Ringling the changes: emerging roles for DASH at the kinetochore–microtubule Interface

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Abstract Regulated interaction between kinetochores and the mitotic spindle is essential for the fidelity of chromosome segregation. Potentially deleterious attachments are corrected during prometaphase and metaphase. Correct attachments must persist during anaphase, when spindle-generated forces separate chromosomes to opposite poles. In yeast, the hetero-decameric DASH complex plays a vital role in maintaining this link. In vitro DASH forms both oligomeric patches and rings that can form load-bearing attachments with the tips of polymerising and depolymerising microtubules. In vivo, DASH localises primarily at the kinetochore, and has a role maintaining correct attachment between spindles and chromosomes in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. Recent work has begun to describe how DASH acts alongside other components of the outer kinetochore to create a dynamic, regulated kinetochore–microtubule interface. Here, we review some of the key experiments into DASH function and discuss their implications for the nature of kinetochore–microtubule attachments in yeast and other organisms.

Keywords Dam1 · DASH · Kinetochore · Microtubule · Spindle.

Abbreviations

CIN	Chromosomal instability
EM	Electron microscopy
GDP	Guanosine diphosphate
GMPCPP	Guanosine [(α,β)-methyleno]triphosphate
GTP	Guanosine triphosphate
SPB	Spindle pole body

Introduction

The failure to correctly segregate chromosomes during mitosis is a major cause of chromosomal instability (CIN), the variations in chromosome number that are a hallmark of many solid tumours (Thompson et al. 2010). CIN correlates with poor prognosis in patients and is often associated with increased drug resistance, presumably due to the increased genetic plasticity of the tumour (McClelland et al. 2009).

In normal cells, sister chromatids are faithfully segregated into daughter cells by the mitotic spindle, a microtubule-based organelle that generates the force required for chromosome segregation. Only when all the chromosomes in a cell are correctly bi-oriented (i.e. each sister chromatid is attached, via the spindle, to the opposite pole) are cells allowed to transition from metaphase into anaphase. Constant monitoring and correction of attachments between chromosomes and

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the spindle are required to prevent the formation of aneuploid daughter cells. This interaction between spindle microtubules and chromosomes occurs at kinetochores: large protein structures that assemble on specialised regions of chromatin termed centromeres (reviewed in Przewloka and Glover 2009).

The kinetochore functions as more than just a simple link between chromosomes and the spindle. Not only must the kinetochore efficiently capture highly dynamic microtubules during prometaphase, it must also detect and correct errors in attachment to facilitate bioriented alignment on the metaphase plate. Kinetochore-based signalling pathways generate the spindle assembly checkpoint signal that delays anaphase onset until all chromosomes are correctly oriented. During anaphase A, when sister chromatids are pulled towards opposite poles, kinetochores must be able to transmit the force generated by depolymerising microtubules into movement. The kinetochore is therefore endowed with many intriguing properties that allow efficient and accurate chromosome segregation. For example, micromanipulation experiments in grasshopper spermatocytes show that kinetochore microtubules become more stable when put under tension (Nicklas and Ward 1994). The ability to sense tension between a centromere pair has since been shown to be sufficient for chromosome biorientation in yeast cells (Dewar et al. 2004). In addition, experiments in yeast cells indicate that kinetochores can link microtubule depolymerisation to chromosome movement in the absence of conventional motors (Tanaka et al. 2005; Grishchuk and McIntosh 2006). An understanding of how kinetochores work is essential to our understanding of the process of chromosome segregation.

Almost all of the components of the yeast kinetochore have been discovered, and a similarly complete view is forming of kinetochores in other model systems. Many of the various subcomplexes that make up the kinetochore are conserved from yeast to humans (McAinsh et al. 2003; Liu et al. 2005; Przewloka et al. 2007; Cheeseman and Desai 2008). The exciting challenge ahead is to discover how different kinetochore components act together and with other proteins to generate the dynamic microtubule–chromosome interface. The fungal DASH complex (often called the Dam1 complex, after one of its components) is one kinetochore component that has been subject to intense study in

recent years. DASH is a heterodecameric complex that binds microtubules and localises to the outer kinetochore (Nogales and Ramey 2009). Purified *Saccharomyces cerevisiae* DASH complex has been shown to oligomerise to form rings around microtubules in vitro and can track depolymerising microtubule plus ends (Miranda et al. 2005; Westermann et al. 2005, 2006). Plus-end tracking DASH complexes can couple cargo movement to microtubule depolymerisation, presenting an attractive model by which kinetochores could harness microtubule dynamics to move chromosomes (Asbury et al. 2006; Efremov et al. 2007).

In *S. cerevisiae* cells, the DASH component Dam1 is phosphorylated by Ip11, an Aurora-related kinase essential for correcting incorrect microtubule–kinetochore attachments (Cheeseman et al. 2002; Pinsky et al. 2006b). This phosphorylation is opposed by the PP1 phosphatase Glc7 and persists in the absence of tension across kinetochores, suggesting that DASH may also be involved in tension-dependent correction of kinetochore–microtubule interactions (Pinsky et al. 2006a; Keating et al. 2009).

A greater understanding of DASH function is rapidly forming through a combination of in vivo and in vitro approaches. In particular, recent advances are beginning to show how DASH functions at the kinetochore and how this function is regulated (Gestaut et al. 2008; Lampert et al. 2010; Tien et al. 2010). Here, we summarise some of the key experiments that have helped establish the role of DASH at the kinetochore and discuss their implications for the nature of the kinetochore–microtubule interface.

