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Molecular analysis of kinetochore architecture in fission yeast

Xingkun Liu[1], Ian McLeod[2], Scott Anderson[2], John R Yates III[2] and Xiangwei He[1,*]

[1]Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, USA and [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, USA

Kinetochore composition and structure are critical for understanding how kinetochores of different types perform similar functions in chromosome segregation. We used affinity purification to investigate the kinetochore composition and assembly in *Schizosaccharomyces pombe*. We identified a conserved DASH complex that functions to ensure precise chromosome segregation. Unlike DASH in budding yeast that is localized onto kinetochores throughout the cell cycle, SpDASH is localized onto kinetochores only in mitosis. We also identified two independent groups of kinetochore components, one of which, the Sim4 complex, contains several novel Fta proteins in addition to known kinetochore components. DASH is likely to be associated with the Sim4 complex via Dad1 protein. The other group, Ndc80–MIND–Spc7 complex, contains the conserved Ndc80 and MIND complexes and Spc7 protein. We propose that fission yeast kinetochore is comprised of at least two major structural motifs that are biochemically separable. Our results suggest a high degree of conservation between the kinetochores of budding yeast and fission yeast even though many individual protein subunits do not have a high degree of sequence similarity.

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Introduction

Central to accurate chromosome segregation is the kinetochore, a multi-protein organelle assembled on centromeric DNA that mediates the interaction between chromosomes and spindle microtubules (MTs). Kinetochores perform multiple functions during chromosome segregation: providing the biochemical affinity between chromosomes and the spindle MTs, generating the physical forces that drive chromosome movement, and acting as sensors of proper MT attachment that couples with the spindle checkpoint pathway. These important biological functions are performed by essentially all types of kinetochores, although in different organisms they have dramatic differences in their composition and morphology—a longstanding enigma in the field of mitosis.

The most significant difference among kinetochores is the centromeres, the size of which varies from 125 bp in budding yeast to several megabases in metazoans. In recent studies, major progress in identifying the protein composition of some kinetochores has been made, particularly in budding yeast in which more than 60 distinct proteins have been identified so far (McAinsh *et al*, 2003). In other species, knowledge of kinetochore proteins is now accumulating at an impressive rate as well (Cleveland *et al*, 2003). Some kinetochore proteins, such as Ndc80p, are highly conserved in their primary sequences and cellular functions (Zheng *et al*, 1999). On the other hand, the similarity between other essential kinetochore proteins is less or not at all obvious (McAinsh *et al*, 2003). Efforts to decipher the architecture of kinetochores have also been initiated, most extensively in budding yeast (De Wulf *et al*, 2003; Westermann *et al*, 2003). Many of the kinetochore proteins in budding yeast are organized into discrete complexes which are likely to be structural and functional modules. Studies on the interdependency of kinetochore localization among the components have led to an emerging hierarchy of kinetochore assembly—inner kinetochore proteins that are in contact with DNA, such as CBF3, form the foundation for the mid-layer proteins, represented by three independent complexes, Ndc80, MIND and COMA, and, further, the outer kinetochore components, such as the motor proteins and the DASH complex (McAinsh *et al*, 2003).

Kinetochores from different organisms behave remarkably differently. In budding yeast, kinetochores are attached to spindle MTs throughout almost the entire cell cycle. Therefore, it can be implied that functional kinetochores are always needed. In vertebrate cells, on the other hand, assembly of kinetochores starts in S phase and continues until early mitosis (Amor *et al*, 2004; Maiato *et al*, 2004). Thus, a thorough investigation in multiple organisms is necessary to elucidate the general principles of kinetochore function and to distinguish the structural and mechanistic features that are universal to all kinetochores from those that are species-specific.

The fission yeast, *Schizosaccharomyces pombe*, provides an excellent model for the study of kinetochores. With repetitive and complex centromeric DNA spanning from 35 to 100 kilobases and the capacity for binding multiple MTs per kinetochore, fission yeast cells have the complex kinetochores, representative of those found in most eukaryotes. Previous studies have led to extensive knowledge of the sequence and organization of centromeric DNA, as well as the identification of a number of kinetochore-specific proteins in fission yeast (Pidoux and Allshire, 2004).

Several intriguing features of kinetochore behavior in fission yeast are noteworthy. The centromeres cluster tightly in the vicinity of the spindle pole body (SPB) throughout the

*Corresponding author. Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA.
Tel.: +1 713 798 2093; Fax: +1 713 798 8142;
E-mail: xhe@bcm.tmc.edu

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interphase (Uzawa and Yanagida, 1992), so that kinetochores are visualized by fluorescence microscopy as a single dot adjacent to or overlapping the SPB (Goshima *et al*, 1999; Nabetani *et al*, 2001; Jin *et al*, 2002; Pidoux *et al*, 2003). Electron microscopy (EM) studies have suggested that the tethering of kinetochores to the SPB may be direct, since no MTs were found between the kinetochores and the β-tubulin of SPB (Ding *et al*, 1997; Kniola *et al*, 2001). Upon entry into mitosis, a short bipolar spindle is assembled, and sister kinetochores become attached to the dynamic plus ends of spindle MTs and establish a bi-orientation configuration in which the sisters are linked to the opposite SPBs via kinetochore MTs (kMTs). At this stage of mitosis, kinetochores are seen as several discrete dots aligned between the two spindle poles (Uzawa and Yanagida, 1992; Nabetani *et al*, 2001). The fact that kinetochores are tethered to the SPB implies that functional kinetochores of a certain form must be assembled in interphase. On the other hand, to accommodate their release from SPBs and subsequent attachment to MTs upon entry into mitosis, kinetochores may be biochemically modified. Consistently, the composition of kinetochores changes from interphase to mitosis; some proteins here referred to as constitutive components are localized to kinetochores throughout the cell cycle, while others, here referred to as transient components, are localized to kinetochores only in mitosis. Typical constitutive components include Nuf2, Mis6, Mis12, Sim4, Mal2, and Cnp1 (Goshima *et al*, 1999; Takahashi *et al*, 2000; Nabetani *et al*, 2001; Jin *et al*, 2002; Pidoux *et al*, 2003). Typical transient components include Dis1, Alp14, Klp2, Klp5, and Klp6, all of which interact with MTs (Nakaseko *et al*, 2001; Troxell *et al*, 2001; Garcia *et al*, 2002a, b; West *et al*, 2002).

In this study, using affinity purification and mass spectrometry, we identified three kinetochore protein complexes in fission yeast and novel kinetochore components, as well as known ones, as the subunits of these complexes. Two complexes, Sim4 and Ndc80–MIND–Spc7 (NMS), are constitutive components. The third complex, DASH, is transiently associated with kinetochores only in mitosis and is required for precise chromosome segregation. We demonstrated that the Sim4 complex functions as a loading dock for the DASH complex.

Results

Identification of the DASH complex as a transient kinetochore component

The DASH (Dam1, DDD) complex, a 10-subunit complex in the budding yeast, functions in securing the sister kinetochore bi-orientation (Cheeseman *et al*, 2001; Janke *et al*, 2002; Li *et al*, 2002). Inactivation of DASH interferes with biorientation, but not with MT capture. Given that DASH mediates kinetochore/MT interaction and that kinetochores interact with MTs only in mitosis in fission yeast, one can speculate that the postulated *S. pombe* DASH may be kinetochore localized specifically during mitosis.

