* (Genbank accession number

13   AB777870,   5'-GATCCGGGAGTACCAGAACAGC-3'   and   5'-

14   CCATGGCGTATATGCCCTTCTTG-3'); *BmDgt6* (Genbank accession number AB777871,

15   5'-GGCGCTACCCTGGCCTTTGTATG-3' and 5'-GTCATCAAATTGTCTTGAATTTA-3').

16   The amplified fragments were cloned into EcoRV-cleaved pZErO-2 (Life Technologies). The

17   T7 promoter sequences were added on both sides of the target fragments by PCR with the

18   primers   (5'-

19   GCGTAATACGACTCACTATAGGGGCCGCCAGTGTGCTGGAATTCTGCAGAT-3'   and

20   5'-GCGTAATACGACTCACTATAGGGTGCTCGAGCGGCCGCCAGTGTGATGGAT-3').

21   The fragments with two T7 promoter sequences were transcribed by T7 RNA polymerase. To

22   perform soaking RNAi in BmN4-SID1, dsRNAs were added to the culture medium directly.

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2.3. Cell Culture and Immunofluorescence

The silkworm cell line BmN4, which is derived from ovary tissues, (a gift from Dr. Chisa Aoki, Kyushu University Graduate School) and its derivative cell line BmN4-SID1 was cultured on concanavalin A (Con A) - coated coverslips in IPL-41 (Sigma Aldrich, Saint Louis, MI, USA; I7760) supplemented with 10% FBS. Con A (Sigma) was used for the better attachment and visualization of cells (Mon et al., 2012; Rogers et al., 2002). The BmN4 cells were immediately fixed with 4% paraformaldehyde for 10 min at room temperature, permeabilized with 0.25% Triton X-100 for 5 min and then blocked for 30 min using blocking buffer (1% BSA). The antibody incubation was done in 1% BSA for 1 h at 37°C. Rabbit anti-BmINCENP polyclonal antibody was used at a dilution of 1:1000 (Mon et al., 2012). Tubulin staining was performed using mouse monoclonal antibody DM1A (Abcam, MA, USA; ab7291) at a dilution of 1:1,500. The coverslips were washed three times and incubated with Alexa 488 conjugated anti-rabbit or mouse IgG (Molecular Probes, Eugene, Oregon, USA) for 1 h at room temperature. DNA was counterstained with Hoechst 33342 (Molecular Probes). The cells were mounted with DABCO (P8136; Sigma). A series of images were obtained using a Nikon A1 confocal imaging system equipped with an oil immersion objective lens (Plan Apo VC 60X 1.4NA) and a dichroic mirror (405/488/561/640). Microscopic data was processed by ImageJ software (National Institutes of Health). 3D reconstruction of confocal images was performed using the NIS-Elements AR-SP software (Nikon, Japan). In order to monitor the localization of BmINCENP on chromosomes in more detail, chromosome spreads were prepared using Cytospin (Thermo Scientific). BmN4 cells diluted in PBS were centrifuged onto Con A - coated slides for 3 minutes at 1,300 rpm. The chromosomes spreads were fixed and processed for immunofluorescence with anti-BmINCENP antibody as described above.

2.4. Immunoprecipitation

The HA-tagged and Flag-tagged proteins were expressed transiently in BmN4 cells for five days (Sugahara et al., 2007; Yamashita et al., 2007). The extracts were subject to immunoprecipitation with anti-Flag antibody (M2, Sigma), followed by Western blot analysis (Sugahara et al., 2007) with anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz, CA; sc-7392).

2.5. RNAi and FACS analysis

RNAi experiments were performed using BmN4-SID1 cells, in which CeSID-1 was ectopically expressed as previously reported (Mon et al., 2012). After the direct addition of the corresponding dsRNA into the culture medium, cell cycle progression was examined using flow cytometry (Guava, Millipore, Billerica, MA, USA). The histogram plots were generated using FlowJo software (Three Star, Ashland, OR, USA).