The DASH complex

The first components of the DASH complex were identified in *S. cerevisiae* over 10 years ago. Initial characterisation focused on their localisation to the mitotic spindle and their role in its function (Hofmann et al. 1998; Jones et al. 1999). Further studies demonstrated that the DASH complex localised to the kinetochore and that mutants had severe biorientation defects, leading to the suggestion that DASH may form the link between the kinetochore and spindle microtubules (Cheeseman et al. 2001a,b). Subsequent work steadily increased the number of DASH components identified. We now know DASH to be a complex of ten subunits: Dam1, Duo1, Ask1, Spc34, Spc19, Dad1,

Dad2/Hsk1, Dad3, Dad4/Hsk2, and Hsk3. Despite the varying sizes of the individual components (Dam1 is almost 40 kDa, and Hsk3 is around 8 kDa), localisation and function of DASH requires the presence of all subunits, as well as the mitotic spindle (Cheeseman et al. 2001b; Enquist-Newman et al. 2001; Janke et al. 2002; Li et al. 2002).

All components of the DASH complex are conserved in the distantly related fission yeast *Schizosaccharomyces pombe*. The *S. pombe* DASH complex was simultaneously discovered through both biochemical and genetic screens (Liu et al. 2005; Sanchez-Perez et al. 2005). Interestingly, whilst all members of the DASH complex are essential in *S. cerevisiae*, DASH is not essential in *S. pombe*. However, DASH mutants display high rates of chromosome missegregation, sensitivity to the microtubule poison thiabendazole and have a long, checkpoint-dependent delay in mitosis (Sanchez-Perez et al. 2005). During anaphase A, poleward movement of kinetochores is much slower in these cells, and some kinetochores frequently 'lag' behind the rest. Severing the spindle by laser ablation reveals that these lagging kinetochores are due to a slower rate of anaphase A, rather than merotelic attachments (Sanchez-Perez et al. 2005; Saitoh et al. 2008; Gao et al. 2010).

Kinetochores capture

The first crucial role for the kinetochore during mitosis is to allow efficient capture of chromosomes by dynamic spindle microtubules. Tanaka et al. developed an elegant system that allows the regulated inactivation of individual centromeres in *S. cerevisiae*. Cells were blocked in metaphase, and then the centromere was reactivated. Fluorescent markers allowed the tracking of individual kinetochore capture events upon centromere reactivation (Tanaka et al. 2005). Consistent with observations in vertebrate cells, kinetochores initially interact laterally with the wall of the microtubule lattice (Rieder and Alexander 1990; Tanaka et al. 2005). Kinetochores are then rapidly retrieved to the spindle poles before re-orientation on the spindle (Tanaka et al. 2005). Retrieval occurs by one of two mechanisms: 'sliding' along the microtubule lattice, mediated by the kinesin-14 family member Kar3, or DASH-dependent 'pulling' by the depolymerising microtubule. In the presence of Kar3, DASH mutants do not appear to have impaired rates of kinetochore retrieval (Tanaka et al. 2005, 2007).

Conversely, in *S. pombe*, DASH is essential for timely kinetochore retrieval (Franco et al. 2007). Cells harbouring a cold-sensitive allele of the gene encoding β -tubulin were blocked in a microtubule-free, prometaphase-like state by prolonged incubation at low temperature. Kinetochore capture and retrieval can be monitored upon shift to permissive temperature. Deleting Klp2, the *S. pombe* kinesin-14 has a small effect in population studies using this assay, indicating that motor-mediated sliding plays only a minor role in retrieval in this system (Grishchuk and McIntosh 2006). DASH is absolutely required for retrieval in this assay (Vanoosthuyse et al. 2009). Live imaging of kinetochore recapture is facilitated by deletion of *mtol*, a gene encoding a centrosomin-like protein that is required for nucleation of cytoplasmic microtubules in *S. pombe* (Sawin et al. 2004). In some Δ *mtol* cells, one centromere pair becomes detached from their characteristic cluster near the spindle pole body (SPB) and must be recaptured by the mitotic spindle before anaphase can progress (Franco et al. 2007). Live imaging of these cells, and cells recapturing kinetochores after microtubule depolymerisation, reveals that deleting either *S. pombe* kinesin-14 only effects kinetochore recapture in a small number of cells (Grishchuk and McIntosh 2006; Franco et al. 2007; Gachet et al. 2008). DASH-deficient cells, however, are severely impaired in these assays, with retrieval only occurring rarely, and after a prolonged delay (Franco et al. 2007; Kobayashi et al. 2007).

This apparent discrepancy in mechanisms of kinetochore retrieval between *S. cerevisiae* and *S. pombe* is perhaps surprising. The extent to which this is due to a fundamental difference between the two species, or differences between the assays used, is unclear. For example, kinetochore capture reported by Tanaka et al. occurs at a greater distance from the SPB than typically seen in the *S. pombe* assays. This 'longer-range' capture may require formation of longer, more stable microtubules better suited to motor-dependent retrieval. Retrieval over shorter distances, as prevalent in the assays used in *S. pombe*, may require shorter and less stable microtubules, better suited to end-on retrieval. Assays in both model systems point to a key role for DASH in kinetochore retrieval and support in vitro experiments that indicate that DASH can couple microtubule depolymerisation to chromosome movement.

DASH structure and function

Detailed analysis of the DASH complex structure and function is complicated by the fact that individual DASH components are insoluble when expressed in *Escherichia coli* (Waldo and Scherrer 2008). However, generation of a polycistronic vector containing the open reading frames (ORFs) of all ten DASH components allowed the purification of complete DASH complexes and was very much the catalyst for the recent insights into DASH function in vitro (Miranda et al. 2005).