Among the DASH subunits, three have readily identifiable homologs in *S. pombe*, one of which, SPBC27.02c, is similar to ScAsk1. We sought to determine the intracellular localization of SPBC27.02c by constructing and expressing a GFP fusion protein from the endogenous promoter as the only functional product of SPBC27.02c. No specific GFP signal was detected in interphase cells. Only in mitotic cells, as indicated by separated SPBs, were localized GFP signals detected (Figure 1A). The multi-dot localization pattern in metaphase cells overlaps closely with the kinetochore marker, Ndc80-CFP (Figure 1A, lower panels). Spindle-like localization (Figure 1A, arrow) and dots in addition to the kinetochore (Figure 1A, arrowhead), but not SPB dots (Figure 1A, middle), were also detected. We thus concluded that SPBC27.02c is a transient kinetochore component and named it Ask1 (or SpAsk1 when needed to be distinguished from ScAsk1).

To identify the postulated SpDASH, we performed tandem affinity purification, using Ask1 as the affinity bait, and identified copurified proteins by mass spectrometry. A fusion of *ask1* with the DNA-encoding TAP, a well-established tag for tandem affinity, was expressed under its endogenous promoter as the only functional *ask1* gene. To reduce the copurification of nonspecific proteins, we performed the purification at high stringency with high salt concentration (see Materials and methods). Among the proteins identified by mass spectrometry (using a 5% sequence coverage cutoff), nine were repeatedly copurified with Ask1-TAP from cells arrested in mitosis either by the *mts2-1* mutation (Seeger *et al*, 1996) or the *nda3-311* mutation (Hiraoka *et al*, 1984) (Figure 1B; Supplementary data).

Among the nine copurified proteins, we identified the other two predicted homologs: Hos2 (later called Dad2) and SPAC14C4.16. Each of the other seven proteins, when aligned pairwise with the ScDASH subunits, shows low but significant sequence similarity (Supplementary Results). We thus adopted the budding yeast protein nomenclature for the homologous proteins in fission yeast. SpSpc19 was not listed as a complete ORF in the Sanger Institute GeneDB database due to a missing start codon, possibly because the first exon was skipped in ORF assignment. We have identified the missing N-terminal by sequencing an Spc19 cDNA (Figure 1C). Mass spectrometry verified the authenticity of the missing N-terminal polypeptide (data not shown). SpDad4 (Figure 1C) was not listed in the GeneDB database either, possibly due to its extremely short length. New ORF numbers have now been assigned: SPCC1223.15c for SpSpc19 and SPBC3B9.22c for SpDad4.

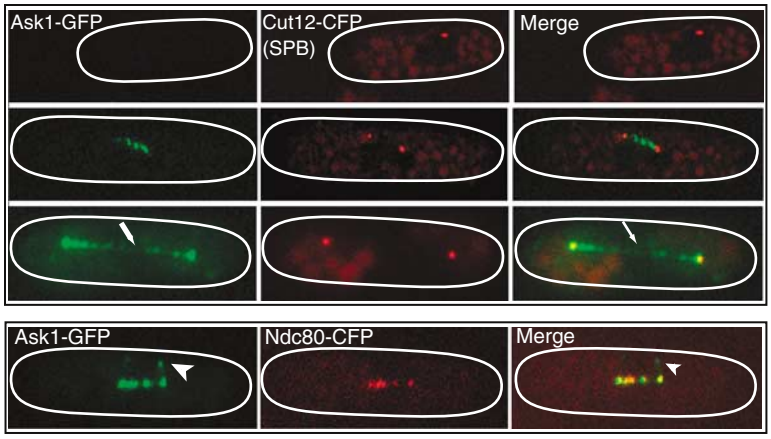
To test whether Ask1 and the copurified proteins form a stable complex, we performed reciprocal purification in mitotic cell extracts using SpDam1-TAP or SpDad1-TAP. Each of the postulated DASH subunits was copurified in most or all independent experiments (Figure 1B). We thus conclude that DASH is conserved in fission yeast.

SpDASH is assembled and localized onto kinetochores specifically in mitosis

SpAsk1-GFP localizes on kinetochores and the spindle only during mitosis (see above). Three other tested DASH components, Dam1, Spc34, and Dad2 (data not shown), exhibited an identical localization pattern, whereas Dad1 did not (see below).

To explore the underlying mechanism of its dynamics, we assessed the protein levels of the DASH subunits during the cell cycle. This was performed using *nda3*^{cs} cells carrying Ask1-TAP that were first arrested at the entry of mitosis by shifting to their restrictive temperature and then allowed to re-enter mitosis synchronously. Cells were harvested at

A



B

Gene name	ORF name	Protein coverage by mass spectrometry in each purification preparation (%)					GFP-fusion kinetochore localization
		Ask1-TAP/ *mts2-1^{ts}*	Ask1-TAP/ *nda3-311*(1)	Ask1-TAP/ *nda3-311*(2)	Dad1-TAP/ *mts2-1^{ts}*	Dam1-TAP/ *mts2-1^{ts}*	
ask1	*SPBC27.02c*	38.4	56.0	69.1	54.7	54.7	Yes
dam1	*SPAC589.08c*	76.8	88.4	96.1	52.9		Yes
spc34	*SPAC8C9.17c*	67.1	59.1	90.9	40.2	26.2	Yes
duo1	*SPBC32F12.08c*	53.0	47.6	53.6	16.9	25.9	ND
dad1	*SPAC16A10.05c*	54.1	52.9	90.6		21.2	Yes
*dad2**	*SPAC1805.07c*	80.9	83.0	94.7	24.5	30.9	Yes
dad3	*SPAC14C4.16*	Δ	87.2	91.9	44.2	24.4	ND
dad4	SPBC3B9.22c	Δ	34.0				ND
spc19	SPCC1223.15c	Δ	74.2	Δ			ND
*hsk3***	*SPCC417.02*		46.8	75.5			ND

Δ : protein detected by mass spectrometry, but coverage percentage not available;
ND: not determined; *: *dad2* identical to *hos2*; **: *hsk3* identical to *hos3*.

C

Spc19
MSYLDGLQQCVDSLQISIGTLSSSIDTLESGIHDFPRIKHILKVQRHFNLISEDELSA
RQAKFEEIVKPILSKAFQRLEDSISSLQRQEDSLKTKYELQEARLDMLKNRPATSAF
SVTTESSEQLKAIIAKRQKLVYTLERYTLQLQQKRGH

Dad4
MNNPMEEQQSALLGRIISNVEKLNESITRLNHSLQVRLRRQIFVYISTFR

Figure 1 Identification and characterization of DASH complex. (**A**) Live-cell images of wild-type cells with Ask1-GFP and other markers. The mid and lower panels were digitally enhanced to show the localizations other than kinetochores (arrow and arrowhead). (**B**) Summary of mass-spectrometric analysis of DASH purifications, indicating the percent sequence coverage for each polypeptide with >5%. Nonspecifically copurified proteins are listed in Supplementary Results. (**C**) Amino-acid sequences of Dad4 and Spc19 deduced from their genomic DNA sequences. The N-terminal sequence of Spc19 in red illustrates the fragment missing from the incomplete ORF in Sanger Center GeneDB.

different time points, corresponding to different stages of mitosis (Hiraoka *et al*, 1984). Cell extracts at each time point were prepared in the presence of trichloroacetic acid (TCA) to cause protein precipitation and prevent degradation. By Western blotting, the total Ask1-TAP protein quantity was found to be constant throughout the cell cycle. In addition, during mitosis (0–30 min in Figure 2A), the intensity of the upper band of Ask1-TAP gradually declined, while the lower band gained prominence and eventually became the only band in interphase (60–120 min in Figure 2A). In budding yeast, Ask1 is phosphorylated extensively (Li and Elledge, 2003). To test whether the upper band represents the phosphorylated SpAsk1, Ask1-TAP was first partially purified from *nda3^{cs}* cells arrested in mitosis by protein A affinity using IgG resins and then treated with protein phosphatase in the absence or presence of phosphatase inhibitors (PI). The upper band was eliminated by phosphatase treatment but present with the addition of PI, demonstrating that SpAsk1 was phosphorylated specifically during mitosis. By the same assay, the protein levels of Dam1-TAP and Dad1-TAP were also found to be constant, although no obvious phosphorylation bands were detected (data not shown).

