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Reconstruction of the Kinetochore during Meiosis in Fission Yeast *Schizosaccharomyces pombe*

Aki Hayashi,* Haruhiko Asakawa,* Tokuko Haraguchi,*† and Yasushi Hiraoka*†

*Kansai Advanced Research Center, National Institute of Information and Communications Technology, 588-2 Iwaoka, Iwaoka-cho, Nishi-ku, Kobe 651-2492, Japan; and †Department of Biology, Graduate School of Science, Osaka University, 1-1 Machikaneyama, Toyonaka, 560-0043, Japan

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During the transition from mitosis to meiosis, the kinetochore undergoes significant reorganization, switching from a bipolar to a monopolar orientation. To examine the centromere proteins that are involved in fundamental reorganization in meiosis, we observed the localization of 22 mitotic and 2 meiotic protein components of the kinetochore during meiosis in living cells of the fission yeast. We found that the 22 mitotic proteins can be classified into three groups: the Mis6-like group, the NMS (Ndc80-Mis12-Spc7) group, and the DASH group, based on their meiotic behavior. Mis6-like group proteins remain at the centromere throughout meiosis. NMS group proteins disappear from the centromere at the onset of meiosis and reappear at the centromere in two steps in late prophase. DASH group proteins appear shortly before metaphase of meiosis I. These observations suggest that Mis6-like group proteins constitute the structural basis of the centromere and that the NMS and DASH group proteins reassemble to establish the functional metaphase kinetochore. On the other hand, the meiosis-specific protein Moa1, which plays an important role in forming the meiotic monopolar kinetochore, is loaded onto the centromere significantly earlier than the NMS group, whereas another meiosis-specific protein, Sgo1, is loaded at times similar to the NMS group.

INTRODUCTION

Meiosis is a process of general importance for sexually reproducing eukaryotic organisms, generating haploid gametes from a diploid cell. During this process, two rounds of chromosome segregation follow a single round of DNA replication. A unique feature of chromosome behavior in meiosis is the pairing and segregation of homologous chromosomes. A homologous set of chromosomes are paired and recombined with each other in meiotic prophase. The paired homologous chromosomes segregate to the opposite poles at the first meiotic division (meiosis I) and the sister chromatids segregate at the second meiotic division (meiosis II). Understanding the mechanisms for proper segregation of chromosomes is clinically important because chromosome missegregation during meiosis is a major cause of human miscarriage and trisomy disease (Hassold *et al.*, 1996).

The kinetochore is a specialized structure formed on the centromere and is essential for faithful segregation of chromosomes, playing an important role in attachment of spindle microtubules to generate forces during chromosome segregation. During mitosis, pairs of sister chromatids produced by DNA replication segregate equally to dividing cells. In contrast, during meiosis, sister chromatids segregate to the same pole (reductional segregation) in meiosis I while they segregate to the opposite poles (equational segregation) in meiosis II as in mitosis. Reductional segregation is achieved by monopolar attachment of the spindle to the kinetochore that is established uniquely during meiosis. Thus, the kinetochore undergoes significant reorganization during the transition from mitosis to meiosis.

These fundamental functions of the kinetochore are conserved from yeasts to humans. In the budding yeast *Saccharomyces cerevisiae*, a 125-base pair sequence called CDE is sufficient for centromere function (Cottarel *et al.*, 1989). In the fission yeast *Schizosaccharomyces pombe*, the centromere spans from 40 to 100 kbp (Chikashige *et al.*, 1989). Human chromosomes have a large centromere consisting of more than 1 Mbp of repetitive alpha satellite sequence flanked by heterochromatin (reviewed in Cleveland *et al.*, 2003; Maiato *et al.*, 2004). In spite of the variation in centromere DNA sequences, kinetochore proteins are well conserved among organisms. The *S. cerevisiae* kinetochore complex is composed of four subcomplexes: MIND, NDC80, COMA, and Ctf19 (De Wulf *et al.*, 2003). In addition, the DASH complex is localized at the kinetochore and the spindle and is required for spindle attachment to the kinetochore (Cheeseman *et al.*, 2001; Li *et al.*, 2002). The *S. cerevisiae* DASH complex is composed of 10 proteins that localize at the kinetochore and the spindle (Miranda *et al.*, 2005; Westermann *et al.*, 2005). Biochemical analyses have revealed that the kinetochore complex is comprised of subcomplexes of proteins. Many of these proteins are conserved in other eukaryotes, from yeasts to humans (De Wulf *et al.*, 2003; Nekrasov *et al.*, 2003; Cheeseman *et al.*, 2004; Obuse *et al.*, 2004; reviewed in Meraldi *et al.*, 2006).

Subcomplex structures of the *S. pombe* kinetochore, similar to that of *S. cerevisiae* and humans, have been reported (Hayashi *et al.*, 2004; Obuse *et al.*, 2004; Liu *et al.*, 2005). The *S. pombe* kinetochore contains the Ndc80 complex (Ndc80, Nuf2, Spc24, and Spc25), which is highly conserved in many organisms from yeasts to humans (Nabetani *et al.*, 2001; Wigge and Kilmartin, 2001). *S. pombe mis12*$^+$, *mis13*$^+$,

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Address correspondence to: Yasushi Hiraoka (yasushi@nict.go.jp).

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Table 1. Strain list

AY160-14D	h^{90}	ade6-216, leu1-32, lys1-131, ura4-D18
H08/E06	h^{90}	AY160-14D w/mis12$^+$::mis12-GFPHA-kan^r
H04/B01	h^{90}	AY160-14D w/mis13$^+$::mis13-GFPHA-kan^r
H02/H11	h^{90}	AY160-14D w/mis14$^+$::mis14-GFPHA-kan^r
H18/D01	h^{90}	AY160-14D w/nnf1$^+$::nnf1-GFPHA-kan^r
H13/B02	h^{90}	AY160-14D w/spc7$^+$::spc7-GFPHA-kan^r
H09/E06	h^{90}	AY160-14D w/ndc80$^+$::ndc80-GFPHA-kan^r
H07/D08	h^{90}	AY160-14D w/nuf2$^+$::nuf2-GFPHA-kan^r
H06/C03	h^{90}	AY160-14D w/mis6$^+$::mis6-GFPHA-kan^r
H17/C04	h^{90}	AY160-14D w/sim4$^+$::sim4-GFP-kan^r
H08/E08	h^{90}	AY160-14D w/mis15$^+$::mis15-GFP-kan^r
H17/H08	h^{90}	AY160-14D w/fta7$^+$::fta7-GFPHA-kan^r
H03/B04	h^{90}	AY160-14D w/mal2$^+$::mal2-GFP-kan^r
H07/E02	h^{90}	AY160-14D w/dad1$^+$::dad1-GFPHA-kan^r
02/E03	h^{90}	cnp1-YFP-LEU2::leu1-32 (gifted by Dr. Yoshida)
H18/A02	h^{90}	AY160-14D w/cnp3$^+$::cnp3-GFPHA-kan^r
H14/A05	h^{90}	AY160-14D w/cnl2$^+$::cnl2-GFPHA-kan^r
H07/F02	h^{90}	AY160-14D w/dam1$^+$::dam1-GFPHA-kan^r
H18/B01	h^{90}	AY160-14D w/dad2$^+$::dad2-GFP-kan^r
H08/D09	h^{90}	AY160-14D w/ask1$^+$::ask1-GFPHA-kan^r
H07/G05	h^{90}	AY160-14D w/spc34$^+$::spc34-GFP-kan^r
AHY217	h^{90}	H14/A05 w/nuf2$^+$::mRFP-ura4$^+$ (pHA143)
AHY68	h^{90}	H05/C12 w/lys1$^+$::nuf2-mRFP (pHA142)
AHY42	h^-	ade6-210, ura4-D18, leu1-32, lys1-131, mis6-302, cnl2$^+$::cnl2-GFPHA-kan^r
AHY43	h^-	ade6-210, ura4-D18, leu1-32, lys1-131, mis6-302, fta7$^+$::fta7-GFPHA-kan^r
AHY99	h^{90}	ade6-216, dam1$^+$::dam1-GFPHA-kan^r, nuf2$^+$::nuf2-mRFP-ura4$^+$, lys1-131, ura4-D18
AHY108	h^{90}	ade6-210, dam1$^+$::dam1-GFPHA-kan^r, sid4$^+$::sid4-mRFP-kan^r, lys1, ura4, leu1-32
AHY172	h^-	leu1-32, mis13$^+$::mis13-GFPHA-kan^r, pat1-114
AHY176	h^-	leu1-32, mis12$^+$::mis12-GFPHA-kan^r, pat1-114
AHY174	h^-	leu1-32, mis14$^+$::mis14-GFPHA-kan^r, pat1-114
AHY178	h^-	leu1-32, nnf1$^+$::nnf1-GFPHA-kan^r, pat1-114
AHY187	h^-	leu1-32, spc7$^+$::spc7-GFPHA-kan^r, pat1-114, ura4-D18
AHY179	h^-	leu1-32, mis13$^+$::mis13-GFPHA-kan^r, pat1-114, lys1$^+$::mat-Pc
AHY180	h^-	leu1-32, mis12$^+$::mis12-GFPHA-kan^r, pat1-114, lys1$^+$::mat-Pc
AHY184	h^-	leu1-32, mis14$^+$::mis14-GFPHA-kan^r, pat1-114, lys1$^+$::mat-Pc
AHY181	h^-	leu1-32, nnf1$^+$::nnf1-GFPHA-kan^r, pat1-114, lys1$^+$::mat-Pc
AHY182	h^-	leu1-32, spc7$^+$::spc7-GFPHA-kan^r , pat1-114, lys1$^+$::mat-Pc, ura4-D18
PZ988	h^{90}	sgo1$^+$-FLAG-GFP leu1 ade6-M210 pREP81 (CFP-atb2$^+$) (distributed from NBRP)
PZ425	h^{90}	GFP-3Pk-moa1$^+$-kan^r, ade6-M216, leu1 (gifted by Dr. Watanabe)
AHY290	h^-	sgo1$^+$-FLAG-GFP, pat1-114
AHY291	h^-	GFP-3Pk-moa1$^+$-kan^r, ade6-M216, pat1-114
AHY295	h^-	sgo1$^+$-FLAG-GFP, pat1-114, aur1^r+::mat-Pc
AHY296	h^-	GFP-3Pk-moa1$^+$-kan^r, ade6-M216, pat1-114, aur1^r::mat-Pc