3. Results

3.1 Organization of chromatin-driven spindles in silkworm cells

To understand how the spindle is formed in silkworm cells possessing holocentric chromosomes, α-tubulin was immunostained under normal conditions. A high density microtubule mesh-like network was observed near chromosomes in prometaphase without forming bipolar microtubule bundles (Fig. 1A and Fig. S1). The 3D reconstruction of BmN4 cells stained with anti-α-tubulin antibody and Hoechst33342 is shown in Figure 1B and in the supplementary material Video 1. Microtubules accumulated at the upper side of the cell in prometaphase and were mostly convex in shape. At metaphase, chromosomes were attached to spindle microtubules from opposite poles (amphitelic attachment) and astral-like microtubules were detected (Fig. S1). Since most somatic animal cells nucleate mitotic spindles from centrosomes, these observations indicated that spindle organization during prometaphase-meteaphase is significantly unique and dynamic in the silkworm cultured cells used in our study.

Spindle assembly has been reported to occur largely through a chromatin-driven pathway in *Xenopus* extracts or in mammalian and fruit fly oocytes, in which functional centrosomes are present (Karsenti et al., 1984). In mitosis of *Drosophila* S2 cells and various cultured vertebrate cells, kinetochore - mediated microtubule nucleation has been observed during spindle assembly after removal of centrosomes in wild type cells or in mutant cells after depletion of proteins essential to centrosome function or after treatment of the cells with nocodazole (Khodjakov et al., 2000; Mahoney et al., 2006; O'Connell and Khodjakov, 2007; Tulu et al., 2006), and even in physiological conditions (Maiato et al., 2004). Depletion of INCENP and Survivin reduces chromatin-driven spindle formation using the in vitro *Xenopus* system and vertebrate cells (Sampath et al., 2004; Tulu et al., 2006). The confocal vertical

views (XZ and YZ) of BmINCENP and α-tubulin localization indicated they accumulated at the upper side of the cell during prometaphase (Figure 1C). These results led us to examine whether BmINCENP has a role in spindle formation near chromosomes in silkworm cells.

3.2. Functional analysis of the chromosome passenger complex

Aurora-B kinase phosphorylates INCENP, which potentiates the interaction between them and activates its own kinase activity (Honda et al., 2003). INCENP seems to be a scaffold protein which also makes a ternary complex with Borealin/Survivin proteins (Ruchaud et al., 2007). In addition, human INCENP interacts with Heterochromatin protein 1 (HP1) through the PXVXL/I motif, which is a consensus motif for binding to HP1 (Kang et al., 2011). To investigate the physical interactions between BmAurora-B, BmHP1α, BmBorealin and BmSurvivin and BmINCNEP, we cloned the four silkworm CPC components (Fig. S2) and carried out co-immunoprecipitation assays (Mitsunobu et al., 2012). BmINCENP was detected when Flag-tagged Aurora-B, HP1α, Survivin or Borealin was co-expressed in BmN4 cells (Fig. S3). These results demonstrated that the interactions between the CPC complex components were conserved in *B. mori*.

To investigate the localization of BmINCENP during the cell cycle, we raised a rabbit antibody against BmINCENP. To probe antibody specificity, a Western blot assay was performed using crude extracts of BmN4 and silkworm testis, which has a high fraction of dividing cells (spermatocytes) at the 5th larval instar. The anti-BmINCENP antibody exhibited a single band corresponding to a protein of approximately 107 kDa (Fig. S4, lane 1). This single band was reported to disappear following treatment by dsRNA against BmINCENP in BmN4-SID1 cells (Mon et al., 2012). In addition, a smaller band was detected in the testis lysate (Fig. S4, lane 2). In prometaphase, condensed chromosomes showed a round shape, lacking distinct primary constrictions, and BmINCENP was

distributed along them. Although we detected some slightly concentrated signal, most BmINCENP signals appeared to be fuzzy (Fig. 2, top panel). In metaphase, BmINCENP showed a punctate distribution within mitotic chromosomes (Fig. 2, second panel). INCENP is known to be less abundant at centromeric and heterochromatic regions and concentrates at the midbody during anaphase and telophase (Adams et al., 2001; Martineau-Thuillier et al., 1998; Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004). In silkworm cells, some portion of the protein left the chromosomes and was concentrated between the sister chromatids during anaphase, whereas the remaining proteins were still retained around the chromosomes (Fig. 2, third panel). At telophase, BmINCENP was clearly abundant in the midbody (Fig. 2, bottom panel).