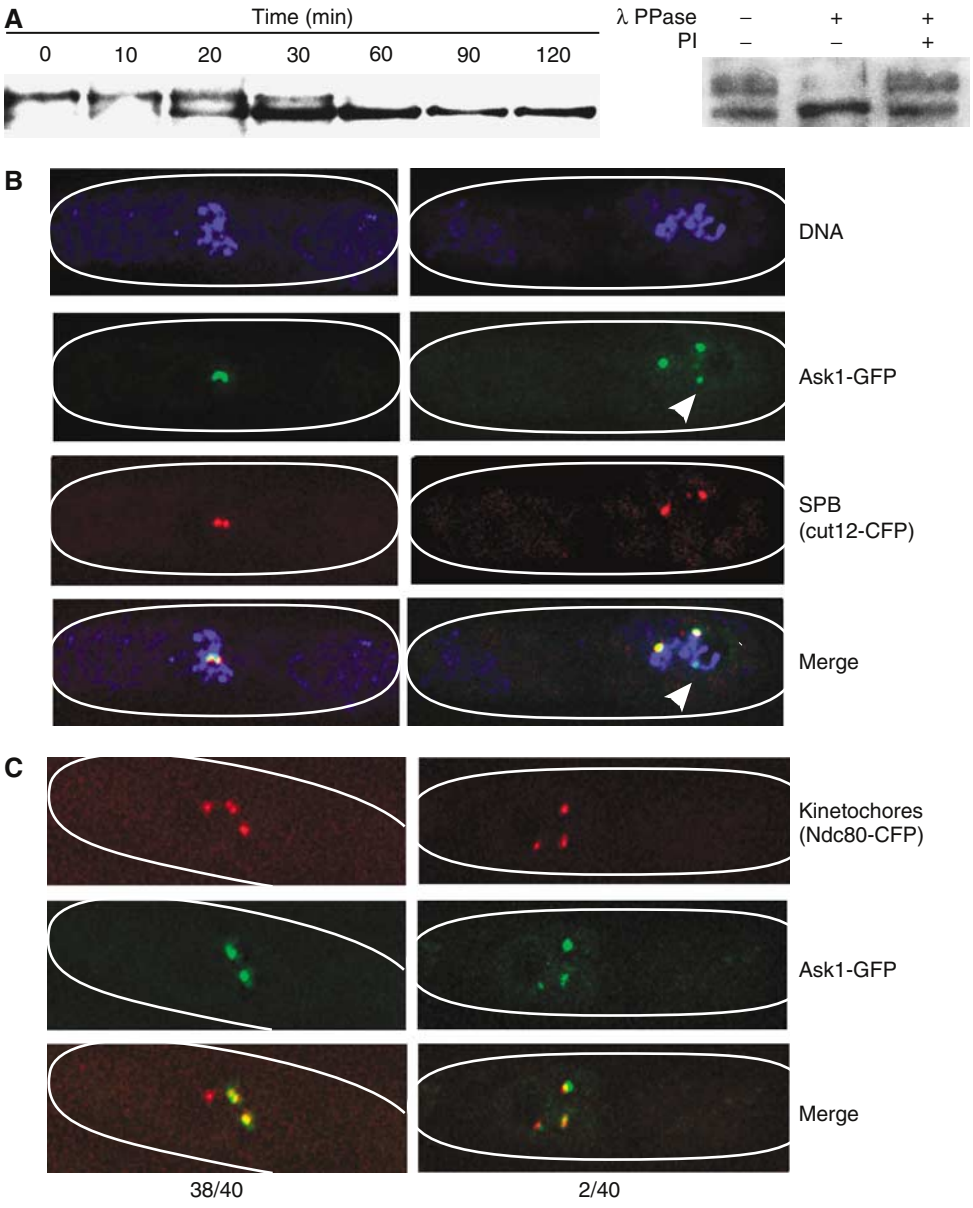


Figure 2 DASH complex dynamics in mitosis. (**A**) Mitosis-specific modification(s) of Ask1. Left, Western blotting with PAP antibody to detect Ask1-TAP in total cell extracts of *nda3* cells incubated at 20°C and then released into 36°C for times as indicated. Time points of 0–30 min correspond to the M phase; 60–120 min correspond to interphase. Right, partially purified Ask1-TAP was either not treated with λ phosphatase (λPPase) or treated with λPPase in the absence or presence of PI. (**B**) Typical localization of Ask1-GFP relative to chromosomal DNA and to SPBs in live *nda3* cells incubated at 18°C for 10 h. Left panels: a cell that has Ask1-GFP signal only near the SPBs; right panels: a cell that has Ask1-GFP near the SPBs and a dot on an unattached chromosome (arrowhead). (**C**) Typical images of *nda3* cells with localization of Ask1-GFP that is associated with only two (left) or with all the three scattered kinetochore dots (right).

Purification of DASH complex by Ask1-TAP or Dam1-TAP was only successful from mitotic cell extracts (Figure 1B). Repeated attempts failed to purify Ask1-TAP and its associated proteins from asynchronous cultures, which were predominantly interphase cells. Indeed, Ask1-TAP protein was stable only in mitotic extracts and was quickly degraded in asynchronous extracts beyond the detection level of Western blotting (data not shown). Ask1-TAP degradation was prevented by protein precipitation through TCA immediately after cell lysis (Figure 2A). These results are consistent with the notion that DASH complex, which is stable in solution, may be assembled during mitosis and that the free Ask1, although present *in vivo* at a constant level, may be prone to nonspecific protein degradation upon cell lysis.

To test whether the binding of DASH to the kinetochores is spindle MT-dependent, we investigated DASH protein localization in $nda3^{cs}$ arrested cells. Over 80% of $nda3^{cs}$ cells had Ask1-GFP (Figure 2B, left) or Dam1-GFP signal (data not shown) localized in the vicinity of the SPBs. Investigation of DASH binding to unattached chromosomes was only possible in a few cells with sufficiently scattered chromosomes. In these cells, Ask1-GFP was occasionally seen to be associated with the unattached chromosome (Figure 2B, right). To better quantify the Ask1-GFP association to unattached kinetochores, we examined *nda3* cells carrying Ask1-GFP and

Ndc80-CFP arrested in mitosis. Among cells that displayed sufficient kinetochore scattering, that is, with 'triangular' Ndc80-CFP dots distribution, 95% (38 out of 40) had Ask1-GFP colocalized with two of the kinetochore dots, but not the third (Figure 2C, left). 5% (2 out of 40) had Ask1-GFP tightly co-localized with all three kinetochores (Figure 2C, right). Together, these results indicate that DASH was bound mostly to the kinetochores associated with the SPBs in *nda3* cells, while only a small fraction of the unattached kinetochores had DASH binding.