The crystal structure of the DASH complex has not yet been solved. However, the architecture of individual DASH complexes has been calculated using electron microscopy (EM) and three-dimensional image reconstruction. This shows DASH to be a bar-shaped complex with a 'bulge' about half-way down its length, from which a protrusion extends. When bound to microtubules, this 'protrusion domain' points towards the microtubule lattice and is likely to be the major site of microtubule attachment on the DASH complex. DASH formed with a truncated form of Dam1 shows a reduced protrusion, indicating that the C-terminus of Dam1 is required for this domain (Wang et al. 2007). Although the precise location of individual subunits within this architecture has yet to be determined, the relative positions of different subunits have been inferred from protein interaction data and structural studies (Nogales and Ramey 2009). In the absence of microtubules, DASH complexes predominately form dimers, with larger oligomers appearing only rarely and in relatively high DASH concentrations (Wang et al. 2007). In the presence of microtubules, however, DASH oligomerises to form rings, and sometimes paired helices, around microtubules (Miranda et al. 2005; Westermann et al. 2005). Careful analysis of negative-stain electron micrographs revealed that oligomers of 16 DASH complexes are required to form rings around microtubules formed of 13 protofilaments (Westermann et al. 2006).

The discovery that DASH forms rings around microtubules captured the imagination of researchers interested in the microtubule–kinetochore interface (Salmon 2005). Rings provide an excellent model for a microtubule plus end tracker that could couple depolymerisation to movement (Efremov et al. 2007; Armond and Turner 2010). A ring-based coupler

bound weakly to the plus end would effectively track the depolymerising tip as the splayed microtubule end would push on the ring as the microtubule depolymerises (Fig. 1a). A DASH ring linked to the kinetochore, possibly via the Ndc80 complex [which it interacts with in vitro and in vivo, albeit weakly (Shang et al. 2003)] presents an enticing candidate for such a coupler (Fig. 1a).

DASH is well suited to a role linking the kinetochore to the plus end of the microtubule, due to its observed affinity to guanosine triphosphate (GTP)-tubulin. On microtubules containing stretches of fluorescently labelled GDP-tubulin grown from seeds of unlabelled tubulin assembled with guanosine-5'-[(α,β)-methylene]triphosphate (GMPCPP—a stable GTP homologue that is only hydrolysed very slowly), DASH was seen to preferentially bind to the GMPCPP-tubulin seed (Westermann et al. 2005). This indicates that DASH will preferentially associate with GTP-tubulin (as GMPCPP-tubulin mimics GTP-tubulin) and provides a mechanism for DASH association (and potentially kinetochore association) with the microtubule plus end.

Further support for DASH as a coupler came with the observation that DASH tracks the plus end of depolymerising microtubules, as predicted by the model of a ring-based coupler with affinity for the plus end. Microtubules decorated with DASH were depolymerised by reducing the local tubulin concentration to a level that causes microtubule depolymerisation, or by adding the microtubule-depolymerising kinesin-13 XKCM1. Fluorescent labels on the DASH complex and the tubulin allowed DASH to be seen tracking the depolymerising microtubule end (Westermann et al. 2006).

In addition, tip-tracking DASH complexes are capable of transporting cargo in the form of small beads (Asbury et al. 2006; Westermann et al. 2006). This demonstrates that DASH is capable of coupling microtubule depolymerisation to force generation. Optical trapping experiments demonstrate that depolymerising microtubules can move DASH-coated beads under moderate levels of tension. Strikingly, increasing the tension on microtubule-attached DASH beads promotes microtubule growth by reducing the frequency and speed of microtubule depolymerisation events and increasing the frequency of microtubule rescue (Franck et al. 2007). In vivo, chromosome biorientation is ensured by destabilising incorrect kinetochore–microtubule attachments and stabilising

Fig. 1 Recent models of DASH function. **a** The properties of DASH in vitro suggest that DASH could form a major microtubule-binding site at the kinetochore, possibly via the Ndc80 complex. Later studies indicate that this is not the case. **b** DASH localises within the Ndc80 complex, with which it cooperates to facilitate its microtubule association. DASH may form rings or smaller oligomers. **c** *S. pombe* kinetochores bind two to four microtubules. DASH does not apparently form rings in these cells and appears to favour smaller oligomeric forms.

correct, amphitelic, attachments (Tanaka and Desai 2008). Tension across kinetochores is a key indicator of correct attachment. In this assay, DASH bound to beads appears to reconstitute some of the key observed kinetochore behaviours, suggesting that DASH has a role in ensuring correct microtubule–kinetochore interactions.

Although the ring-based coupler is undoubtedly an attractive model for DASH function, the presence of rings remains controversial. Crucially, rings have never been seen around microtubules at yeast kinetochores in vivo (McIntosh 2005). The absence of rings may be

due to technical difficulties inherent in electron tomography of yeast kinetochores. However, until direct evidence of DASH rings is seen in vivo, the biological relevance of DASH rings remains in doubt.

Interestingly, many of the observed properties of the DASH complex do not require oligomerisation into a ring. Individual GFP-tagged DASH complexes are sufficient to form dynamic attachments to microtubules. Small oligomers corresponding to one to four DASH complexes are also capable of processive tracking of depolymerising plus ends (Gestaut et al. 2008). Non-ring forming DASH complexes also

appear to be competent to couple microtubule depolymerisation to cargo transport. To observe tip-tracking by smaller DASH oligomers, Grishchuk and colleagues coated microbeads with DASH and observed their tracking on shortening microtubules in the presence or absence of soluble DASH. In the presence of soluble DASH, bead-bound DASH is able to form rings around microtubules. In the absence of soluble DASH, beads prevent the formation of encircling DASH rings. Perhaps surprisingly, DASH-coupled beads still track depolymerising microtubule ends in the absence of soluble DASH, but move more rapidly, and with apparently different kinetics from when DASH is allowed to form rings (Grishchuk et al. 2008).