mis14$^+$, *nnf1*$^+$, and *spc7*$^+$ genes exhibit genetic interactions (Obuse *et al.*, 2004), and their respective proteins have been copurified with the Ndc80 complex (Liu *et al.*, 2005). Mis12, Mis13, Mis14, and Nnf1 likely compose the Mis12 complex, corresponding to the *S. cerevisiae* MIND complex. A supercomplex containing the Ndc80 and Mis12 complexes and the Spc7 protein is also called NMS complex collectively (Liu *et al.*, 2005). Recently 13 proteins were purified as a Mis6-containing complex by biochemical purification (Liu *et al.*, 2005). These proteins include Sim4 and Mis15, which have been reported to depend on the Mis6 protein for their centromere localization (Takahashi *et al.*, 2000; Pidoux *et al.*, 2003; Hayashi *et al.*, 2004). Thus, it is likely that these proteins compose the Mis6 complex (also called the Sim4 complex in Liu *et al.*, 2005), which corresponds to the *S. cerevisiae* COMA and Ctf19 complexes. Ten *S. pombe* proteins, which are conserved in the *S. cerevisiae* DASH complex, were purified as a complex by biochemical purification (Liu *et al.*, 2005). The DASH complex is localized at the kinetochore and the spindle at mitotic phase (Liu *et al.*, 2005) and functions with Klp5/6 to capture the kinetochore (Sanchez-Perez *et al.*, 2005), indicating that it plays a role in spindle attachment during chromosome segregation. In addition to these mitotic centromere proteins, meiosis-specific centromere proteins, Sgo1 and Moa1, that play an important role in meiotic chromosome segregation have been characterized in *S. pombe*. Moa1 is essential to establish the monopolar kinetochore together with the meiotic cohesin Rec8 (Yokobayashi and Watanabe, 2005), and Sgo1 protects Rec8 at the centromere to maintain cohesion between sister centromeres until meiosis II (Kitajima *et al.*, 2004; Rabitsch *et al.*, 2004).

S. pombe provides a useful experimental system in which to study the reorganization of chromosomes during meiosis. In this organism, the centromeres cluster near the spindle pole body (SPB; a centrosome-equivalent structure in fungi) throughout mitotic interphase; however, during meiotic prophase centromeres detach from the SPB, and instead telomeres cluster to the SPB (Chikashige *et al.*, 1994). During this period of meiosis, the nucleus elongates and oscillates between the cell poles, with telomeres clustered at the SPB located at the leading edge of the moving nucleus. The elongated nucleus is often called the "horsetail" nucleus. This striking repositioning of centromeres may be associated with meiotic reorganization of the kinetochore, which occurs during the horsetail stage when the centromeres are separated from the SPB. Analysis of centromere proteins in meiotic prophase would improve our

Table 2. Summary of *S. pombe* kinetochore proteins

Group	Subgroup	*S. pombe* gene	*S. cerevisiae* genes	*S. cerevisiae* complex[a]
Group 1	Mis6-like group	*mis6$^+$/SPAC1687.20c*	*CTF3*	Ctf19 complex
		sim4$^+$/SPBC18E5.03c	—	—
		mis15$^+$/SPBP22H7.09c	*CHL4*	Ctf19 complex
		fta7$^+$/SPCC1235.07	—	—
		mal2$^+$/SPAC25B8.14	*MCM21*	COMA complex
		dad1$^+$/SPAC16A10.05c	*DAD1*	DASH complex
		cnp1$^+$/SPBC1105.17	*CSE4*	CENP-A
		cnp3$^+$/SPBC1861.01c	*MIF2*	CENP-C
		SPAC23H4.11c/cnl2$^+$	—	—
	NMS group	*mis12$^+$*/SPBC409.04c	*MTW1*	MIND complex
		mis13$^+$/SPBC409.09c	*DSN1*	MIND complex
		mis14$^+$/ nsl1$^+$/SPAC68.02c	*NSL1*	MIND complex
		nnf1$^+$/SPAC29E6.04	*NNF1*	MIND complex
		spc7$^+$/SPCC1020.02	*SPC105*	—
		ndc80$^+$/SPBC11C11.03	*NDC80*	NDC80 complex
		nuf2$^+$/SPAC27F1.04c	*NUF2*	NDC80 complex
		spc24$^+$/SPBC336.08	*SPC24*	NDC80 complex
		spc25$^+$/SPCC188.04c	*SPC25*	NDC80 complex
Group 2	DASH group	*dam1$^+$/SPAC589.08c*	*DAM1*	DASH complex
		dad2$^+$/hos2$^+$/SPAC1805.07c	*DAD2*	DASH complex
		ask1$^+$/SPBC27.02c	*ASK1*	DASH complex
		spc34$^+$/SPAC8C9.17c	*SPC34*	DASH complex

S. cerevisiae complexes are reviewed by Chan *et al.* (2005).

understanding of the mechanisms controlling centromere reorganization during meiosis.

Recently we found that the Ndc80 complex proteins and Mis12 disappear during meiotic prophase (Asakawa *et al.*, 2005). To further investigate this finding in the current study we have observed 22 centromere proteins in living cells during meiosis. Time-lapse observation of living cells can provide a unique opportunity to follow the dynamic appearance and disappearance of proteins directly in individual cells. Our observations indicate that the mitotic centromere proteins may be classified into three groups that each behaves differently during meiosis. The behaviors of the meiosis-specific centromere proteins, Sgo1 and Moa1, were also followed during meiosis and compared with those of the mitotic centromere proteins.

MATERIALS AND METHODS

Strains and Plasmids

The *S. pombe* strains used in this study are listed in Table 1. Culture media and handling of *S. pombe* cells are described in Moreno *et al.* (1991). YES medium was used for routine culture. EMM2 liquid medium depleted of nitrogen sources (EMM2-N) and ME agar plates were used to induce meiosis and sporulation.

GFP-3HA tagging at the carboxy terminus of the ORF on the chromosome was performed by PCR-mediated integration as previously described (Bähler *et al.*, 1998). The PCR template pAH90 plasmid for GFP-3HA tagging was constructed by inserting the GFP gene into the pFA6a-3HA-kanMX plasmid. A strain carrying Cnp1-YFP is a gift of Dr. M. Yoshida (RIKEN, Saitama, Japan), in which Cnp1-YFP is integrated at the *leu1$^+$* locus, and is expressed under the *nmt1* promoter (Matsuyama *et al.*, 2006). A strain carrying Sid4 fused with monomeric RFP (mRFP) was crossed with a GFP-HA tagged strain for double staining of the SPB and the kinetochore (Chikashige *et al.*, 2006). For tagging the Nuf2 carboxyl terminal with mRFP, the promoter region and ORF region of the *nuf2$^+$* gene were cloned by PCR using genomic DNA as the template and fused with the mRFP coding gene in a vector containing the *S. pombe lys1$^+$* gene. The resulting plasmid, pHA142, was integrated at the *lys1$^+$* locus to produce a strain carrying an additional copy of the *nuf2$^+$* gene. A fragment of pHA142, which contains the Nuf2-mRFP coding sequence and the *nmt1$^+$* terminator, was ligated to pYC6 carrying the *S. pombe ura4$^+$* marker gene (pHA143). The fusion construct coding Nuf2-mRFP was integrated at the chromosomal *nuf2$^+$* locus. Chromosomal integration was confirmed by PCR. The *nnf1$^+$* gene was isolated by RT-PCR using a 3′-RACE kit (TaKaRa, Shiga, Japan).