SpDASH is not essential for viability, but necessary for accurate chromosome segregation

S. pombe cells in which *dad2* (initially called *hos2*) was deleted were viable, but hypersensitive to high osmotic pressure (Nakamichi *et al*, 2000). We deleted *ask1* and *dad1* and found that the cells were viable. Thus, none of the tested DASH subunits is essential for viability, in contrast to ScDASH. To test whether the whole SpDASH complex is dispensable for cell viability, we examined the impact of these deletions on the localization of other DASH subunits. In each deletion strain, all other DASH subunits tested, except Dad1-GFP, lost their kinetochore localization (Figure 3A–C). Uniquely, Dad1-GFP was constitutively kinetochore localized. In anaphase, Dad1-GFP was also localized on spindle MTs similar to, albeit less intensively than, other tested DASH subunits (Figure 3B, left panels, arrowheads). In *ask1*-Δ and *dad2*-Δ cells, Dad1-GFP remained kinetochore associated, but was no longer spindle localized (Figure 3B, arrows). However, in *dad1*-Δ cells, none of the tested DASH subunits was kinetochore associated. These results show that SpDad1 alone is sufficient for its own kinetochore association and that other DASH proteins require each other, and possibly the assembly of the whole complex, for kinetochore association. Furthermore, since *dad1*-Δ cells are viable and that in these cells DASH fails to be kinetochore localized in these cells, we conclude that the whole DASH complex is dispensable for viability.

Deletion of DASH genes often leads to abnormality in mitosis progression and chromosome segregation. In normal anaphase cells, the segregated kinetochores tightly cluster near the SPBs so that kinetochore proteins appear as two single dots overlapping with SPBs (Nabetani *et al*, 2001). However, in *ask1*-Δ cells, 86% of anaphase cells (43/50) in an asynchronous population displayed kinetochore lagging and/or mis-segregation (Figure 3D). To quantify the chromosome loss rate, we generated a strain with *dad1*-Δ and chromosome I tagged with GFP using the LacO tandem array and LacI-GFP (Rabitsch *et al*, 2003). In cells with a septum, which have finished mitosis, about 86% displayed a normal pattern of equal segregation of chromosome I and the overall nuclear mass (Figure 3E, left). Less than 4% showed mis-segregation of chromosome I but still had nuclear division (Figure 3E, middle), while 10% had the undivided nucleus displaced to one end (Figure 3E, right).

The discrepancy between the high percentage of anaphase cells with lagging kinetochore and the relative low percentage of septated cells with chromosome loss suggests that many anaphase cells correct the defect and eventually execute a successful mitosis. To provide direct evidence for this hypothesis, we followed the mitosis progression in *dad1*-Δ cells by time lapse microscopy. In all six cells that clearly displayed kinetochore lagging during anaphase, the lagging kinetochore (arrowhead in Figure 3F) caught up with the other two in late anaphase, resulting in a normal mitosis.

Identification of a network of interacting kinetochore proteins containing Sim4, Mal2, Mis6, Mis15, Mis17, Dad1, and Fta1–7

Sim4 coimmunoprecipitates with Mis6, suggesting that they might be part of a complex (Pidoux *et al*, 2003). We sought to identify other potential Sim4 interacting proteins by affinity purification. Using the tandem purification strategy described above, Sim4-interacting proteins were copurified with Sim4-TAP from an asynchronous cell extract and identified by mass spectrometry. Among them, Mis6 and two recently identified Mis6 interacting proteins, Mis15 and Mis17 (Hayashi *et al*, 2004), were found, validating our purification strategy (Figure 4A; Supplementary data). In addition, a kinetochore protein, Mal2 (Jin *et al*, 2002), and Dad1, a DASH complex subunit (see above), were also identified, suggesting a close interaction between these proteins. Other copurified proteins were novel, most with no noticeable sequence similarity except for uncharacterized ORFs in other lower eukaryotes (Figure 4A; Supplementary data).

To test whether copurification of these proteins was specific, we performed the reciprocal purification using Mal2-TAP. Sim4, as well as most of the above proteins were repeatedly copurified with Mal2-TAP. We thus name the novel proteins that copurify with Sim4 and Mal2, Fta1–7 (Sim4 and Mal2 associating proteins) (Figure 4A; Supplementary data).

To verify whether the novel Fta proteins are authentic kinetochore components, we examined their localization with GFP fusion, some together with Cut12-CFP, an SPB marker, or Ndc80-CFP, a kinetochore marker. We constructed GFP fusion for Fta1–5 by inserting GFP at the C-terminus of the chromosomal gene. Fta1, 2, 3, and 4 displayed the characteristic kinetochore localization, namely, multiple dots spread between the two SPBs, overlapping the Ndc80-CFP dots in the early mitotic nucleus (Figure 4C). Fta6 and Fta7 were found to be kinetochore localized in a systemic, genomewide characterization of protein localization (M Yoshida, personal communication).

We further tested whether Fta proteins were associated with the centromeric DNA by chromatin immunoprecipitation (ChIP) (Jin *et al*, 2002). Fta1–5 GFP strains were tested using anti-GFP antibody, and Fta2, Fta3, and Fta4 were found to be associated with the central core *cnt* and the inner repeats *imr* region of the centromere, but not with the outer repeats *otr* region or the chromosome arm locus *fbp*. These results are identical to those for the known kinetochore components, such as Mal2 (Figure 4D) (Jin *et al*, 2002). Although Fta1-GFP convincingly displayed a kinetochore localization pattern (Figure 4C, top), chromatin IP of Fta1-GFP had a very weak and not always repeatable association with cen DNA (data not shown), perhaps because GFP on the C-terminal of Fta1 within kinetochores was not easily accessible by the antibodies.

We were unable to obtain direct evidence of kinetochore association for Fta5. Results of protein localization and chromatin IP by various tagging strategies (at N- or C-terminal; with -GFP or -TAP as the tag) were inconclusive.