The existence of two different tip-tracking forms of DASH oligomer in vitro, albeit with different properties, has obvious implications on the nature of the kinetochore–microtubule interface. The physiological relevance of the different oligomerisation states has yet to be determined (Fig. 1b). Potentially, different oligomerisation states could represent DASH at different times during mitosis. The fact that DASH complexes containing Dam1 with mutations on its C-terminus that mimic Ipl1 phosphorylation oligomerise less readily may support this hypothesis (Wang et al. 2007).

DASH at the yeast kinetochore

Knowing the relative abundance and localisation of DASH at the kinetochore allows us to make some inferences about the likelihood of DASH forming rings physiologically.

Recently, high-resolution microscopy studies by Salmon and colleagues have greatly enhanced the understanding of the composition and architecture of the kinetochore (Joglekar et al. 2006, 2008, 2009). Joglekar et al. were able to take advantage of the ease with which proteins expressed under their endogenous promoter can be fluorescently tagged at their C-termini in yeast. This allowed quantitative assessment of protein number via the relative fluorescent intensity of different tagged proteins. Due to the low protein turnover of kinetochore proteins, Joglekar et al. were able to estimate copy number of different kinetochore components, relative to the *S. cerevisiae* CENP-A homologue, Cse4, in both metaphase and anaphase (Joglekar et al. 2006).

This method estimates that there are 16–20 DASH complexes per kinetochore during metaphase and 10–11 during anaphase-telophase (Joglekar et al. 2006). As each kinetochore binds a single microtubule in *S. cerevisiae*, this indicates 16–20 DASH complexes per microtubule during metaphase. DASH decorates the spindle in anaphase, and this could account for the discrepancy in DASH complex numbers between metaphase and anaphase (Hofmann et al. 1998; Joglekar et al. 2006). These observations are consistent with the model of DASH acting as a ring. There are sufficient quantities of DASH to form rings in metaphase, prior to chromosome segregation. After chromosomes have been separated, during late anaphase and telophase, DASH function at the kinetochore may be less critical, and rings could disperse.

Applying the same technique in *S. pombe* suggests fewer DASH complexes per microtubule (Joglekar et al. 2008). *S. pombe* centromeres are typically much larger than those in *S. cerevisiae* (40–100 kb, rather than 150 bp) and bind two to four microtubules (Ding et al. 1993). Interestingly, the protein composition of an individual microtubule-binding site is largely conserved between *S. pombe* and *S. cerevisiae*, despite the large difference in centromere size between the two species. *S. pombe* kinetochore protein numbers were estimated at G2/M transition and in anaphase. During anaphase, there are approximately seven DASH complexes per kinetochore, corresponding to two to three complexes per microtubule (Joglekar et al. 2008). Even if all DASH complexes were clustered onto a single microtubule, there would not be enough to form a single microtubule ring (Fig. 1c).

Only one component of the *S. pombe* DASH complex, Dad1, is present at the kinetochore before entry into mitosis (Sanchez-Perez et al. 2005). During G2/M, there are one to two copies of Dad1 per kinetochore, less than one per microtubule (Joglekar et al. 2008). However, levels of Dad1 appear significantly reduced throughout the cell cycle in the absence of other DASH components (GB, unpublished observations), suggesting that more Dad1 may be recruited to the kinetochore in the presence of other DASH components. Measuring levels of Dad1 at G2/M may therefore be a poor indicator of DASH copy numbers during metaphase. The precise level of DASH at metaphase kinetochores remains to be determined. However, looking at DASH copy numbers during

anaphase in *S. pombe*, and comparing anaphase and metaphase levels of DASH in *S. cerevisiae*, we can infer that DASH is unlikely to be present in sufficient numbers to form rings around microtubules in *S. pombe*. The most likely form of DASH at *S. pombe* kinetochores appears to be as small oligomers of two to three complexes, although larger oligomers concentrated on a single microtubule are not ruled out by these observations (Fig. 1c).

Recently, DASH complexes have been seen decorating interphase microtubules in *S. pombe*. In this context, DASH appears to be organised into small ‘patches’ of two to four complexes (Gao et al. 2010). Interphase patches presumably lack any mitotic regulation that may affect kinetochore DASH. However, the presence of these DASH patches, which move along microtubules and track depolymerising microtubule ends, provides more evidence that DASH oligomers in *S. pombe* do not form rings around microtubules (Gao et al. 2010).

Mapping DASH at the kinetochore

High-resolution fluorescence microscopy has also enabled the creation of a one-dimensional ‘map’ of the budding yeast kinetochore. Using pairs of proteins fused to enhanced green fluorescent protein and tdTomato (a red fluorescent protein), the relative positions of different kinetochore proteins along the kinetochore–microtubule axis were estimated with up to 10 nm accuracy. DASH was localised to the outer kinetochore, around 60 nm away from the Cse4 nucleosome during metaphase. During anaphase, the whole kinetochore contracts, presumably due in part to loss of tension after cohesin degradation. Consistent with this contraction, DASH moves to around 40 nm away from Cse4, but maintains its position relative to the rest of the kinetochore. Surprisingly, DASH is not the most distal kinetochore component. The microtubule-binding N-terminus of Ndc80 is consistently 5–10 nm further from the centromere (Joglekar et al. 2009). The localisation of DASH within an Ndc80 ‘cage’ at the kinetochore has implications for DASH function (Fig. 1b). The geometry the Ndc80 complex appears to preclude a DASH ring between the microtubule and Ndc80. The authors propose that DASH either forms a ring outside the Ndc80 complex or that smaller oligomers form a ‘broken ring’ interrupted by Ndc80 complexes

(Joglekar et al. 2009). Clearly, both these models have different implications for DASH function, and further study is required to determine the correct arrangement of DASH within the kinetochore.