Fluorescence Microscopy of Living S. pombe Cells

Live-cell observation was carried out as described in Ding *et al.* (2004) with some modifications. For observation of vegetative cells, cells were cultured in YES liquid medium at 26°C. Early log phase cells were collected, washed with distilled water, and then transferred to EMM2 medium. For observation of meiotic cells, meiosis was induced by transferring log phase cells to an ME plate. After a 16-h incubation at 20°C, the cells were suspended in EMM2-N medium supplemented with appropriate amino acids. For staining chromosomes in living cells, cells were washed twice with distilled water and treated with Hoechst 33342 (at a final concentration of 25 μg/ml in distilled water) for 15 min at room temperature. For live observation, cells were placed on a 35-mm glass-bottom culture dish coated with 0.2% (wt/vol) concanavalin A (MatTek, Ashland, MA). Fluorescence microscope images were obtained using SoftWoRx software on the DeltaVision microscope system (Applied Precision, Seattle, WA) set up in a temperature-controlled room as described previously (Haraguchi *et al.*, 1999). A set of images taken at 10 focal planes with 0.3-μm intervals were obtained every 5 min for observation of centromere proteins throughout meiosis. The intensity was corrected by subtracting the background that measured outside cells. For cells double-stained for Nuf2 and Sid4, or Dam1 and Sid4, images were obtained every 2 min.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Jin *et al.*, 2002, Katou *et al.*, 2003). Cnl2-GFP-3HA cells were grown at 26°C to log phase and shifted to 18°C for 30 min. Cells were then fixed with 1% of formaldehyde for 1 h at 18°C. Cell extracts were prepared using Multibeads Shocker (Yasui Kikai, Osaka, Japan). Magnetic beads conjugated with protein A (Dynabeads Protein A, Dynal, Norway) and anti-HA antibody (3F10, Roche, Indianapolis, IN) was used for ChIP. ChIP analysis was carried out by quantitative PCR, with ABI PRISM 7000 and ABsolute QPCR SYBR green ROX Mix (Abgene, Epsom, United Kingdom). The sequences of primers used were described previously: by Saitoh *et al.* (1997) for the cnt and *lys1$^+$* regions and by Jin *et al.* (2002) for the imr and otr regions.

Preparation of Cell Extracts for Immunoblot Analysis

Synchronized cultures of meiotic cells were prepared using a temperature-sensitive *pat1-114* or *pat1-114 mat-Pc* strain as described in Yamamoto and Hiraoka (2003). Approximately 0.5×10^7 cells/ml culture in EMM2-N medium were incubated at 26°C for 16 h and were induced to enter meiosis by shifting the temperature to 34°C. For preparation of cell extracts, cells were

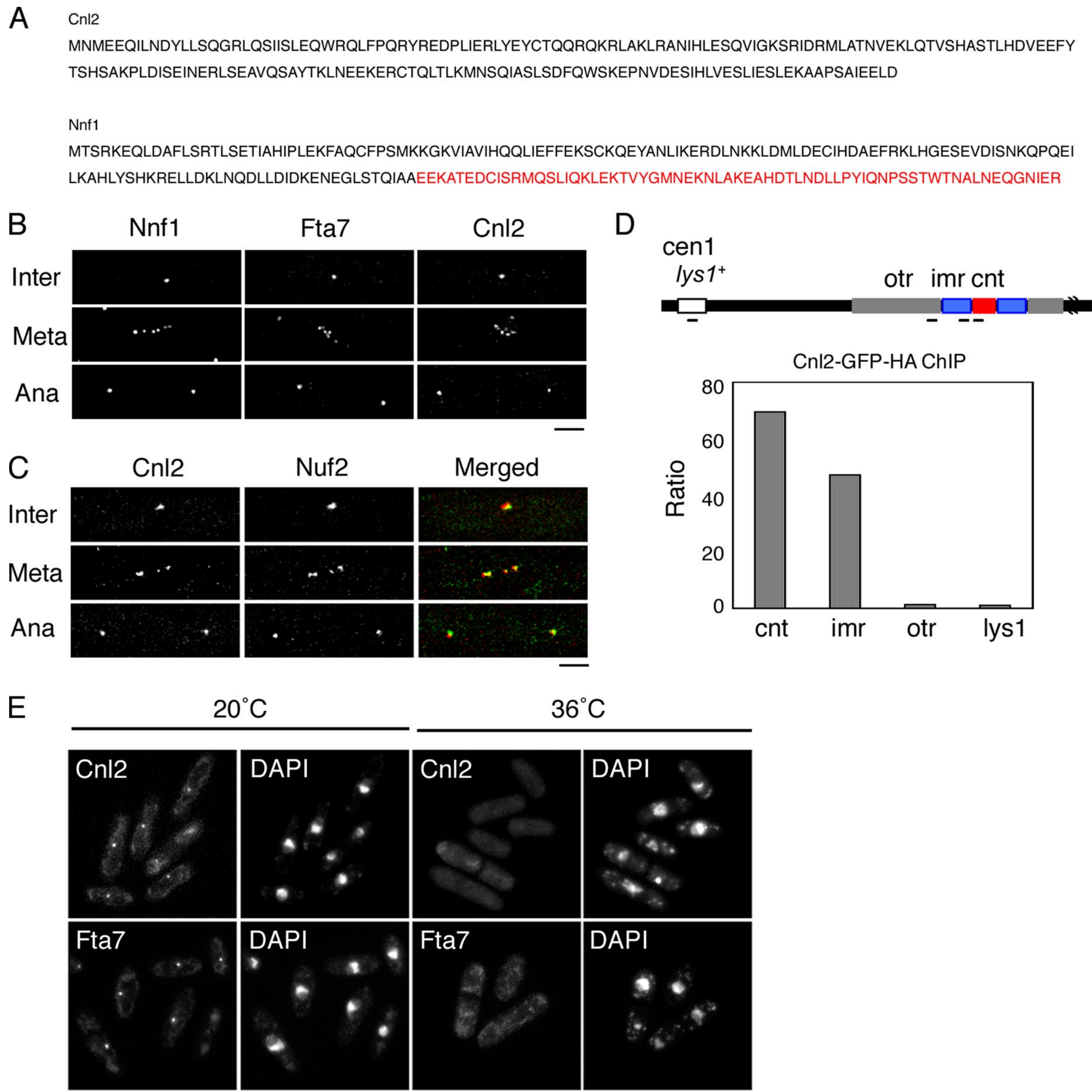
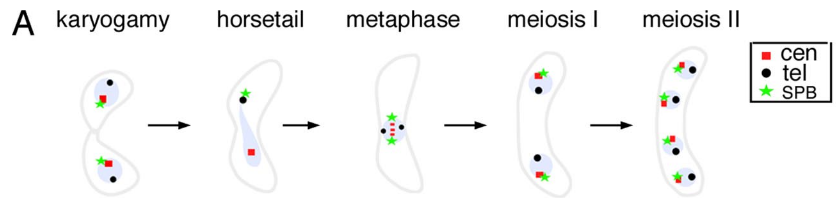


Figure 1. Behavior of centromere proteins in mitosis. (A) Amino acid sequences of newly identified proteins from the GFP fusion library. The location of the carboxy terminus of Nnf1 was determined from the *nnf1*$^+$ ORF, which was amplified by RT-PCR. The Nnf1 protein sequence was found to be longer (shown in red) than was predicted in the Sanger Center database. (B) Mitotic localization of centromere proteins in living cells. Inter, interphase; Meta, metaphase; Ana, anaphase. Bar, 5 μm. (C) Colocalization of Nuf2 protein with the Cnl2 protein. Bar, 5 μm. (D) ChIP analysis with anti-HA antibody was used to measure Cnl2-GFP-3HA levels associated with the indicated chromosome sites: the central core (cnt; indicated in red), the inner repeats (imr; indicated in blue), the outer repeats (otr; indicated in gray), and the chromosome arm (*lys1*$^+$; indicated in white) on the chromosome I, as shown in the upper diagram. Bars below the diagram show the position of PCR primers for the cnt, imr, otr, and *lys1*$^+$ sites. DNA isolated from chromatin immunoprecipitated with anti-HA antibody or whole cell extract (WCE) was subjected to quantitative PCR to measure the DNA amount of a corresponding region. The ratio of DNA amount in ChIP fractions with respect to that in WCE was calculated for each chromosome site and normalized with the value for the *lys1*$^+$ site. The normalized ratios were plotted in the lower panel. DNA amount in ChIP fractions prepared with no antibody were measured as a negative control, and the normalized ratios were negligible (0.1–0.3) for the cnt, imr, otr, and *lys1*$^+$ sites. (E) Localization of Cnl2 and Fta7 proteins in the *mis6-302* mutant. Mutant cells were incubated at 26°C for 4 h and then incubated for 6 h at 20 or 36°C. Both proteins disappeared from the centromere of the *mis6-302* mutant cells at the restrictive temperature, 36°C. Bar, 15 μm.

collected at appropriate times and incubated with 1 mM PMSF for 10 min at room temperature. Cells were washed three times with lysis buffer (50 mM HEPES, pH 7.5, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 1× protease inhibitor cocktail [Roche], and 1 mM PMSF) and disrupted using a Multibeads Shocker (Yasui Kikai). The resulting cell extracts were centrifuged at 15,000 rpm for 15 min. Cell extracts, each containing ~70 μg total protein, were separated on a 10% SDS-PAGE gel. GFP-3HA–tagged proteins were detected using the 3F10 rat monoclonal anti-HA antibody (Roche) at 1:1000 dilution. To confirm equal loading, the Cdc2 protein was detected with anti-PSTAIR (a gift from Dr. Yamashita, Hokkaido University).