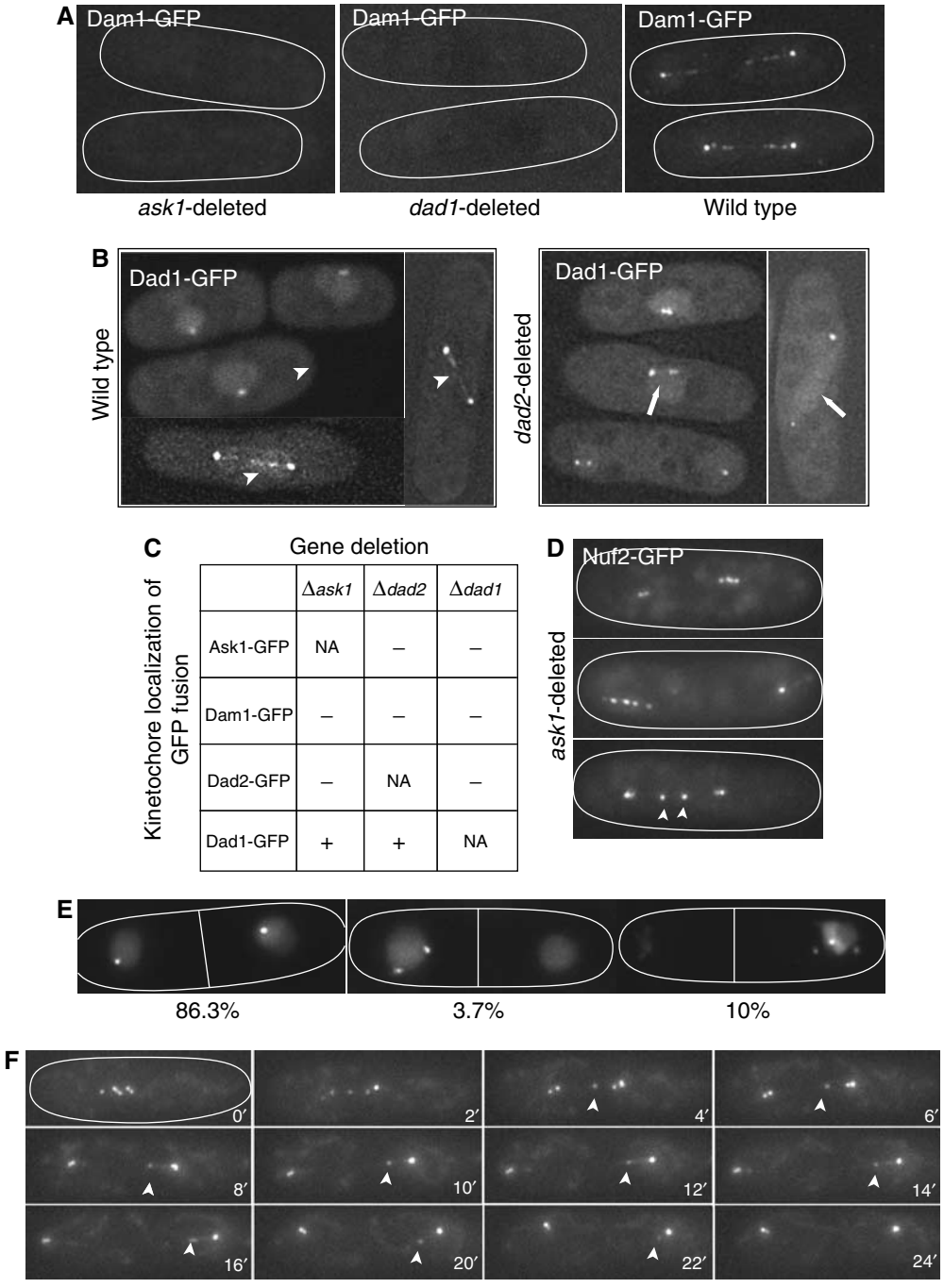


Figure 3 Characterization of DASH-Δ cells. (**A**) Dam1-GFP is mislocalized in *ask1*-Δ or *dad1*-Δ cells. (**B**) Dad1-GFP retains its kinetochore localization, but not spindle localization, in *dad2*-Δ cells. Arrowheads: spindle localization in WT; arrows: the absence of spindle localization in *dad2*-Δ. (**C**) Summary of interdependency of DASH proteins for kinetochore localization. (**D**) Kinetochore mis-segregation (top and middle) or lagging (arrowheads in the bottom) in *ask1*-Δ cells. (**E**) Quantification of chromosome loss in *dad1*-Δ cells with chromosome I labeled with GFP. Left, a cell with equal segregation of chromosome I; middle, a cell with nuclear division, but with chromosome I mis-segregated; right, a cell with an undivided nucleus displaced to one end. The numbers indicate the percentage among the septated cells. (**F**) Images of a time-lapse sequence (in min) of an *ask1*-Δ cell undergoing anaphase with a lagging chromosome (arrowhead).

However, due to its repeated copurification with Sim4 and Mal2, we tentatively include SPAC1F8.06 in the Fta protein collection to reflect its binding to Sim4 and Mal2 in solution. Further investigation is required to verify its identity as a kinetochore protein. Overall, copurification and protein localization and chromatin IP results lead to the conclusion that an interacting network, which we name as the Sim4 complex, encompasses the previously identified kinetochore proteins, Sim4, Mal2, Mis6, Mis15 and Mis17, and Dad1, as well as a group of novel proteins.

The Sim4 complex functions as the loading dock for DASH

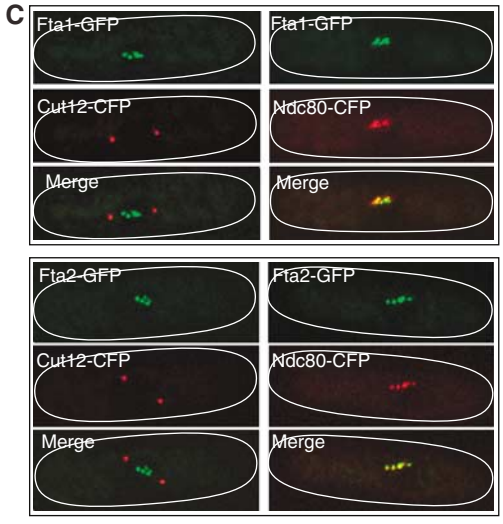
A DASH component, Dad1, repeatedly copurified with Sim4 and Mal2 (Figure 4A). This result prompted us to postulate that Dad1 is a component of both the DASH and Sim4

A

Gene name	ORF name	*S. cerevisiae* homolog	Protein coverage by mass spectrometry in each purification preparation (%)				GFP-fusion kinetochore localization
			sim4-TAP	mal2-TAP (1)	mal2-TAP (2)	dad1-TAP	
sim4	*SPBC18E5.03c*		27.4	16.2	35.0	23.5	Yes
mis6	*SPAC1687.20c*	*CTF3*	23.5	6.1	5.2	12.2	Yes
mal2	*SPAC25B8.14*	*MCM21*	43.9	11.2	16.2	32.0	Yes
mis15	*SPBP22H7.09c*	*CHL4*	38.9			12.4	Yes
fta1	*SPAC4F10.12*		38.2		9.5	9.3	Yes
fta2	*SPAC1783.03*		12.5		11.1		Yes
fta3	*SPBP8B7.12c*		29.5	14.1		9.1	Yes
fta4	*SPCC1393.04*		38.2		35.2	21.5	Yes
fta5	*SPAC1F8.06*		16.4		13.2	13.0	N.D
fta6	*SPAC11H11.05c*		37.3				Yes
fta7	*SPCC1235.07*		27.9				Yes
mis17	*SPBC21.01*		23.6		23.9		Yes
dad1	*SPAC16A10.05c*	*DAD1*	56.5	16.5		38.8	Yes

B

Gene name	Mass spec. coverage (%)
ask1	63.2
dam1	70.3
spc34	68.9
duo1	23.5
dad1	38.8
dad2	56.4
dad3	55.8
dad4	
spc19	
hsk3	25.5