The revelation that DASH is not the most distal component of the kinetochore also suggests a subtler role for DASH at the kinetochore than early models (e.g. Fig. 1a). Localisation within the Ndc80 complex implies that, rather than forming the major kinetochore–microtubule attachment site, DASH has less major role, acting in concert with other kinetochore components.

Cooperation between DASH and Ndc80 complexes

In higher eukaryotes, a highly conserved network consisting of the Ndc80 and Mis12 complexes, and the outer kinetochore component Blinkin/Sp105/KNL-1, has been proposed as the major microtubule-binding site in the kinetochore (Cheeseman et al. 2006). Whilst these complexes (termed the KMN network) are conserved across eukaryotes, DASH homologues have not been found outside fungi (Fig. 3). Recently, purified *S. cerevisiae* Ndc80 complex has been shown to be sufficient to form load-bearing attachments to microtubule tips in vitro, supporting the assertion that the KMN network is the major linker between kinetochores and microtubules in all eukaryotes (Powers et al. 2009). In *S. cerevisiae* and, to a lesser extent, *S. pombe*, DASH is essential for correct end-on interaction between microtubules and kinetochores. The precise role of DASH in mediating these interactions has, until recently, been unknown.

Two recent, independent studies demonstrate that the major role of DASH at the kinetochore may be to facilitate microtubule tip-tracking by the Ndc80 complex (Lampert et al. 2010; Tien et al. 2010). Both studies show, in agreement with previous work, that Ndc80 complex alone forms relatively transient associations with microtubules (Powers et al. 2009; Lampert et al. 2010; Tien et al. 2010). Adding increasing concentrations of DASH reduces the rate of Ndc80 dissociation from microtubules. In the presence of DASH, Ndc80 forms prolonged associations with assembling and disassembling microtubule tips (Lampert et al. 2010; Tien et al. 2010). This tip-bound Ndc80 forms load-bearing attachments that can transport beads on both assembling and disassembling microtubules in the presence of DASH.

By recruiting Ndc80 to microtubules, and facilitating its plus-end tracking, DASH could allow dynamic, regulated attachment between the kinetochore and the microtubule plus end. This model for DASH function explains the observed defects in kinetochore retrieval observed in DASH-deficient yeast cells. It also explains how DASH can be essential for kinetochore function, yet only apparently interact weakly with the rest of the kinetochore. The interaction sites between Ndc80 and DASH have not yet been characterised. Doing so will show how DASH can help recruit Ndc80 to microtubules and potentially how this link is regulated.

Whole kinetochores purified from *S. cerevisiae* also exhibit microtubule-binding activity in vitro and can track depolymerising microtubule ends. Intriguingly, these attachments are stabilised when subjected to levels of tension of a similar order to that generated by the mitotic spindle. Purified kinetochores deficient in either Ndc80 or Spc105 show severely impaired microtubule binding. DASH-deficient kinetochores have near wild-type affinity for microtubules, indicating that there are other mechanisms for generating stable kinetochore–microtubule interactions. Functional DASH is still essential to stabilise attachments under tension, however, suggesting that this may be a major role for DASH in vivo (Akiyoshi et al. 2010).

DASH as a template for building a kinetochore ‘top–down’

Recent attempts to build a ‘minimal’ functional kinetochore in yeast provide interesting insights into interaction between DASH and the outer kinetochore. ‘Natural’ kinetochores are thought to be assembled ‘bottom–up’ on centromeres; specialised regions of chromatin. In order to establish the minimal kinetochore required for chromosome biorientation, two different groups employed broadly similar techniques to generate artificial kinetochores in *S. cerevisiae*. Briefly, plasmids were generated that did not contain centromeres, but were artificially tethered to a variety of different kinetochore components and microtubule binding proteins. Tethering components of the inner kinetochore or the KMN network did not produce a functioning kinetochore. Remarkably, artificially tethering DASH component Ask1 to an acentric plasmid not only allows the plasmid to interact with microtubules but also allows it to biorient and segregate with

an accuracy approaching that of ‘natural’ kinetochores (Kiermaier et al. 2009; Lacefield et al. 2009).

Accurate chromosome segregation with an artificial kinetochore requires components of the DASH, Ndc80 and Mis12 complexes. This suggests that a DASH-based artificial kinetochore recruits outer kinetochore components, and these components cooperate with DASH to facilitate chromosome biorientation. The ‘top–down’ formation of these artificial kinetochores has interesting consequences for kinetochore function. Tethering inner kinetochore components to an acentric plasmid does not support effective biorientation. This means that centromeric DNA is absolutely required for ‘bottom–up’ building of a working kinetochore. Perhaps, the centromere imposes geometry on the nascent kinetochore that is essential to build a functional kinetochore in this way. Top–down kinetochores do not require the inner kinetochore CBF3 complex (Lacefield et al. 2009), indicating that the major role for this complex is to act as a platform upon which to build the outer kinetochore.

The in vivo significance of a DASH-based artificial kinetochore remains to be seen. However, the observation that DASH can recruit components of the KMN network to form a working kinetochore suggests that DASH may play a key role in organising and regulating the microtubule attachment site at the kinetochore.