Preparation of Fixed Cells

S. pombe cells were fixed with 3% of formaldehyde for 5 min at room temperature. Fixed cells were washed twice with PBS containing 0.05% Triton X-100 and stained with 4′,6-diamidino-2-phenylindole (DAPI) at a final con-



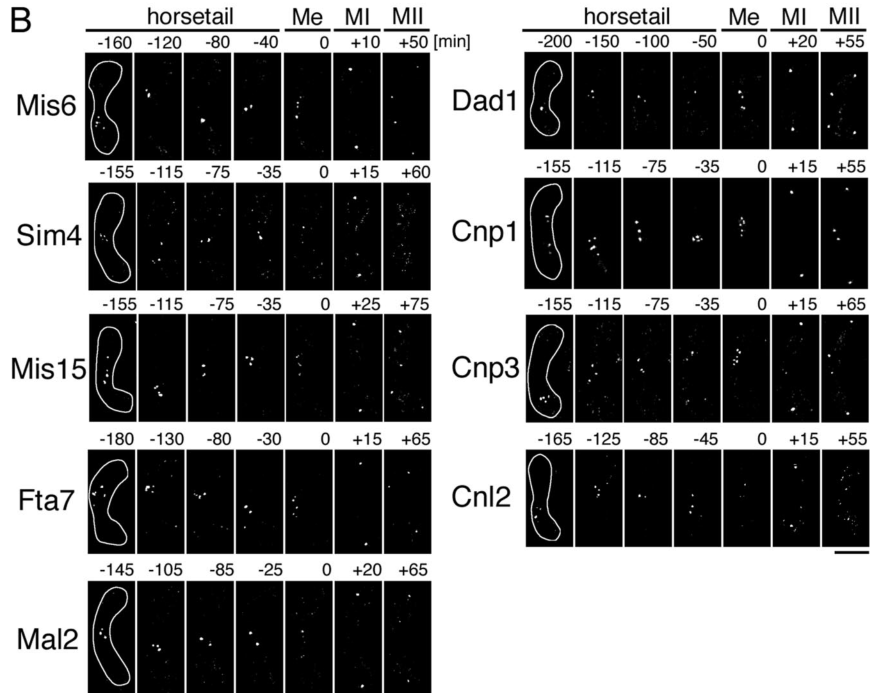


Figure 2. Behavior of Mis6-like group proteins during meiosis. (A) Nuclear dynamics during meiosis in *S. pombe*. On nitrogen starvation, the haploid cells of the opposite mating type conjugate to form a diploid zygote and immediately enter meiosis. During karyogamy and through the horsetail stage, the centromeres separate from, and telomeres cluster at, the SPB. The elongated horsetail nucleus shows oscillated movement during meiotic prophase. After the nucleus ceases this movement at metaphase, it undergoes two rounds of division to generate four spores. At meiosis I, the telomeres de-cluster, and centromeres recluster to the SPB. The horsetail stage was calculated to continue for 142 ± 22 min and metaphase for 37 ± 9 min (average and SD for 170 cells; Ding *et al.*, 2004). (B) GFP-tagged Mis6-like group proteins (with an exception of YFP-tagged Cnp1) were observed in living meiotic cells: Mis6, Sim4, Mis15, Fta7, Mal2, Dad1, Cnp1, Cnp3, and Cnl2. The number of cells examined: 21 for Mis6, 13 for Sim4, 30 for Mis15, 20 for Fta7, 27 for Mal2, 16 for Dad1, 10 for Cnp1, 10 for Cnp3, and 19 for Cnl2. Me, metaphase I; MI, meiosis I; MII, meiosis II. Numbers at the top of each panel indicate times in minutes relative to the metaphase–anaphase transition. Bar, 5 μm.

centration of 2–5 μg/ml. For the synchronized cultures of meiotic cells, cells of the *pat1-114 mat-Pc* mutant that had been induced to enter meiosis were collected at appropriate times after the temperature shift and fixed with cold 70% ethanol.

RESULTS

Mitotic Behaviors of Kinetochore Proteins Observed in Living Cells

We constructed GFP fusions of 22 kinetochore-localized proteins (Table 2). These GFP fusion constructs constitute part of a library containing ~1000 GFP fusion constructs, in which the coding sequence of GFP and 3HA is integrated at the 3′-end of the chromosomal ORF to express the fusion construct under the control of its own promoter (A. Hayashi and Y. Hiraoka, unpublished results). This library contains a group of uncharacterized genes that are predicted to be nuclear proteins (Wood *et al.*, 2002). Microscopic screening of these GFP-fusion gene products assigned 22 of them as centromere proteins; their localization at the centromere was confirmed by colocalization with the well-characterized centromere protein, Nuf2. Of these 22 proteins, three proteins were newly identified and named the Cnl proteins (centromere localized protein). During the course of our study, Cnl1 and Cnl3 were independently identified as Mis13 protein (Obuse *et al.*, 2004) and Fta7 (Liu *et al.*, 2005), respectively. Cnl2 (ORF ID: SPAC23H4.11c) is yet uncharacterized and has no obvious homologues in other organisms (Figure 1A). Gene disruption experiments have revealed that *cnl2⁺* is nonessential for mitotic cell growth (data not shown).

First we examined the mitotic behaviors of these 22 centromere-localized proteins in living cells and classified them into two groups based on their mitotic behaviors. Of the 22 proteins, 18 proteins were localized at the centromere throughout the mitotic cell cycle (Table 2; group 1). Examples of this group (Nnf1, Fta7, and Cnl2) are shown in Figure 1B. These proteins were always localized at a single spot during interphase and as six spots in metaphase. Centromere localization of the newly identified Cnl2 protein was examined by colocalization with Nuf2 (Figure 1C). Furthermore, ChIP experiments confirmed that Cnl2 was localized at the central region (cnt and imr), but not at the outer repeats (otr), of the centromere (Figure 1D). In contrast, four proteins (Dam1, Dad2, Ask1, and Spc34) were localized at the centromere only at the M phase (Table 2; group 2). These 4 proteins share homology with the *S. cerevisiae* DASH complex, DAM1, DAD2, ASK1, and SPC34 (Miranda *et al.*, 2005), and the observation that their centromere localization is limited to the M phase has been previously reported in *S. pombe* (Liu *et al.*, 2005). Thus, we assigned these four proteins to the DASH complex (Table 2).

Meiotic Behaviors of Kinetochore Proteins Observed in Living Cells

Next we examined the meiotic behavior of these 22 centromere proteins. In *S. pombe*, haploid cells of the opposite mating type conjugate upon nitrogen starvation; two haploid nuclei fuse together during karyogamy, and meiotic prophase is characterized by the elongated horsetail nucleus

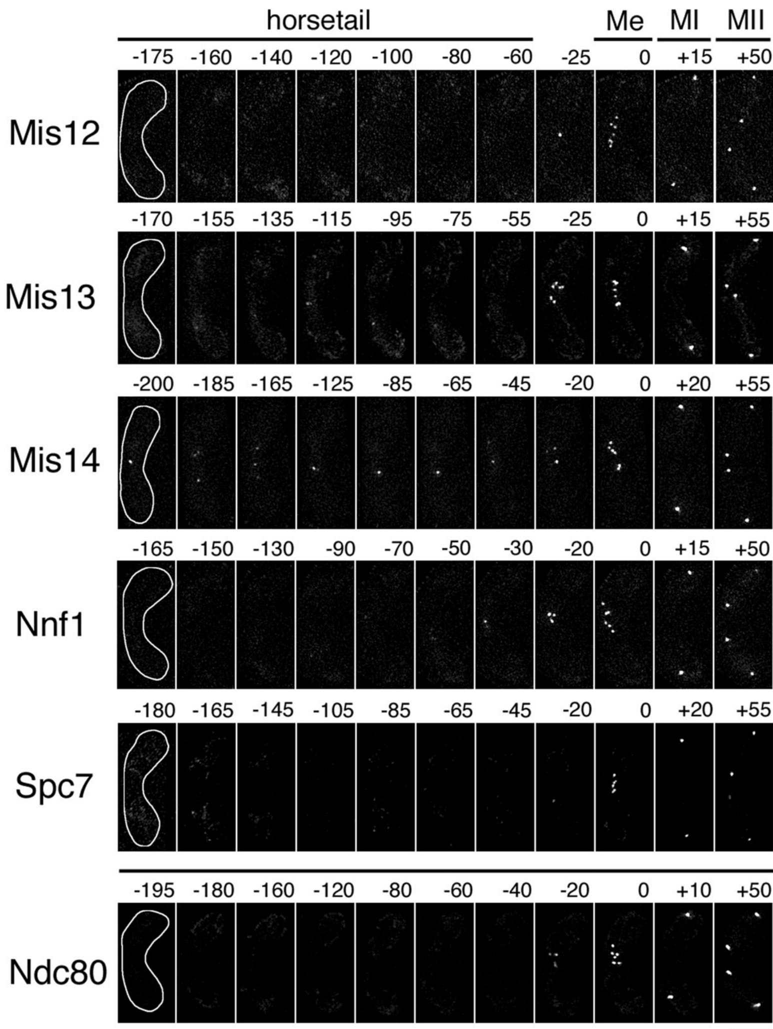


Figure 3. Behavior of NMS group proteins during meiosis. GFP-tagged NMS group proteins were observed in living meiotic cells: Mis12, Mis13, Mis14, Nnf1, and Spc7. Ndc80 is a control for the Ndc80 complex. The number of cells examined: 36 for Mis12, 18 for Mis13, 19 for Mis14, 28 for Nnf1, and 21 for Spc7. Me, metaphase I; MI, meiosis I; MII, meiosis II. Numbers at the top of each panel indicate times in minutes relative to the metaphase-anaphase transition. Bar, 5 μm.