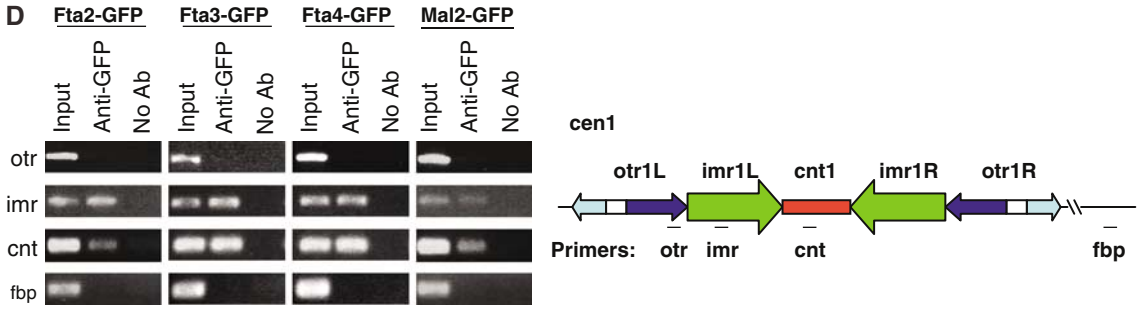


Figure 4 Characterizations of the Sim4 complex. (**A**) Summary of mass-spectrometric analysis of Sim4 complex purifications, indicating the percent sequence coverage for each polypeptide >5%. DASH subunits detected in Dad1-TAP purification are listed in (**B**). (**C**) Kinetochore localization of Fta proteins. GFP fusion of Fta1 (top) and Fta2 (bottom) localizes to multiple dots that are between the SPBs (left), and overlap with the kinetochores (right) in live cells in metaphase. (**D**) ChIP of Fta proteins. Fta2, 3, and 4-GFP proteins are associated with the central core (cnt) and the inner repeats (imr) of the centromere I, but not the outer repeats (otr) or the chromosome arm locus (fbp).

complexes. To test this idea, we performed TAP purification using Dad1-TAP. From an asynchronous cell extract, most of the components of both the DASH and Sim4 complexes, but not other kinetochore proteins, copurified with Dad1 (Figure 4A–C). These results provide strong evidence that Dad1 mediates the interaction between the DASH and Sim4 complex. We propose that DASH, when assembled in mitosis, is loaded on kinetochores specifically on the Sim4 complex via Dad1. Supporting evidence was also gained in determining the protein localization of Dad1 and the dependency of other DASH proteins on Dad1 for kinetochore localization (Figure 3A–C).

Ndc80 complex, MIND complex, and Spc7 are conserved kinetochore components that physically interact with one another

Mis12 and Nuf2 are two constitutive kinetochore proteins. Mutations in these two proteins cause chromosome mis-segregation or complete detachment of kinetochores from the spindle (Goshima *et al*, 1999, 2003; Nabetani *et al*, 2001; Obuse *et al*, 2004). In budding yeast, Mtw1, the Mis12 homolog, and Nuf2 are integral components of the MIND and Ndc80 complexes, respectively (Janke *et al*, 2001; Wigge and Kilmartin, 2001; De Wulf *et al*, 2003; Nekrasov *et al*, 2003). These two complexes are biochemically separable and are assembled on the kinetochores independently (De Wulf *et al*, 2003).

To test whether these two complexes are conserved in fission yeast, we performed tandem affinity purification using SpMis12-TAP and SpNuf2-TAP. From an asynchronous cell extract, eight specific proteins were copurified with SpNuf2-TAP (Figure 5A). In addition to SpNuf2, homologs of three other budding yeast Ndc80 complex components, SpNdc80, SpSpc24, and SpSpc25, were identified. This result shows that the Ndc80 complex is conserved in fission yeast.

Unexpectedly, the other remaining copurified proteins include the homologs of all four subunits in the MIND complex and of Spc105 (Figure 5A), a kinetochore protein in budding yeast (Nekrasov *et al*, 2003). In the reciprocal purifications using Mis12-TAP or Spc7-TAP, Spc7 and all subunits of the Ndc80 and MIND complexes, but no other kinetochore proteins, were copurified (Figure 5A). No homolog of the Spc105-interacting protein, Ydr532p (Nekrasov *et al*, 2003), was identified. These results strongly suggest that the MIND, Ndc80 complexes, and Spc7 tightly associate with each other in fission yeast. To further test the tight association between the MIND and Ndc80 complexes, we carried out chromatographic analysis of Mis12-TAP and Nuf2-TAP. Total extract was prepared from cells carrying both Mis12-TAP and Nuf2-TAP and subjected to gel filtration or ion-exchange chromatography. A super-complex containing the MIND and Ndc80 complexes and Spc7 would have a minimum calculated molecular weight of 460 kDa. Consistently, Mis12-TAP and Nuf2-TAP were coeluted, with one elution peak of a size over 500 kDa (Figure 5B). By MonoQ ion-exchange chromatography with a linear elution salt concentration gradient, these two proteins were also coeluted (Figure 5C).

In summary, these results show that, in fission yeast, the MIND, Ndc80 complexes, and Spc7 are conserved kinetochore components that physically interact with one another and form a super-complex. We name this super-complex as the NMS complex.

Discussion

Correlation between kinetochore assembly kinetics and functions

In metazoan cells, kinetochores are not fully assembled until mitosis. In interphase human cells, centromeres are dispersed within the nucleus, and several kinetochore proteins, hMis12, CENP-A, -B, -C, -H, and -I (hMis6), are constitutively associated with the centromeric DNA loci (Amor *et al*, 2004). Most transient kinetochore components, such as motor proteins or passenger proteins, are associated with kinetochores only after the entry into mitosis (McIntosh *et al*, 2002; Maiato *et al*, 2004). One exception among the transient components is the Ndc80 complex, which associates with the kinetochores in late G2 phase and mitosis (Hori *et al*, 2003).

In budding yeast, however, kinetochores remain attached to kMTs throughout the cell cycle (Knop *et al*, 1999; McIntosh and O'Toole, 1999). Interestingly, other than the spindle checkpoint proteins, most identified kinetochore proteins, including those that interact with MTs, are constitutively kinetochore-localized (He *et al*, 2001; McAinsh *et al*, 2003).

In contrast, fission yeast kinetochores maintain a static attachment directly to the SPB in interphase, while in mitosis kinetochores are attached to the dynamic ends of MTs (Uzawa and Yanagida, 1992; Ding *et al*, 1993, 1997; Kniola *et al*, 2001). Thus, fission yeast cells need to assemble and maintain 'basic' functional kinetochores in interphase and fully functional kinetochores in mitosis. Consistently, some kinetochore components in fission yeast are constitutive, while the others are transient.

Comparisons across species suggest a pattern of kinetochore assembly in which specific components are loaded onto kinetochores only when they are needed. Even for conserved components, their kinetochore association dynamics varies, possibly coinciding with the timing of their functions. For example, the Ndc80 complex, which plays a structural role, is transiently associated with kinetochores in vertebrate cells (Wigge and Kilmartin, 2001; Hori *et al*, 2003; McCleland *et al*, 2003), but is constitutively associated in fission yeast (Nabetani *et al*, 2001), whereas the MT-interacting DASH is constitutively kinetochore associated in budding yeast (Cheeseman *et al*, 2001; He *et al*, 2001; Janke *et al*, 2002; Li *et al*, 2002), but transiently in fission yeast.

A nonessential DASH is associated with kinetochores only in mitosis in fission yeast

DASH is an essential component of the kinetochore in budding yeast that is required for the bi-orientation of sister chromatids (Cheeseman *et al*, 2001; Janke *et al*, 2002; Li *et al*, 2002). Such an activity is required for equal chromosome segregation in all species, and, yet, homologs of DASH subunits are scarce in other organisms and absent in vertebrate genomes. Identification of SpDASH suggests that the complex is conserved at least between budding yeast and fission yeast. For most of the DASH subunits, the sequence divergence is pronounced between the two species. It is possible that more similarity may be identified at a higher order of structure. It is also possible that, in other model organisms, homologs of DASH proteins are too diverged to be identified simply by sequence comparison. In-depth structural and functional studies are needed to identify the possible orthologs of DASH in metazoans.