Phospho-regulation of DASH

Timely phosphorylation and dephosphorylation of kinetochore components is essential for correct biorientation. A screen for in vivo phosphorylation sites on different *S. cerevisiae* kinetochore complexes revealed 13 phosphorylation sites on the DASH complex (Cheeseman et al. 2002). Dam1 was shown to be phosphorylated on four sites: one at the N-terminus of the protein and three sites in the C-terminal domain believed to correspond to the ‘protrusion domain’ (Cheeseman et al. 2002; Wang et al. 2007); Fig. 2). All four of these sites were shown to be targets of the budding yeast Aurora-like kinase, Ipl1. Ipl1 has a role in detecting and correcting incorrect microtubule attachments and is essential for correct biorientation (Biggins et al. 1999; Pinsky et al. 2006b). Changing most of these sites to alanine confers sensitivity to the microtubule poison benomyl and has an additive effect

S.p. Dam1 MEKYQKATQN-----PL-----ENVDNVKESENAIPSNLQAF--KSL
 S.c. Dam1 MSE---DKAKLGTTSATEYRLIGSAPTSRRSSMGSSSLMKFADQGLTSSVGYENNTIQQLLPKI
 N.s. Dam1 MPDSQTPAPQRTSRPTNPTPLRPSSRS-----LRASRESIHGSSASF---PLNAFE-PAF
 G.z. Dam1 MSMTPKDAKRPNRSRTS-RPTTLPRLPSSRS-----FRDSARSAH-HDAFF---PLNAFE-PAF
 E.n. Dam1 MDAA--S---RSSSRRAVSRTTLPRLPSSRS-----VREAHGYDKRISVPPYTPAINALE-PQF
 A.o. Dam1 MDPTAFS---RSSSRPRPSSRTTLPRLPSSRS-----LREAHGYGGSISNAGYTQAINALE-PQF

S.p. Dam1 AVLDDNVSEFRKRMHLSATQILDNFNMFSSFLYGLQINAFVVDYENAP--LSESL-
 S.c. Dam1 RELSDSIITLDSNTRNLNFIHESLADLNESLGLLYGLMNSWCVEFSQAPHDQDDLAIKQLKSLDE
 N.s. Dam1 AELADAMLEANMHFOLMHESLARFSDFASFLYGLNMNAFCVDFPEGP--LTSFK-----
 G.z. Dam1 AELSDAMADLETNMHFMHESLARFSDFASFLYGLNMNAFCVDFPEGP--VPESFR-----
 E.n. Dam1 ADLADRMAELEANFDKQLMHESLRFSEFASFLYGLNMNAFCVDFPEA-----
 A.o. Dam1 AELADSMADLEANFMHLMHESLRFSEFASFLYGLNMNAFCVDFPEA--IPESFK-----

S.p. Dam1 -----LQAKKDPQKATLMTRTG--HSISDPPYDGGV-----ISHDPN-----FAT
 S.c. Dam1 KNNLVHLSNMERGIKKRDEQENDLAKASQNKQFNQPLFPSSQVKYRSYDNRDKRPSKIGNNLQVE
 N.s. Dam1 -----RMKEKEVREAAAGGGGQNNMETGVMGTGTGMMAVTKQERE-----GFD
 G.z. Dam1 -----RDRTRQEQPAVTPSKSEADAEM-----
 E.n. Dam1 -----EEEEESAK-----GNE
 A.o. Dam1 -----RAKQAEAKGKINV-----TLHLME--LYGTNTLQAEVETQTRQ-----ANE

S.p. Dam1 ADETF---ATNDTSFIERPETY-----SAS-----
 S.c. Dam1 NEEDYEDDTSSEAFVLNPTNIGMSKSGHVTKTLRLNNTNSKLRKRLTLTIRNSLQAGADLPEND
 N.s. Dam1 GETTF---MTDTSFVNDPTSN-----ISGTPRKPLVAPKTPAPRQSRVPAPSG--TTRGT-----
 G.z. Dam1 ---TF---MTDGSFVENPPT-----TTKTSKFTTPEPKRQSRPLAAPGRGTTARG-----
 E.n. Dam1 ADMTF---MTSDTTFIEHPST-----VS-----TKQVFK-PGTSRGRDCGNF-KGADGALK-----
 A.o. Dam1 GETTF---MTDTSFVENPST-----TP-----PKPVRKPTSSRGTRTGSSTGRGTYTRG-----

S.p. Dam1 -----R
 S.c. Dam1 NVNVLGDLHPNNRIIGSGAARVVNGPVTKNNRSMFSGRAERKPTESRHSVAKTEKKINTRPFFR
 N.s. Dam1 -----SGRGVGRVTPSSRTTSRGAITRSTSNRGVGG-----KGLR
 G.z. Dam1 -----STRGVGR-----RPSGLARARG-----RGVR
 E.n. Dam1 -----SSRAI-----KTPFGRRATLDDFCLSH-----HGAL
 A.o. Dam1 -----TSRAR-----PSALPRGRGLR

<i>S. cerevisiae</i>	Site	<i>S. pombe</i>	Kinase (<i>S. cerevisiae</i>)	Regulatory role (<i>S. cerevisiae</i>)
S20 (-RLSIG-)		Not conserved		Microtubule binding
S257 (-RKSLI-)		Not conserved	Ipl1	
S265 (-RNSIA-)		Not conserved		Co-operativity with Ndc80 complex
S292 (-RLSIG-)		Not conserved		
S218 (-TSSEA-)		Not conserved	Mps1 (<i>in vitro</i>)	Kinetochore association with microtubule plus-end
S221 (-EASFV-)		S143? (-DTSFI-)		

Fig. 2 Alignment of Dam1 homologues from different fungi. Dam1 from *S. pombe*, *S. cerevisiae*, *Neurospora crassa*, *Gibberella zeae*, *Aspergillus nidulans* and *Aspergillus oryzae*. Yellow circles denote Ipl1-dependent phosphorylation sites. Blue circles highlight characterised putative Mps1 sites. Table indicates conservation of

Ipl1 and putative Mps1 phosphorylation sites between *S. cerevisiae* and *S. pombe*. The long C-terminus of *S. cerevisiae* Dam1 is absent in *S. pombe*, and none of the Ipl1 sites are conserved.

with temperature-sensitive *ipl1* mutants. Changing the same sites to aspartic acid, in an attempt to mimic constitutive phosphorylation, partially rescues the temperature sensitivity of the same *ipl1* mutant (Cheeseman et al. 2002).