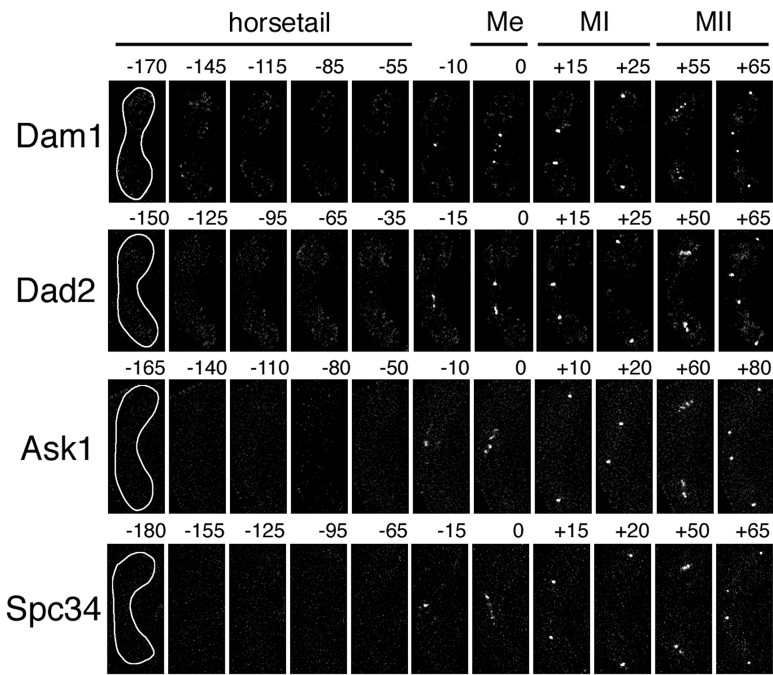


Figure 4. Behavior of DASH group proteins in meiosis. GFP-tagged DASH group proteins were observed in living meiotic cells: Dam1, Dad2, Ask1, and Spc34. The number of cells examined: 28 for Dam1, 32 for Dad2, 21 for Ask1, 27 for Spc34. Me, metaphase I; MI, meiosis I; MII, meiosis II. Numbers at the top of each panel indicate times in minutes relative to the metaphase–anaphase transition. Bar, 5 μm.

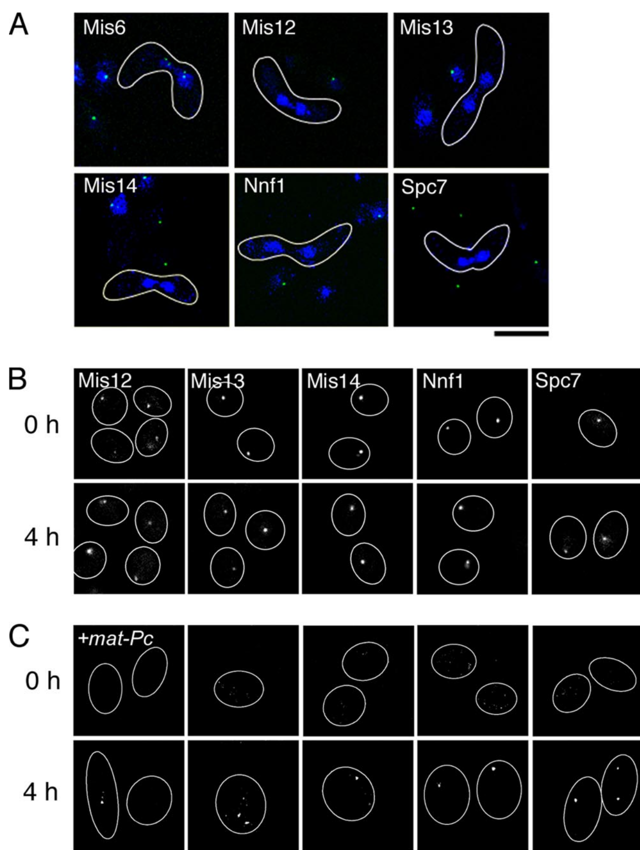


Figure 5. Disappearance of Mis12-Spc7 complex proteins upon mating pheromone signaling. (A) A conjugated diploid zygote is outlined by the white line. Green, GFP fused protein. Blue, DAPI-stained nuclei. Mis12-Spc7 complex proteins (Mis12, Mis13, Mis14, Nnf1, and Spc7) reduce centromere localization at karyogamy. Included as a control, Mis6 remains at the centromere. Bar, 5 μm. (B) Localization of Mis12 group proteins in *pat1* mutant at 0 and 4 h after the temperature shift-up. (C) Localization of Mis12 group proteins in a *pat1 mat-Pc* strain at 0 and 4 h after the temperature shift-up. The time course of nuclear division is shown in Figure 6B.

moving back and forth between the cell ends (Figure 2A). This oscillatory movement continues for some hours; after stopping at the center of the cell, the nucleus condenses and initiates meiotic divisions. During nuclear movements, centromeres are separated from the SPB (Figure 2A). Eighteen group 1 proteins behaved differently during meiosis and were further classified into two subgroups: the Mis6-like and NMS groups (Table 2). Proteins of the Mis6-like group remained at the centromere throughout meiosis (Figure 2B), whereas those of the NMS group disappeared from the centromere or their presence was significantly reduced, during meiotic prophase (Figure 3).

The Mis6-like group contains nine proteins that behave like Mis6 (Table 2; Figure 2B). Among these proteins, six proteins (Mis6, Sim4, Mis15, Fta7, Mal2, and Dad1) were found to be part of a Mis6-containing complex isolated by biochemical purification (Liu *et al.*, 2005). Cnp1 and Cnp3/Mif2 are homologues of metazoan CENP-A and CENP-C, respectively. *S. pombe* Dad1 was localized at the centromere throughout mitosis as shown previously (Liu *et al.*, 2005). Dad1 remained at the centromere during meiosis (Figure 2B) as well as mitosis and was thus classified as a Mis6-like group protein, despite the fact that Dad1 belongs to the DASH complex in *S. cerevisiae*. We classified Cnl2 and Fta7 as members of this group because they remained at the centromere during mitosis and meiosis. In addition, their centromere localization depended on Mis6: Cnl2 and Fta7 proteins lost their centromere localization in a *mis6-302* temperature-sensitive mutant at the restricted temperature of 36°C (Figure 1E).

The NMS group contains nine proteins that disappear from the centromere during meiotic prophase (Table 2; Figure 3). This group of proteins correspond to the biochemically defined NMS supercomplex, which is comprised of the Ndc80 complex (Ndc80, Nuf2, Spc24, and Spc25), the Mis12 complex (Mis12, Mis13, Mis14, and Nnf1), and Spc7 (Liu *et al.*, 2005). In this report, hereafter we refer to the Mis12 complex and the Spc7 protein as the Mis12-Spc7 complex. These proteins showed similar, but slightly different, behaviors of disappearance and reappearance during meiotic prophase. The Ndc80 complex proteins and Spc7 disappeared from the centromere during karyogamy and reappeared in late meiotic prophase (Asakawa *et al.*, 2005; Figure 3). In contrast, levels of Mis12 complex proteins were significantly reduced at the centromere during meiotic prophase, with only residual faint signals detected (Figure 3). Different localization patterns were observed when these proteins