3.3. Diffuse distribution along the chromosome arms of BmINCENP

In monocentric chromosomes, the CPC protein complex is known to localize at the inner centromeres and is detected as two spots along the inner axis of the chromosome (Cooke et al., 1987). To determine the location of BmINCENP on the silkworm holocentric chromosomes, we prepared chromosome spreads of mitotic cells immunostained with anti-BmINCENP antibody. At prometaphase, BmINCENP was diffused along the chromosomes, although we also detected many clear spot-like signals (Fig. 3A). At higher magnification BmINCENP appeared in rodlike discontinuous zones along the chromosomes (Fig. 3B, top panel). However, some of the immunostained structures were more intense and patchily distributed along the whole length of the chromosomes (Fig. 3B, middle and bottom panels). These results established that BmINCENP persisted along the entire length of silkworm chromosomes.

3.4. The chromosomal passenger complex is a conserved cytokinesis regulator

1   Previously, we reported that cultured silkworm cells depleted of BmINCENP

2   accumulate in G2/M phase accompanied by a dramatic increase in the fraction of polyploid

3   cells (Mon et al., 2012). In other organisms, RNAi knockdown of the four CPC components

4   causes multinucleation phenotypes (Gao et al., 2008; Honda et al., 2003; Vader et al., 2006).

5   Light microscope analysis showed that the depletion of BmINCENP for two weeks resulted

6   in an increased cell size (Fig. 4A). We measured the alteration of cell size directly by forward

7   scatter intensity using flow cytometry. Compared with the curve for the control dsRNA using

8   dsCDC27 (red), we observed a right shift of the curve for dsRNA against BmINCENP (blue)

9   or BmBorealin (blue), indicating an increase in average cell size (Fig. 4B). In addition, after

10   RNAi-induced depletion of these proteins the number of cells in the G2/M phase was greater

11   than in the control (Fig. 4C). These results indicated that the CPC plays a crucial role in

12   cytokinesis.

13

14   *3.5. BmINCENP is required for stable spindle formation near chromosomes*

15   Long term knockdown of BmINCENP causes growth arrest in G2/M, multinucleation

16   phenotypes and chromosome decondensation (Mon et al., 2012). To investigate whether a

17   relationship exists between spindle assembly and INCENP, silkworm cells were incubated

18   with dsRNA for five days and immediately fixed and immunostained with anti-αTubulin

19   antibody. Abnormal spindle assembly near chromosomes was observed in BmN4-SID1 cells

20   after the addition of dsRNA against BmINCENP (Fig. 5A and B). Several studies have

21   reported that Aurora-B phosphorylates mitotic centromere-associated kinesin (MCAK) and

22   regulates its depolymerization activity (Kline-Smith et al., 2004; Maney et al., 1998). The

23   silkworm has one kinesin-13 family protein (referred to below as BmMCAK), which has a

24   microtubule-destabilizing activity (Wang et al., 2010). Depletion of BmMCAK caused cell

25   cycle arrest in mitosis, especially in prometaphase (Fig. S5). We observed that a reduction in

1 BmMCAK expression led to a slight increase in microtubule nucleation around chromosomes

2 in prometaphase (Fig. 5C). A double knockdown of BmINCENP and BmMCAK expression

3 restored spindle formation, indicating that BmINCENP plays a crucial role to regulate the

4 assembly of the spindle near the chromosomes in silkworm cells.

5   Many results presented in this study, such as co-localization of microtubules and

6 INCENP at the upper side of the cell and lack of visible bipolar microtubule bundles in

7 prometaphase suggested that chromatin-driven spindles, not bipolar microtubules, play an

8 important role in metaphase congression of silkworm chromosomes. To validate this

9 hypothesis, the effect of inhibition of bipolar microtubule formation on congression of

10 chromosomes in metaphase was examined. We conducted RNAi-mediated knockdown of

11 Dgt6, which is responsible for microtubule nucleation from within the mitotic spindle

12 (Goshima et al., 2008). In silkworm cells, depletion of the augmin component BmDgt6

13 provokes cell cycle arrest in metaphase, but not in prometaphase. At metaphase, the amount

14 of spindle was significantly decreased and anomalous spindle formation was observed in

15 BmDgt6 knockdown cells (Fig. 6). These data suggest that BmDgt6 is indispensable for

16 microtubule nucleation from postmetaphase to anaphase, but not in prometaphase.