Our results have shown that SpDASH is transiently associated with the kinetochores, and that the complex is likely to be assembled specifically during mitosis. Except for Dad1 (see below), most DASH components depend on each other for their kinetochore localization, suggesting that the holocomplex of DASH is required for its kinetochore association. We postulate that complex assembly is a major form of regulation of DASH *in vivo*. Consistently, we have observed specific phosphorylation on Ask1 in mitosis, concomitant with DASH assembly and kinetochore association. Multiple phosphorylation sites have been identified in ScDASH pro-

A

Gene name	ORF name	*S. cerevisiae* homolog	Protein coverage by mass spectrometry (%)		
			nuf2-TAP	mis12-TAP	spc7-TAP
nuf2	*SPAC27F1.04c*	*NUF2*	31.1	48.1	54.0
ndc80	*SPBC11C11.03*	*NDC80*	61.9	55.0	55.3
spc24	*SPBC336.08*	*SPC24*	65.2	74.7	68.2
spc25	*SPCC188.04c*	*SPC25*	56.3	39.1	33.6
mis12	*SPBC409.04c*	*MTW1*	29.7	36.7	43.2
mis14	*SPAC688.02c*	*NSL1*	39.0	68.1	54.3
nnf1	*SPAC29E6.04*	*NNF1*	37.9	68.3	35.2
mis13	*SPBC409.09c*	*DSN1*	30.1	47.7	24.9
spc7	*SPCC1020.02*	*SPC105*	15.6	27.3	30.2

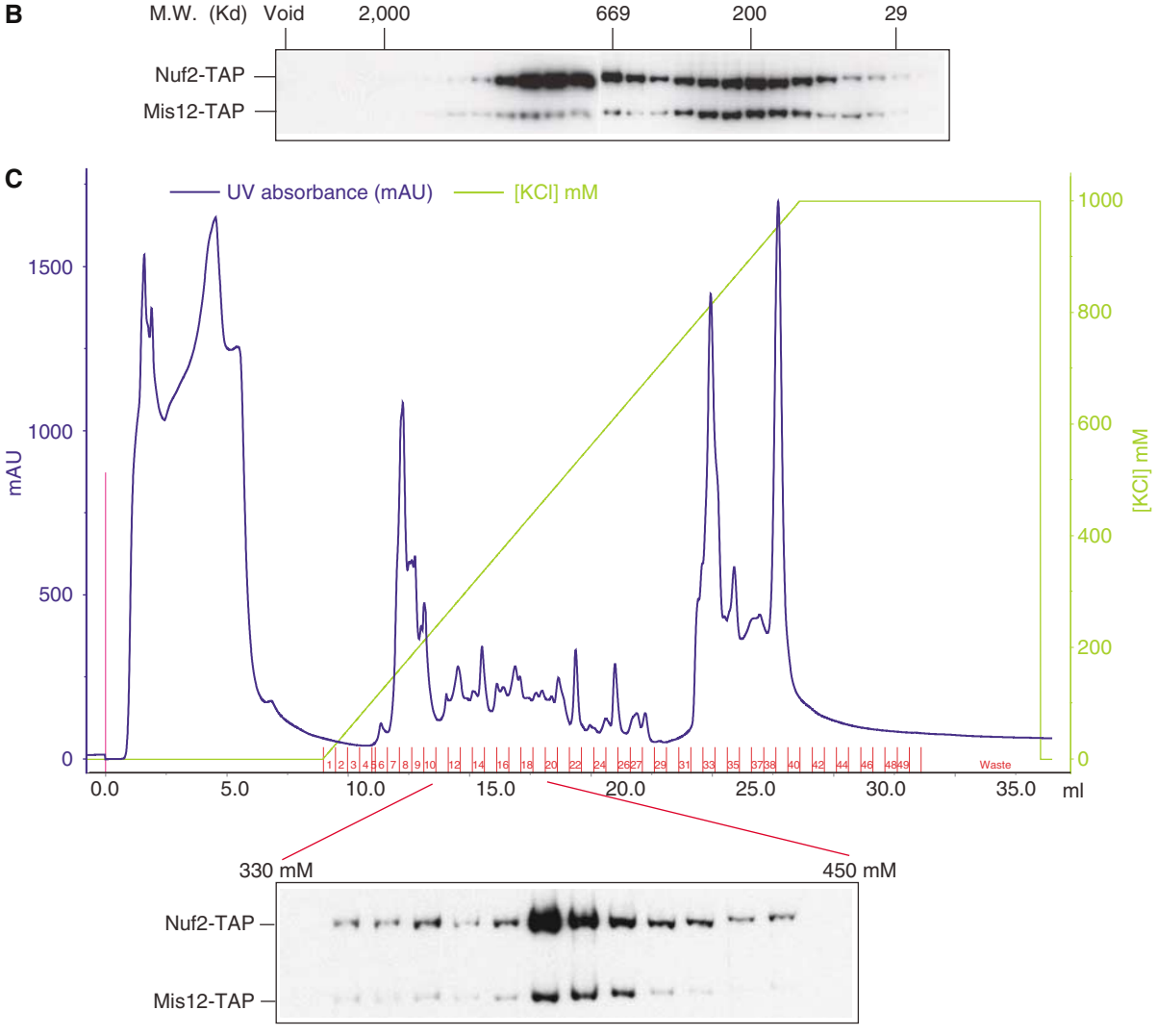


Figure 5 Characterization of the NMS complex. (**A**) Summary of mass-spectrometric analysis of reciprocal purification of the complex, indicating the percent sequence coverage for each polypeptide >5%. (**B**) Coelution of Nuf2-TAP and Mis12-TAP in size exclusion chromatography. SDS–PAGE electrophoresis and Western blotting of fractions of a Superose-6 size exclusion column. The peaks of the elusion of the molecular weight markers are labeled at the top. The identities of the upper and lower bands as Nuf2-TAP and Mis12-TAP were confirmed by Western blotting of cell extracts containing Nuf2-TAP or Mis12-TAP only (data not shown). (**C**) Coelution of Nuf2-TAP and Mis12-TAP in ion exchange chromatography. Chromatogram of MonoQ ion exchange chromatographic analysis of total cell extract containing Nuf2-TAP and Mis12-TAP. The salt gradient in elution buffer is illustrated by the green line. Concentration of eluted proteins, measured by 280 nm UV absorbance, is illustrated by the blue line. The Western blot includes the fractions in which Nuf2-TAP and Mis12-TAP were eluted.

teins; some are critical for the activity of the whole complex (Cheeseman *et al*, 2002; Li and Elledge, 2003; Shang *et al*, 2003). Whether the same phosphorylation events play a role in SpDASH awaits further investigation. At least for ScDam1, the critical phosphorylation in the C-terminal region (S257, S265, and S292) by the Ipl1 kinase (Cheeseman *et al*, 2002) does not seem to be conserved, since the corresponding domain is missing in SpDam1.

It was postulated that ScDASH is delivered to the kinetochores by the spindle MTs (Li *et al*, 2002). Our results in fission yeast show that DASH is predominantly associated with kinetochores that are in the vicinity of the SPBs, and noticeably to a smaller extent with the unattached kinetochores in the same cells. We postulate that DASH is capable of binding to kinetochores in the absence of MTs or SPBs. However, a strong or stable binding needs the presence of SPBs, or short MTs derived from the SPBs.