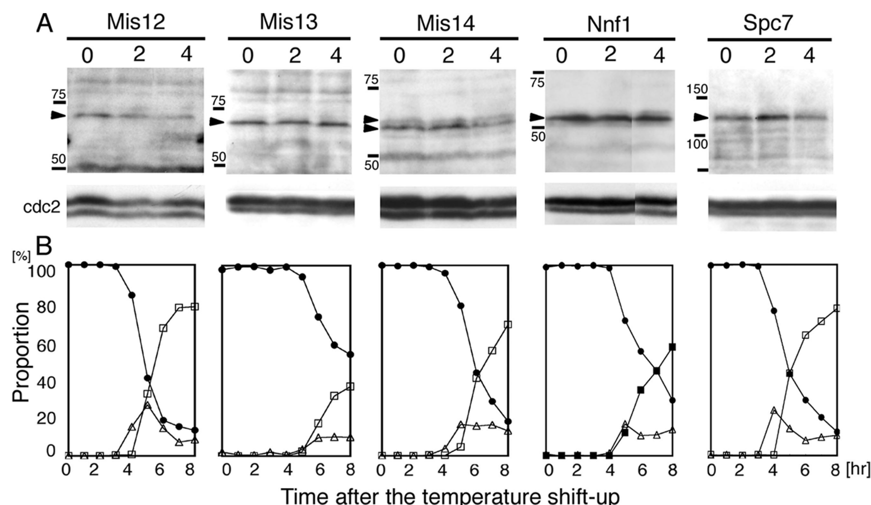
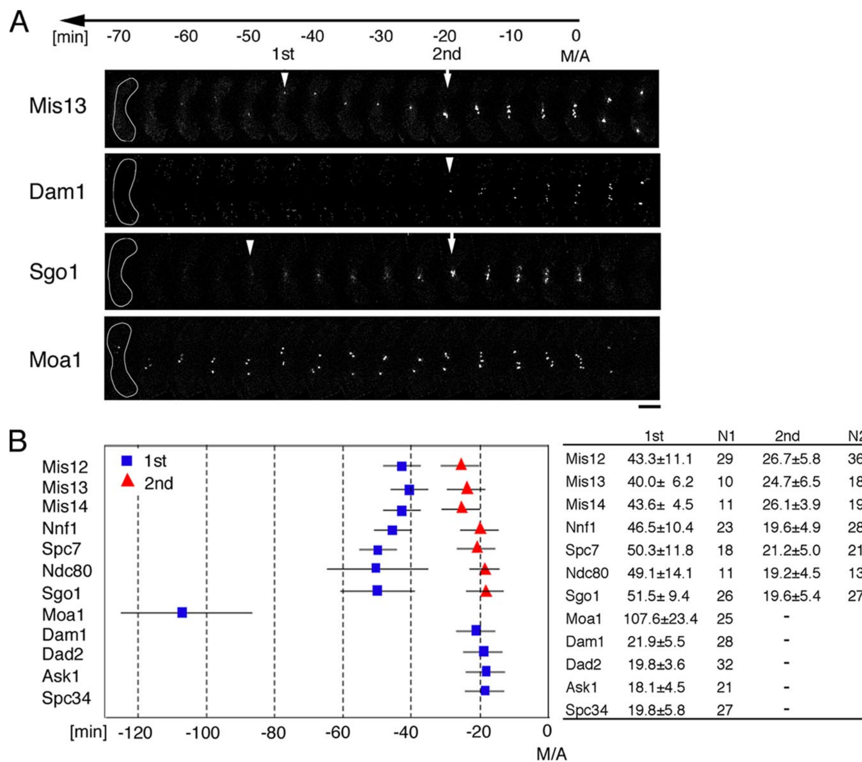


Figure 6. Expression of Mis12-Spc7 complex proteins during meiotic prophase. (A) Mis12-Spc7 complex proteins were fused to GFP-HA tags and detected with anti-HA antibody. Molecular weights (kDa) are shown on the left of each panel. The number at the top of each column indicates the time after the temperature shift-up; cells of a *pat1 mat-Pc* strain were collected at the indicated times, and cell extracts were prepared for immunoblot analysis. Cdc2 was used as a loading control for each of the extracts. (B) Time course of nuclear division in a *pat1 mat-Pc* strain after temperature shift-up. One nucleus, ●; two nuclei, Δ; three/four nuclei, □.



	1st	N1	2nd	N2
Mis12	43.3±11.1	29	26.7±5.8	36
Mis13	40.0± 6.2	10	24.7±6.5	18
Mis14	43.6± 4.5	11	26.1±3.9	19
Nnf1	46.5±10.4	23	19.6±4.9	28
Spc7	50.3±11.8	18	21.2±5.0	21
Ndc80	49.1±14.1	11	19.2±4.5	13
Sgo1	51.5± 9.4	26	19.6±5.4	27
Moa1	107.6±23.4	25	-	
Dam1	21.9±5.5	28	-	
Dad2	19.8±3.6	32	-	
Ask1	18.1±4.5	21	-	
Spc34	19.8±5.8	27	-	

Figure 7. Times of reappearance of NMS and DASH complex proteins at the centromere in meiosis. (A) Times were measured in minutes relative to the metaphase–anaphase transition (expressed as negative values). The NMS complex proteins and Sgo1 reappeared at the centromere at two steps in late prophase. The arrowhead indicates the time of the first reappearance at the centromere, and the arrow indicates the time of the second increase of the GFP signal. The DASH group immediately appeared before meiosis I. M/A. metaphase–anaphase transition. Bar, 5 μm. (B) Time of reappearance before the metaphase-anaphase transition. The blue rectangle indicates the time of the first-step increase, and the red triangle indicates the time of the second-step increase. Error bar, SD. The table at the right shows the number of observed cells and measured minutes of proteins. N1, the number of cells that showed the first-step reappearance; N2, the total number of cells examined. The first-step reappearance was not detected in a portion of the cells (the number of these cells corresponds to N2 minus N1). Average times of the first-step relocalization of the NMS complex proteins and Sgo1 ranged between 40 and 50 min from the metaphase–anaphase transition. DASH group proteins reappeared at the centromere at approximately the same time as the second-step increase in the Mis12 group and Sgo1 signals. Moa1 and the proteins of the DASH group appeared at a single step.

were overexpressed under the control of the *nmt1* promoter in meiotic prophase; although overexpression of Nuf2 of the Ndc80 complex showed diffuse cytoplasmic localization (Asakawa *et al.*, 2005), overexpression of Mis13 and Mis14 of the Mis12 complex showed diffuse nuclear localization (data not shown).

The DASH complex proteins (Dam1, Spc34, Dad2, and Ask1) were not detected during meiotic prophase. They reappeared at the centromere shortly before metaphase of meiosis I (Figure 4), as is seen at metaphase in the mitotic cell cycle. Centromere localization limited to the period of chromosome segregation supports their role in spindle attachment. Taken together, the behavior of these groups was generally consistent with the subcomplex structures that have been identified from genetic interaction and biochemical purification studies (Hayashi *et al.*, 2004; Obuse *et al.*, 2004; Liu *et al.*, 2005).

Mis12-Spc7 Complex Proteins Disappear from the Centromere in Response to Mating Pheromone Signaling

During meiotic prophase, signals of the Mis12-Spc7 complex were significantly reduced, whereas the Mis6 signal remained at the centromere (Figure 5A). Because the Ndc80 complex is known to disappear from the centromere in response to mating pheromone signaling during meiosis (Asakawa *et al.*, 2005), we examined if the Mis12-Spc7 complex proteins are regulated by the same signaling pathway. To this end, we used h^- haploid cells carrying the temperature-sensitive *pat1-114* mutation. Cells of the *pat1-114* mutant can be induced to enter meiosis by shifting to a restrictive temperature (Iino and Yamamoto, 1985). In this mutant, in contrast to the wild type, centromeres remain clustered at the SPB during meiotic prophase (Chikashige *et al.*, 2004). Importantly, centromeres become separated from the SPB in response to activation of mating pheromone signaling by *mat-Pc* gene expression (Asakawa *et al.*, 2005). We observed localization of the Mis12-Spc7 complex proteins in h^- *pat1-114* mutant cells and h^- *pat1-114* mutant cells carrying the *mat-Pc* gene at the restrictive temperature of 34°C. In the *pat1-114* mutant strains, meiotic division I starts 4–5 h after the temperature shift-up. Cells were observed at 0 and 4 h after the temperature shift-up. Observation revealed that all the Mis12-Spc7 complex proteins were localized at the centromere both at 0 and 4 h in *pat1-114* mutant cells not expressing the *mat1-Pc* gene (Figure 5B). In contrast, in *pat1-114* mutant cells expressing the *mat-Pc* gene, centromere localization of the Mis12-Spc7 complex proteins was decreased at 0 h (Figure 5C). At 4 h, some cells entered meiosis I, and in those cells Mis12-Spc7 complex proteins were relocated to the centromere, as was observed in wild-type cells (Figure 5C). These results indicate that the disappearance of the Mis12-Spc7 complex, as well as the Ndc80 complex, from the centromere is regulated by the same pathway via mating pheromone signaling.

To test whether Mis12-Spc7 complex proteins undergo proteolytic degradation when they exhibit reduced centromere localization, we performed immunoblot analysis by preparing cell extracts from synchronous cultures of the *pat1 mat-Pc* strain described above. Cells of *pat1 mat-Pc* expressing a Mis12-Spc7 complex protein were taken at 0, 2, and 4 h after induction of meiosis, and the extracts were separated by SDS-PAGE and analyzed by immunoblotting (see *Materials and Methods*). The Mis12-Spc7 complex proteins, which were fused to GFP-3HA at their carboxyl termini, were detected by anti-HA antibody. All of the fusion proteins, except for Spc7-GFP-3HA, were detected at their predicted molecular weights, which include the 31 kDa GFP-3HA tag. The Spc7-GFP-3HA fusion protein showed an apparent molecular weight of 130 kDa, significantly smaller than its predicted molecular weight of 185 kDa. The levels of each