Phosphomimetic DASH complexes generated in vitro (hereafter referred to as 4D DASH) show no significant change changes in DASH monomer structure. However, they show a much-reduced tendency to oligomerise into rings around microtubules: At concentrations where oligomeric DASH rings are normally common, 4D DASH typically remains as monomers and dimers (Wang et al. 2007).

DASH complexes phosphorylated by purified Ipl1 also show reduced affinity for microtubules. In this assay, changing the N-terminal Ipl1 site of Dam1 to Alanine (S20A DASH) reversed this effect. Ipl1-phosphorylated S20A DASH was indistinguishable from unphosphorylated DASH in its microtubule binding kinetics, demonstrating that phosphorylation of Dam1 at Serine 20 alone regulates microtubule binding (Gestaut et al. 2008).

Ipl1 phosphorylation of Dam1 also regulates the interaction between DASH and the Ndc80 complex. 4D DASH binds less well to Ndc80 than 4A or wild-type DASH in in vitro pull-downs or by yeast two-hybrid (Shang et al. 2003). Unphosphorylated S20A

DASH enhances microtubule tip tracking by the Ndc80 complex in vitro. However, Ipl1 phosphorylated S20A DASH does not (Tien et al. 2010). As there is no phosphorylation at Serine 20, DASH phosphorylation by Ipl1 is not regulating DASH association with microtubules in this experiment. Ipl1 phosphorylation of the three C-terminal phosphorylation sites of Dam1 must therefore be regulating the interaction between DASH and Ndc80.

Phosphorylation of Dam1 in *S. cerevisiae* is opposed by the PP1 phosphatase Glc7 (Pinsky et al. 2006a). PP1 phosphatases are targeted to kinetochores and have been shown to regulate chromosome bio-orientation, congression and spindle checkpoint silencing (Pinsky et al. 2009; Vanoosthuysen and Hardwick 2009; Kim et al. 2010; Liu et al. 2010). Dam1 phosphorylation by Ipl1 requires microtubules, but is reduced in metaphase. In the absence of sister chromatid cohesion, and therefore inter-kinetochore tension, Dam1 phosphorylation persists (Keating et al. 2009). The tension-dependent mechanism of Ipl1-dependent phosphorylation at the kinetochore remains unknown. However, Ipl1 phosphorylation of Dam1 presents a useful paradigm for Aurora B-mediated correction mechanisms in other systems.

Interestingly, none of the four Ipl1 sites in *S. cerevisiae* are conserved in *S. pombe*. In part, this is

because *S. pombe* Dam1, along with other fungal Dam1 homologues, lacks the long C-terminal domain of *S. cerevisiae* Dam1 (Fig. 2). Phosphorylation of these sites is implicated in regulating DASH ring formation (Wang et al. 2007), so this may be a consequence of DASH not apparently forming rings in *S. pombe*. Mutations that change, or completely remove, the C-terminal 27 amino acids of *S. pombe* Dam1 confer resistance to thiabendazole (Sanchez-Perez et al. 2005; Griffiths et al. 2008). This suggests that the C-terminus of *S. pombe* Dam1 is important for DASH function. It would be of interest to see whether it represents a major site of DASH regulation, as in *S. cerevisiae*.

S. cerevisiae Dam1 is also phosphorylated in vitro by the kinase Mps1 (Shimogawa et al. 2006). Mps1 is required for spindle assembly checkpoint activity as well as SPB duplication in *S. cerevisiae* (Weiss and Winey 1996). Shimogawa et al. describe in vitro Mps1 phosphorylation of Dam1 at Serines 218 and 221, which are within a region conserved in *S. pombe* (Fig. 2), suggesting that this may represent a broadly conserved mechanism for regulating DASH. Mutating these sites to alanine appears to cause kinetochores to cluster nearer spindle poles during metaphase and alters the relative distributions of kinetochores and the microtubule plus end protein Bik1 (the budding yeast CLIP170 homologue). The authors conclude that Mps1 phosphorylation of Dam1 is required to link kinetochores to microtubule plus ends. As DASH appears to preferentially bind the microtubule plus end in the absence of phosphorylation in vitro (Westermann et al. 2005), and there is no direct evidence for Mps1 phosphorylation of Dam1 in vivo, the significance of this observation remains unclear. More work is needed to determine the effect of Mps1 phosphorylation on DASH in vitro, as well as determine its significance in vivo.

The only recorded phosphorylation of an *S. pombe* DASH component is phosphorylation of T136 in *S. pombe* Ask1 (Wilson-Grady et al. 2008). This threonine is part of a consensus Cdk1 phosphorylation motif. Ask1 is phosphorylated in a cell-cycle specific manner consistent with Cdk1 phosphorylation (Liu et al. 2005). *S. cerevisiae* Ask1 is also phosphorylated by Cdk1, although phospho-null mutants of these sites have comparatively mild phenotypes, suggesting that this is not an essential mechanism by which DASH is regulated (Li and Elledge 2003).