17

4. Discussion

In this work, we identified the core components of the CPC, including INCENP, Aurora-B, Borealin and Survivin in the silkworm, *B. mori*, using BLAST searches of the silkworm genome. The C-terminus region of BmINCENP, a coiled-coil and IN box domain, is well conserved among species and the N-terminal and middle domain are less conserved. Recently, the genome of the migratory monarch butterfly (*Danaus plexippus*) was published (Zhan et al., 2011). The N-terminus of BmINCENP shares 53% sequence identity with the putative *D. plexippus* INCENP. This supports our prediction for the position of the first ATG codon.

BmINCENP interacted with BmAurora-B, BmBorealin and BmSurvivin and could serve as a scaffold for the CPC. To study the interactions among the CPC components, we first utilized the insect two-hybrid (I2H) system (Mon et al., 2009) whereby target proteins are fused with the DNA-binding domain (DBD) of the yeast GAL4 or NF-κB transcriptional activation domain (p65AD). The interaction between target proteins was evaluated as the activation of the UAS promoter by measuring luciferase activity. Unlike reports in other organisms, we could not detect any interactions between the silkworm CPC proteins by the I2H system (Jeyaprakash et al., 2007; Klein et al., 2006) or the yeast two-hybrid system. The interactions were detected even when we used DBD or p65AD fused proteins in co-immunoprecipitation experiments. These results might indicate that some of the silkworm CPC components have transcriptional repression activity.

CPC proteins are known to move dynamically during the cell cycle (Ruchaud et al., 2007). During mitotic prometaphase and metaphase, silkworm CPC proteins concentrated on chromosomes which were undergoing segregation. After the onset of anaphase, they also localized to the central spindle. This dynamic movement was conserved in silkworm cells in

almost the same manner as in other organisms (Ruchaud et al., 2007). In anaphase, BmINCENP was still observed on the outer regions of the chromosomes. This kind of signal was also detected when we immunostained BmHP1 proteins at metaphase (Mitsunobu et al., 2012). It is unclear whether this kind of localization is related to the holocentricity of silkworm chromosomes. Many spindles appear to be enriched around the holocentric kinetochores and might be tangled at the outer regions of the chromosomes. Given this complicated situation, the CPC might function in the surveillance for proper microtubule-kinetochore attachments in anaphase.

It is believed that holocentric chromosomes possess centromeric activity along the entire chromosomal axis. Thus far, cytological observation of holocentric chromosomes in insect cells has been limited to heterochromatin using sequence specific staining with the GC-specific chromomycin A_3(CMA$_3$) and the AT-specific 4'-6-diamidino-2-phenylindole (DAPI) or by fluorescence *in situ* hybridization (FISH) with a (TTAGG)n telomeric probe (Mandrioli et al., 2011; Mandrioli and Borsatti, 2007). Nevertheless, molecular analysis has been little studied and thus far there are no useful markers for centromeres of holocentric chromosomes. In this paper, we showed that the inner centromere protein, BmINCENP, was localized along the length of silkworm holocentric chromosomes. In monocentric chromosomes, INCENP is enriched in the inner centromere between a pair of kinetochores (Martineau-Thuillier et al., 1998; Ruchaud et al., 2007). ICP-1, a *C. elegans* homolog of INCENP, is also localized between the two kinetochore plates (Oegema et al., 2001). The images we obtained for localization of BmINCENP in prometaphase and metaphase are significantly different from that of human, *Drosophila* and *C. elegans* INCENP. It is reported that Survivin is a reader of phosphorylation of Thr3 in the N-terminal tail of histone H3 (H3-pT3) (Kelly et al., 2010; Yamagishi et al., 2010) and recruits the other CPC proteins to chromosomes. Most of the residues involved in H3-pT3 recognition are conserved in

BmSurvivin, indicating that BmSurvivin could recognize H3-T3ph. This suggests that the difference of INCENP localization between silkworm and other species could be due to variation in the modification of histone H3 at silkworm centromeres.