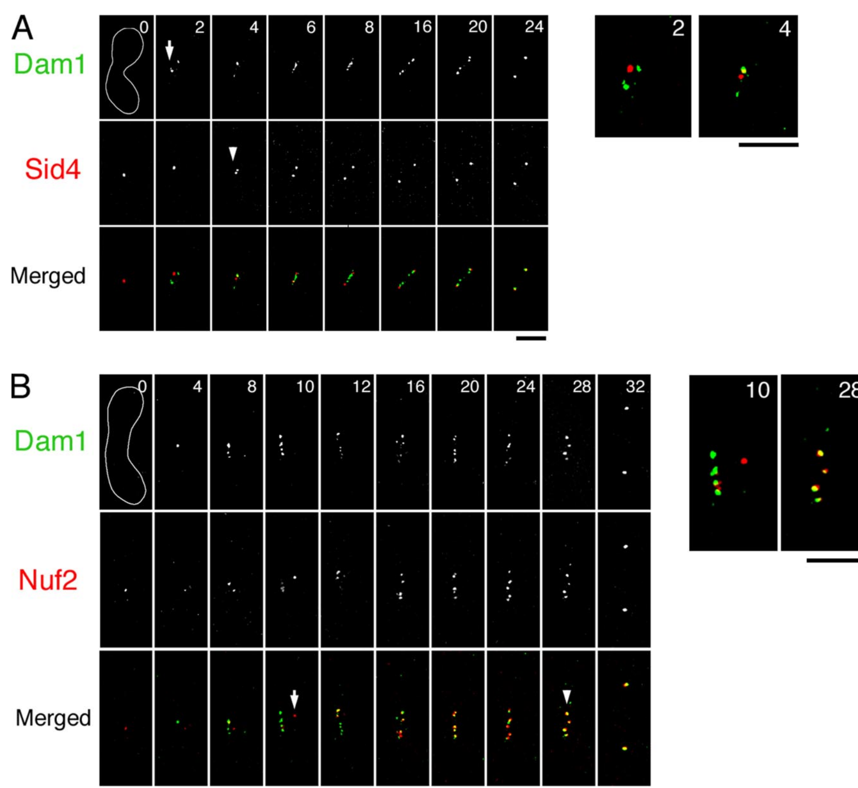


Figure 8. Reappearance of Dam1 protein immediately before SPB separation. (A) Dam1 appearance is related to the time of SPB separation. Dam1 appears within a nucleus (arrow, 2 min) immediately before SPB separation (arrowhead, 4 min). Bar, 5 μm. (B) Nuf2 protein relocalized before Dam1 appearance (0 min) and did not colocalize with Dam1 at early meiosis I (arrow, 10 min). At metaphase, Nuf2 protein colocalized with Dam1 (arrowhead, 28 min). Right panels show magnified images at the two time points (10 and 28 min). Bar, 5 μm.

Mis12-Spc7 complex protein were not significantly different at the various time points (Figure 6). Thus, reduced localization of Mis12-Spc7 complex proteins involves relocalization but not degradation.

Mis12-Spc7 and DASH Complexes Sequentially Reappear at the Centromere

To determine the temporal sequence of kinetochore reassembly during meiosis, times of reappearance of Mis12-Spc7 and DASH complex proteins at the centromere were measured in living cells. Results showed that Mis12-Spc7 complex proteins reappeared at the centromere in two steps: first fluorescent signals reappeared at the centromere in late prophase, and this was followed by a further increase in signal intensity shortly before meiosis I (Figure 7A). The first increase in the intensity of the fluorescent signals occurred 40–50 min and the second increase 19–27 min before the metaphase-anaphase transition of meiosis I (Figure 7B).

The DASH complex proteins reappeared about the same time as the second increase of the Mis12-Spc7 complex, ranging from 18 to 23 min before the metaphase-anaphase transition of meiosis I (Figure 7B). We further compared the time of reappearance of Dam1 with that of SPB separation by simultaneous observation of Dam1-GFP and the SPB stained with Sid4-mRFP. Dam1 protein reappeared within the nucleus immediately before SPB separation, and formed foci between separated SPBs (Figure 8A). These foci of Dam1 were not colocalized with Nuf2 or Sid4 at the time of reappearance, but became colocalized with Nuf2 at several spots on the centromere in metaphase, and then converged to a single spot on the SPB at each pole in anaphase (Figure 8B). Thus, Dam1 reappears about the same time as the second increase in Mis12-Spc7 complex signal, but separately. Dam1 probably first appears at the tip of spindle microtubules as is in mitosis (Liu *et al.*, 2005), and then accumulates at the centromere in metaphase probably at the time of spindle attachment to the kinetochore.

Loading of Meiosis-specific Centromere Proteins

To examine when meiosis-specific centromere proteins are loaded onto the centromere during meiotic reconstruction of the kinetochore, we determined the times for appearance of Sgo1 and Moa1. Sgo1 protein signal intensity increased in two steps (52 and 20 min before the metaphase–anaphase transition of meiosis I) in a way similar to the NMS (Ndc80-Mis12-Spc7) complex proteins (Figure 7B). On the other hand, Moa1 protein signal appeared at the centromere 108 min before the metaphase–anaphase transition of meiosis I, significantly earlier than any of the NMS complex proteins (Figure 7B). Taken together, these results demonstrate that Moa1 is loaded onto the Mis6-containing centromere, followed by Sgo1 together with the NMS complex, and then by the DASH complex (summarized in Figure 10B).

Next, to examine loading of Moa1 and Sgo1 in response to mating pheromone signaling, we observed localization of these proteins in h^- *pat1-114* mutant cells and h^- *pat1-114* mutant cells carrying the *mat-Pc* gene. Sgo1-GFP did not localized at the centromere before the temperature shift-up (Figure 9A, 0 h). After the shift-up to the restrictive temperature of 34°C, bright signals of Sgo1-GFP appeared at the centromere in *pat1 mat-Pc* cells (Figure 9A), and proportion of the cells with Sgo1-GFP signals reached the peak at 3 h (Figure 9C), corresponding to meiotic prophase as estimated in Figure 9B. In contrast, only faint signals of Sgo1-GFP were observed in *pat1* cells at 5 h (Figure 9, A and C). Fluorescence intensity of Sgo1-GFP was significantly dimmer in *pat1* cells than in *pat1 mat-Pc* cells: 98% of the Sgo1-GFP signals were below 30 in *pat1* cells, whereas 77% of the Sgo1-GFP signals were above 30 in *pat1 mat-Pc* cells (Figure 9D). At 8 h, Sgo1-GFP disappeared from the centromere (Figure 9, A and

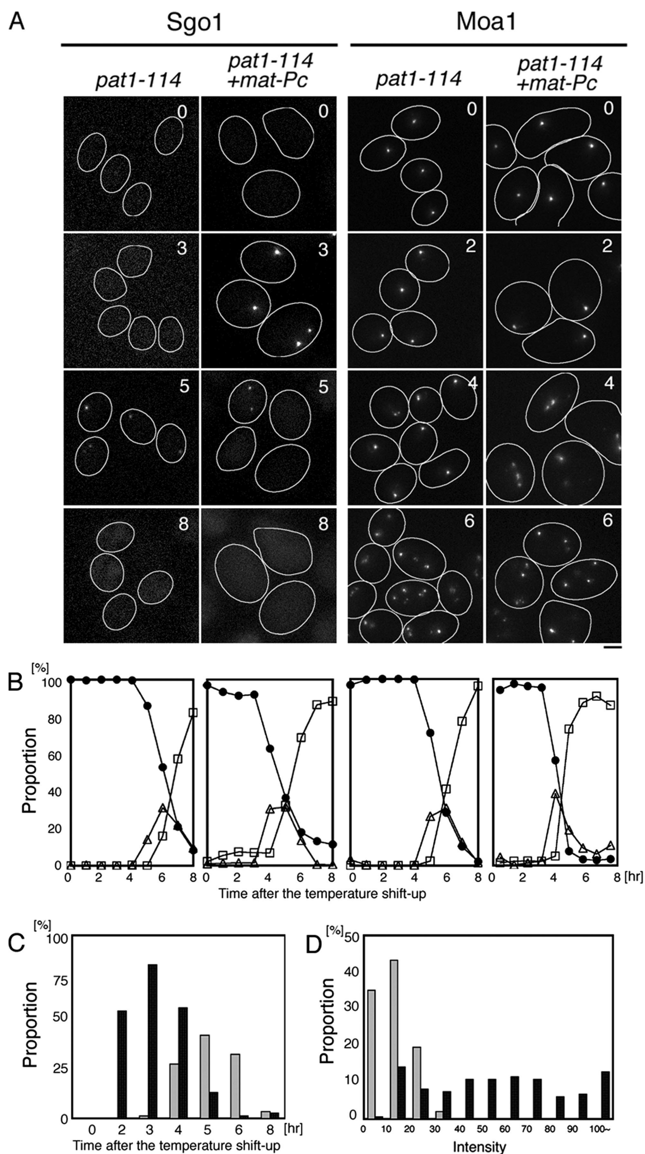


Figure 9. Localization of meiotic centromere protein Sgo1 and Moa1 in *pat1* mutant. (A) Localization of Sgo1-GFP and Moa1-GFP proteins in *pat1-114* and *pat1-114 mat-Pc* mutant strains during meiosis. The number in each panel indicates time after the temperature shift-up in hours. Results were reproduced in two or three independent experiments. Bar, 5 μm. (B) Time course of nuclear division. ●, one nucleus; Δ, two nuclei; □, three or four nuclei. Meiotic nuclear division occurs ~2 h later in the absence of mating pheromone signaling. (C) Proportion of the cells showing Sgo1-GFP signals as a function of time (hours) after the temperature shift-up in the *pat1 mat-Pc* strain (■) or in the *pat1* strain (▨). About 200 cells were counted at each time point. Sgo1 appearance reaches its peak at 3 h in the *pat1 mat-Pc* strain and at 5 h in the *pat1* strain. This difference in time is likely due to slower progression of meiosis in the absence of mating pheromone signaling as shown in B. (D) Histogram of fluorescence intensity of Sgo1-GFP signals. Peak intensity of the GFP signal was measured using the SoftWoRx software. Proportion of the cells in each range of intensity is shown as a bar graph in the *pat1 mat-Pc* strain at 3 h (■; n = 148) or in the *pat1* strain at 5 h (▨; n = 97).