Recently, purified recombinant ScDASH was shown to form a ring structure around the MT *in vitro*, preferably at the GTP-bound region of the MT. Such a distinct binding pattern points to an intriguing mechanism for how the DASH complex may secure the binding of kinetochores to the dynamic ends of MTs (Miranda *et al*, 2005; Westermann *et al*, 2005). Surprisingly, given the prominent roles of ScDASH, DASH is not essential for cell viability in fission yeast. Instead, cells without DASH components frequently undergo mitosis with lagging chromosomes, which, to a much lesser extent, results in chromosome mis-segregation. Most cells with a lagging chromosome in early anaphase are able to 'rescue' the defect and execute a successful mitosis. The defect of chromosome mis-segregation, albeit in a low percentage of the population, is consistent with the postulated role of SpDASH in chromosome bi-orientation. It further implies that in fission yeast there is an alternative mechanism responsible for chromosome bi-orientation that is functionally redundant with DASH. The lagging chromosome defect may be caused by merotelic attachment—MTs derived from both SPBs attached to the same kinetochore, which, presumably, can slow down kinetochore migration toward the destination SPB. Alternatively, it may suggest that SpDASH has another role in chromosome segregration in addition to ensuring bi-orientation, perhaps in modulating the kinetics of k-MT depolymerization.

In addition to its kinetochore localization, we also detect DASH localization as multiple dots along the spindle axis and a couple of extra dots away from the axis in early mitotic cells. These dots may represent DASH at nonkinetochore spindle MT tips (Zimmerman *et al*, 2004; Sanchez-Perez *et al*, 2005). This would seem reasonable, given that DASH favors binding to GTP-bound region of MT *in vitro* (Westermann *et al*, 2005). At this stage, the functional implication of DASH on spindle is unclear. No gross spindle morphological defects or significant change in spindle elongation kinetics were detected in DASH deletion cells (data not shown).

Two biochemically independent motifs comprise the core components of the fission yeast kinetochore

In this paper, we have reported a series of affinity purification that led to the identification of two groups of kinetochore proteins: the Sim4 complex that is comprised of Sim4, Mal2, several Mis proteins and a group of novel Fta proteins, and the NMS complex that consists of the Ndc80 and MIND complexes and Spc7.

Direct interactions among the Ndc80 and MIND complexes and Spc7 may be conserved in other organisms. Ample evidence of extensive genetic and biochemical interactions among proteins in these complexes has been reported in budding yeast (Nekrasov *et al*, 2003). In *Caenorhabditis elegans*, as well as in mammalian cells, copurification of different combinations of these proteins has also been reported (Cheeseman *et al*, 2004; Obuse *et al*, 2004). The significance of protein-interacting networks is clear for dissecting kinetochore architecture. We propose that each protein-interacting network corresponds to a discrete motif within the kinetochore. Although it was clearly demonstrated that the budding yeast Ndc80 and MIND are two independent complexes in solution (McAinsh *et al*, 2003), it does not necessarily imply a major difference in kinetochore architecture between budding yeast and fission yeast. It is possible that the structural motif formation is similar, but that the interaction between these proteins in solution is weak in budding yeast.

Sequence conservation of the Sim4 complex between fission yeast and budding yeast is only limited to several pairs of proteins, namely, SpMal2/ScMcm21, SpMis15/ScChl4, and SpMis6/ScCtf3 (Figure 4A). Mcm21 is a subunit of the COMA complex. Chl4 and Ctf3 also directly interact with other kinetochore proteins in budding yeast (for a review, see McAinsh *et al*, 2003). Although it is tempting to speculate that these budding yeast complexes collectively may be equivalent to the Sim4 complex, no significant sequence similarity has been identified between the rest of the Sim4 complex proteins and other COMA subunits and Chl4, Ctf3 interacting proteins. Perhaps, the similarity resides in the higher-order structure rather than the primary amino-acid sequence. Further investigation is needed to test this model.

Finally, we provide evidence that the Sim4 complex is the loading site for the DASH complex via Dad1. First, Dad1 is found in both the Sim4 complex and DASH complex. Secondly, all other tested DASH subunits require Dad1 as well as each other for kinetochore localization, whereas Dad1 kinetochore localization is independent of other DASH subunits. Finally, by Dad1 affinity purification, DASH and Sim4 complexes are copurified from the extract of an asynchronous culture, which is a mixture of mainly interphase cells and about 10% mitotic cells. As of yet, we have not found the specific cell synchronization conditions to detect the association of the Sim4 and DASH complexes during mitosis. It may be possible that the association occurs at a specific stage during mitosis that is different from the arresting point of the *mts2-1* mutation used for our purifications. Direct evidence for the interaction between the Sim4 and DASH complexes awaits future studies through other biochemical means.

It is possible that the Sim4 complex binds to other MT-interacting proteins as well since the Sim4 complex subunits are essential (Goshima *et al*, 1999; Jin *et al*, 2002; Pidoux *et al*, 2003), while DASH is not. It is likely that the NMS complex serves as the loading dock for other MT-interacting proteins such as EB1-like protein Mal3 (Kerres *et al*, 2004). How other MT-interacting proteins are loaded onto kinetochores in mitosis is unclear. However, it is possible that the two protein interacting networks serve as the loading docks for different MT-interacting kinetochore components. The

identity and specificity of other MT-interacting proteins that bind to the two core kinetochore motifs and how they coordinate their functions will be the interest of future study.

Materials and methods

For more details on Materials and methods, see Supplementary data online.

Yeast strain construction and growth condition

All strains used are listed in Supplementary data. COOH-terminal TAP or GFP fusions and deletions were generated with standard methods as described in detail in Supplementary data. Yeast cultures were grown in standard YE media following established procedure.

Fluorescence microscopy

Living cells carrying various GFP or CFP markers were used for microscopic imaging. Fluorescence microscopy and image analyses were carried out on a Deltavision deconvolution microscope as described (He *et al*, 2000).

Tandem affinity purification and mass spectrometry

An established procedure was modified for tandem affinity purification (Gould *et al*, 2004). After the first-step affinity by IgG agarose, the beads were washed with IPP buffers (10 mM Tris–Cl, pH 8.0, 0.1% NP40) with various salt concentrations, 400, 600, and 400 mM NaCl before TEV protease treatment. Mass spectrometric analysis was performed essentially as described (Cheeseman *et al*, 2001).

Protein techniques and chromatography

Protein extracts for Western blotting were prepared from TCA-treated cells as described (Foiani *et al*, 1994). Peroxidase–antiperoxidase (PAP) antibody (Sigma P-1291) was used for detecting TAP tagged proteins. Western blots were developed using ECL reagents (Amersham Biosciences). Phosphase treatment was performed as described (Li and Elledge, 2003). An AKTA FPLC system (Amersham Biosciences) was used for chromatographic analysis. A Superose 6 column (10/300 mm) was used for gel filtration, with elution buffer (300 mM NaCl, 50 mM Tris–Cl, pH 8.0, 5 mM EDTA, 1 × protease inhibitors). A MonoQ column (5/50 mm) was used for ion exchange, with elution buffer (20 mM HEPES, pH 7.5, 1 mM EDTA, 0.5 mM DTT) and a linear salt gradient in 20 ml, spanning 0–1 M KCl.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described previously (Jin *et al*, 2002).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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