The CPC participates in cytokinesis during anaphase and telophase. In *Drosophila* and human cells, most INCENP proteins relocate from the centromeres to the central spindle (Adams et al., 2001; Martineau-Thuillier et al., 1998; Ruchaud et al., 2007; Vagnarelli and Earnshaw, 2004). BmINCENP also localized at the central spindle, and knockdown experiments showed that BmINCENP could play important roles in the cytokinesis process. The increase we observed in the >4N population could be due to cells that underwent two rounds of DNA replication without cytokinesis. The Kinesin-6 family members MLKP1 (*H. sapiens* KIF23, *D. melanogaster* PAV, *C. elegans* ZEN-4/CeMKLP1) is required for formation of the midbody matrix (Barr and Gruneberg, 2007; Fededa and Gerlich, 2012). In *C. elegans*, AIR-2 (CeAurora-B) and ICP-1 (CeINCENP) are required for ZEN-4/CeMKLP1 localization at the central spindle. BmKif23/BmMLKP1 knockdown causes cytokinesis failure and an increase in the number of binucleate silkworm cells (Mon et al., 2012). This could imply that the signaling pathway between the CPC and BmKif23/BmMLKP1 is conserved between nematode and silkworm. Recently, it has been shown that the CPC prevents cell division while chromatin bridges persist at the midbody (abscission checkpoint) (Carlton et al., 2012; Norden et al., 2006; Steigemann et al., 2009). We frequently observed intercellular chromosome bridges in silkworm BmN4 cells in cytokinesis, which were due to tangles of duplex DNA (Fig. S6). A mechanistic interpretation of the high frequency of lagging chromosomes remains unexplained, but we speculate that chromosome holocentricity may be one of the reasons for entanglements between chromosomal DNAs.

Assembly of the mitotic spindle appears to depend on two microtubule formation pathways; one is dependent on centrosomes and the other on chromosomes. Unexpectedly,

we detected the accumulation of tubulin around chromosomes in silkworm BmN4 cells in prometaphase, which is similar to the assembly of acentrosomal spindles observed during meiosis in oocytes (Dumont and Desai, 2012). Astral microtubules and γ-tubulin are observed in the centrosomes of *B. mori* eupyrene spermatocytes. Moreover, distinct non-centrosomal microtubules are detected which are separate from astral microtubules (Matsuda and Yamashiki, 2007) indicating that the tubulin nucleation around chromosomes could be induced in a centrosome independent manner. Depletion of the CPC in *D. melanogaster* induces defects in meiotic acentrosomal spindle assembly (Radford et al., 2012); however, it has been unclear whether the CPC proteins are involved in spindle assembly in mitosis. We found that depletion of BmINCENP inhibited normal microtubule accumulation near the chromosomes in silkworm cells. Recently, the third pathway of microtubule nucleation was discovered, and the microtubule nucleation takes place within the spindle in an augmin-dependent manner. In *Drosophila* S2 cells, the augmin complex is required for kinetochore-driven microtubule and chromosome alignment (Goshima et al., 2008; Kline-Smith et al., 2004; Maney et al., 1998). In human HeLa cells, depletion of the Dgt6 subunit of the augmin complex arrests the cell cycle in prometaphase or metaphase (Uehara et al., 2009). In silkworm cells, knockdown of BmDgt6 did not cause chromosome misalignment between prometaphase to metaphase. These suggest that chromosome-driven spindle assembly might be an effective way to capture holocentric chromosomes. Further investigation is needed to understand the molecular basis of spindle dynamics in BmN4 cells.