C). These results suggest that mating pheromone signaling promotes loading of Sgo1 to the centromere. On the other hand, Moa1-GFP was localized at the centromere before the temperature shift-up both in *pat1* and *pat1 mat-Pc* strains (Figure 9A), although the fluorescence intensity of Moa1-GFP was slightly higher in *pat1 mat-Pc* cells (213 ± 58.5 for 100 cells) than in *pat1* cells (154.2 ± 30.6 for 100 cells). Interestingly, after temperature shift-up Moa1-GFP remained at the centromere throughout meiosis (Figure 9A). This persistent centromere localization of Moa1 in the *pat1* haploid strains differed from that in wild-type diploid cells, in which Moa1 appears at an early horsetail stage and disappears at anaphase I during meiosis (Yokobayashi and Watanabe, 2005; Figure 7A). These results suggest that localization of Moa1 is regulated independently of mating pheromone signaling in the *pat1* mutant background.

DISCUSSION

In this article, we observed *S. pombe* centromere proteins in living cells and classified them into three groups based on their mitotic and meiotic behaviors. Mis6-like group proteins always localize at the centromere, forming the basic architecture of the kinetochore. NMS group proteins reassemble to the kinetochore during prophase and toward metaphase in meiosis, and subsequently DASH group proteins localize at the centromere during chromosome segregation. These groupings are generally consistent with the complex structures revealed by genetic interactions and proteomic analyses (De Wulf *et al.*, 2003; Cheeseman *et al.*, 2004; Obuse *et al.*, 2004; Liu *et al.*, 2005).

Mis6 Complex: Basic Architecture of the Kinetochore

The Mis6 complex forms the constitutive structure of the kinetochore in meiosis as well as mitosis, providing a framework for the centromere. Thirteen proteins were identified in a Mis6-containing complex that was isolated by biochemical purification. Interestingly, only four of them had homologues in *S. cerevisiae* (Liu *et al.*, 2005). This contrasts with the highly homologous components of the Ndc80 and DASH complexes. The less conserved nature of the Mis6 complex may reflect variations in the DNA sequences among species.

Nevertheless, this complex seems to play a conserved role in forming a biorientation kinetochore in mitosis or a mono-orientation kinetochore in meiosis I in a cohesin-mediated manner. Recently, it has been reported that *S. pombe* Moa1 functions in meiotic cohesin Rec8-mediated monopolar spindle attachment at meiosis I and that its centromere localization depends on Cnp3, a CENP-C homolog (Yokobayashi and Watanabe, 2005). In *S. cerevisiae*, centromere localization of meiotic cohesin Rec8 is reduced by loss of CHL4 (Marston *et al.*, 2004), which is a homolog of *S. pombe* Mis15, and Mis15 requires Mis6 for its centromere localization (Hayashi *et al.*, 2004). Mis6 is also required for loading of Cnp1, a CENP-A homolog (Takahashi *et al.*, 2000). Thus, the Mis6 complex forms a "foothold" for the Rec8-mediated mono-orientation kinetochore, most likely through interactions with CENP-A– and CENP-C–associated regions of the centromere (Figure 10A).

Mis12 and Ndc80 Complexes: Facultative Components of the Kinetochore

Mis12 and Ndc80 complexes remain at the centromere throughout the mitotic cell cycle in both yeasts, but they change their localization during the mitotic cell cycle in some other organisms. Chicken Hec1(Ndc80 homolog) and Nuf2 are localized at the kinetochore during the mitotic phase and relocate to the centrosome in interphase (Hori *et al.*, 2003). In *C. elegans*, HIM-10 protein (Nuf2 homolog) is also localized at the kinetochore only in the mitotic phase

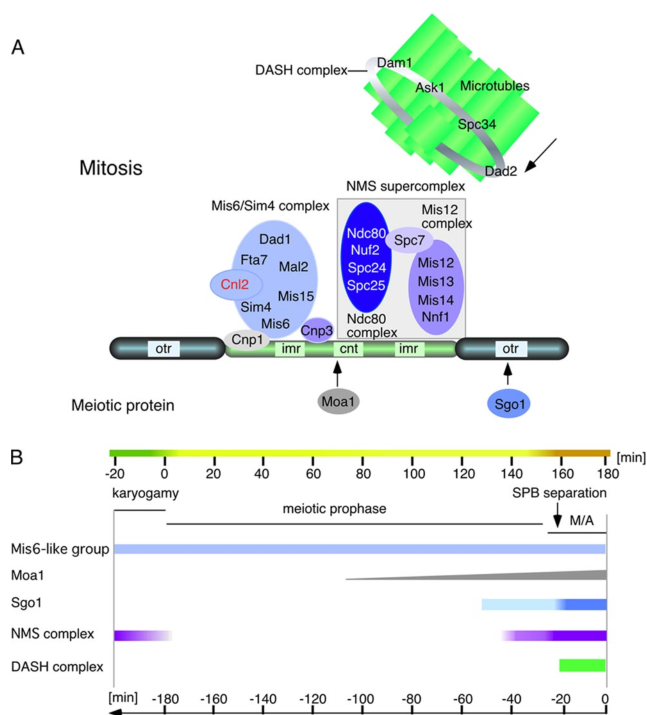


Figure 10. Temporal sequence of the reassembly of centromere proteins during meiosis. (A) Summary of *S. pombe* kinetochore proteins. Mis6 and NMS complexes locate at the inner (imr and cnt) region, but not at the outer region (otr) of the centromere in the mitotic cell cycle. The DASH complex appears at the M-phase, and locates at a tip of the spindle to capture the kinetochore. During meiosis, Moa1 is localized at the inner region of the centromere, and Sgo1 is localized at the outer region of the centromere. (B) Diagram of the meiotic behavior of kinetochore proteins: Mis6, NMS, and DASH complex as well as Moa1 and Sgo1. Bars indicate schematically the period when the respective protein is detected at the centromere during meiosis. Time scale at the top of the diagram shows the progression of meiosis in minutes after the end of karyogamy based on Ding *et al.* (2004). Scale at the bottom indicates the time in minutes to the metaphase-anaphase transition of meiosis I (expressed as negative values) as measured in this article.

and relocates to the cytoplasm in interphase (Howe *et al.*, 2001). Similarly, in humans, hNuf2 protein is localized at the kinetochore in the mitotic phase and relocates to the cytoplasm in interphase (Nabetani *et al.*, 2001), whereas hMis12 remains at the centromere throughout the mitotic cell cycle (Goshima *et al.*, 2003). Thus, localization of the Ndc80 and Mis12 complexes is regulated during the cell cycle differently among organisms, probably reflecting different mechanisms of spindle formation. Also *S. pombe* Ndc80 and Mis12 complexes locate at the centromere independently of each other because Mis12 protein is still localized at the centromere in the *nuf2-1* mutant (Saitoh *et al.*, 2005) and Nuf2 is also localized to the centromere in the *mis12* mutant (H. Asakawa and Y. Hiraoka, unpublished results).

In *S. pombe*, Mis12 and Ndc80 complexes dissociate from the centromere during meiotic prophase. *S. cerevisiae* Nuf2 also disappears from the centromere during meiosis (Hayashi *et al.*, 1998; Asakawa *et al.*, 2005). The biological significance of dissociation of the Ndc80 and Mis12 complexes during meiotic prophase remains unknown. In *S. pombe*, when *pat1-114* cells are induced to enter meiosis in the absence of mating pheromone signaling, the Ndc80 and Mis12 complexes remain at the centromere and fail in reductional segregation in meiosis I (Asakawa *et al.*, 2005; this article). Action of the mating pheromone on these *pat1-114* cells dissociates the Ndc80 and Mis12 complexes from the centromere and results in reductional segregation in meiosis I (Yamamoto and Hiraoka, 2003; Asakawa *et al.*, 2005; this article). Thus, there is an interesting correlation between the centromere dissociation of the Ndc80 and Mis12 complexes and the formation of monopolar spindle attachment downstream of mating pheromone signaling. Removal of the Ndc80 and Mis12 complexes from the centromere under mating pheromone signaling may be a prerequisite for reconstruction of the kinetochore during meiosis, allowing meiotic centromere proteins to be incorporated into the kinetochore. Alternatively, formation of monopolar kinetochore may be regulated by mating pheromone signaling, but independently of removal of the Ndc80 and Mis12 complexes. In this context, it should be noted that Sgo1 is loaded to the centromere in response to mating pheromone signaling. On the other hand, it has been shown that Rec8 and Moa1 are loaded to the centromere in the absence of mating pheromone signaling in *pat1* mutant strains, but chromosomes fail reductional segregation under these circumstances (Yamamoto and Hiraoka, 2003; this article). Therefore, we can conclude that loading of Rec8 and Moa1 to the centromere is not sufficient for reductional segregation of chromosomes. We can also conclude that disappearance of Ndc80 and Mis12 complexes from the centromere is not necessary for loading Rec8 and Moa1 because Ndc80 and Mis12 complexes remain at the centromere in the absence of mating pheromone signaling in *pat1* mutant strains (Asakawa *et al.*, 2005; this article). Thus, yet-unknown factors are likely involved in regulation of monopolar kinetochore formation under mating pheromone signaling.

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