Most knowledge about holocentric chromosome function has been derived from experiments in *C. elegans* (Dernburg, 2001; Maddox et al., 2004). On the other hand, a large number of species have been reported to possess holocentric chromosomes, and holocentric chromosomes have arisen independently multiple times through evolution in plants and animals (Melters et al., 2012). This could imply that detailed structures and proteins involved

1   in the regulation of holocentric chromosomes have arisen independently or diverged among

2   species. For instance, d'Alençon *et al*. identified a CENP-B homolog from the holocentric

3   lepidopteran insect *Spodoptera frugiperda* and found that SfCENP-B localized in the nucleus

4   and bound to retrotransposon DNA (d'Alençon et al., 2011). Knockdown of SfCENP-B in Sf9

5   cultured cells resulted in a slight increase of binucleated cells. Four putative CENP-B-like

6   genes are present in silkworm, and we performed gene knockdown experiments of BmCENP-

7   Bs on BmN4-SID1 cells. In contrast to the results of SfCENP-B, we could not detect any

8   apparent effects on cell growth, morphology or chromosome aberration (data not shown).

9   These results might be due to a functional redundancy among BmCENP-Bs and difficulties to

10   silence all four paralogs, and/or BmCENP-B-like proteins might be less important for

11   establishing holocentric kinetochores in *B. mori*, similar to mice (Hudson et al., 1998; Kapoor

12   et al., 1998; Ohzeki et al., 2002).

13   In the present study, we found a new set of marker proteins for silkworm holocentric

14   chromosomes. The work reported here is a first step for further investigation of holocentric

15   kinetochores in silkworm. With the recent rapid increase in genomic information for many

16   insect species, comparative studies between various Lepidoptera and other arthropods with

17   holocentric or monocentric chromosomes can be made in the near future.

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1 **Acknowledgements**

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Figure Legends

Fig. 1. Organization of chromatin-driven spindles in silkworm cells.

(A) Subcellular localization of α-Tubulin (green) in prometaphase. Scale bar: 10 μm. (B) Confocal three-dimensional reconstructions of chromosomes and spindles in prometaphase. The XY images were selected by rotating on the X axis. BmN4 cells were stained for α-Tubulin (green) and DNA (magenta). See also supplementary material Video 1. Scale bar: 10 μm. (C) Confocal vertical views (XZ and YZ) of BmN4 cells labeled for BmINCENP (C, left panel) and α-Tubulin (C, right panel), followed by Alexa 488-conjugated secondary antibody (green). Hoechst 33342 was used as a counterstain for chromosomes (magenta). Scale bar: 10 μm.

Fig. 2. Subcellular localization of BmINCENP during the cell cycle in BmN4 cells. Cells were fixed and stained with anti-α-Tubulin (green). Hoechst 33342 was used as a counterstain for chromosomes (magenta). Scale bar: 10 μm.

Fig. 3. Localization of BmINCENP along mitotic chromosome arms. (A) Chromosome spreads were prepared from BmN4 cells and stained with anti-BmINCENP (green) antibody and Hoechst 33342 (magenta). Scale bar: 10 μm. (B) A higher magnification view of representative chromosomes. Scale bar: 5 μm.

1 **Fig. 4. Depletion the CPC components causes defects in cytokinesis.** (A) Microscopic

2 observation of BmN4 cells after depletion of BmINCENP. Scale bar: 50 μM. (B) Flow

3 cytometry analysis to determine cell size. Cell size distribution of the control is indicated by

4 the red line. (C) Effects of dsRNA-mediated knockdown of BmBorealin, BmSurvivin and

5 BmAurora-B. Cell cycle analysis was performed by flow cytometry.

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8 **Fig. 5. Depletion of BmINCENP induces abnormal spindle formation in prometaphase.**

9 BmN4 cells were fixed and stained with anti-α-Tubulin (green) antibody and Hoechst 33342

10 (magenta). (A) Untreated BmN4-SID1 cell. (B) BmINCENP RNAi cell. (C) BmMCAK

11 RNAi cell. (D) BmINCENP and BmMCAK RNAi cell. Scale bar: 10 μM.

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14 **Fig. 6. BmDgt6 is not required for mitotic chromosome congression.** BmN4 cells were

15 fixed and stained with anti-αTubulin (green) antibody and Hoechst 33342 (magenta). (A)

16 Untreated BmN4-SID1 cell. Scale bar: 10 μM. (B) BmDgt6 RNAi cell. Scale bar: 10 μM.

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