A non-fungal DASH analogue?

Given the observed role of DASH in facilitating microtubule tracking by the yeast kinetochore, and the lethality of DASH deletions in *S. cerevisiae*, it is perhaps surprising that no direct homologues of the DASH complex have been observed outside of fungi. The other essential microtubule-binding component of the kinetochore, the KMN network, is absolutely conserved from yeast to humans (Fig. 3).

Higher eukaryotes would not need a DASH complex if the KMN network alone were capable of linking kinetochores to dynamic microtubules in a processive manner. Purified *C. elegans* Ndc80 complex can link cargo to dynamic microtubules; however, much like *S. cerevisiae* Ndc80, the movement is not very processive (McIntosh et al. 2008). This suggests that the Ndc80 complex in higher eukaryotes still requires cofactors to allow processive interaction with microtubules. The need would be even more pressing in large cells such as human cells, given their size relative to the small yeast nucleus.

Electron tomography of microtubule ends at kinetochores in PtK1 cells reveals fine 'fibrils' that appear to extend from the inner kinetochore to the inside edge of splayed microtubule protofilaments. These kinetochore fibrils, which have not been reported in yeast, could facilitate attachment of dynamic microtubules to the kinetochore (McIntosh et al. 2008). In the absence of DASH, a fibrillar coupler could facilitate a processive kinetochore–microtubule attachment in concert with the KMN network. As yet, the identity of the fibrous coupler is unknown. McIntosh et al. (2008) suggest that CENP-E could perform such a role. CENP-E is a kinetochore-associated kinesin that is essential for chromosome congression. EM reveals that CENP-E dimers form long (~230 nm, similar to the longest observed fibrils) flexible fibres, due to a discontinuous coiled-coil separating its globular microtubule and kinetochore binding ends (Kim et al. 2008; McIntosh et al. 2008). A recent study has shown that Aurora B and PP1 cooperate to regulate CENP-E (Kim et al. 2010). Crucially, CENP-E is not conserved in yeast. As yet, however, there is no evidence that a CENP-E mediated interaction would allow processive microtubule tracking of the Ndc80 in the same way that DASH does.

Recently, the Ska complex was shown to share many properties with DASH and has been proposed

	<i>S. cerevisiae</i>	<i>S. pombe</i>	Human
Ndc80 Complex	Ndc80, Nuf2, Spc24, Spc25	Ndc80, Nuf2, Spc24, Spc25	Hec1, Nuf2, Spc24, Spc25
Mis12 Complex	Mtw1, Nsl1, Dsn1, Nnf1	Mis12, Nsl1, Dsn1, Nnf1	Mis12, Nsl1, Dsn1, Nnf1
Blinkin	Spc105	Spc7	Blinkin/KNL-1
DASH	Dam1, Duo1, Ask1, Spc34, Spc19, Dad1, Dad2/Hsk1, Dad3, Dad4/Hsk2, Hsk3	Dam1, Duo1, Ask1, Spc34, Spc19, Dad1, Dad2/Hos2, Dad3, Dad4, Dad5/Hsk3/Hos3	-
CENP-E	-	-	CENP-E
Ska	-	-	Ska1, Ska2, Ska3/Rama1

Fig. 3 Conservation of the core microtubule-binding site of the kinetochore between yeast and humans. Components of the KMN network are highly conserved; however, DASH is only

found in fungi. Schematic shows the major microtubule-binding components of the yeast kinetochore and their approximate relative location.

as a metazoan DASH analogue (Welburn et al. 2009). Accordingly, there are no Ska homologues in fungi (Fig. 3). Ska is a three-subunit outer kinetochore complex required for correct mitosis. All three subunits are required for Ska localisation and full activity (Gaitanos et al. 2009; Theis et al. 2009). Ska-depleted cells show prolonged checkpoint-dependent mitotic arrest and mitotic defects (Hanisch et al. 2006; Gaitanos et al. 2009). Interestingly, Ska complex binds cooperatively to microtubules in vitro and, in a striking parallel to the DASH complex, forms ring-like assemblies around microtubules (Welburn et al. 2009). As with DASH, there is no evidence as yet that these in vitro rings represent a functional form of Ska in vivo. In addition, Ska complex-coated beads can undergo microtubule-linked movement of similar duration to those observed by DASH-coated beads (Asbury et al. 2006; Welburn et al. 2009).

The role of Ska during mitosis is still controversial. Some studies report weaker kinetochore–microtubule interactions in Ska-depleted cells, which may be consistent with a role analogous to DASH (Gaitanos et al. 2009); however, other studies detect robust kinetochore–microtubule interactions in Ska-depleted cells (Daum et al. 2009). In these studies, the main effect reported is prolonged spindle checkpoint delay coupled with loss of sister-chromatid cohesion. The extent to which the observed defects in Ska-depleted cells represent the absence of a DASH analogue remains to be seen. The key question to be answered is whether Ska can facilitate processive Ndc80

attachments to dynamic microtubules like DASH. Ska can only be considered a ‘like-for-like’ DASH analogue if the functional cooperation with Ndc80 can be recapitulated using the Ska complex.

In vitro studies of kinetochore–microtubule interactions have focused on interactions of kinetochore components with single microtubules (McIntosh et al. 2008; Akiyoshi et al. 2010; Lampert et al. 2010; Tien et al. 2010). This is a good model for *S. cerevisiae* kinetochores, which capture and track a single microtubule throughout mitosis. Given the dire consequences of failing to correctly segregate even a single chromosome, this represents a ‘high stakes’ mode of kinetochore capture. The essential role of the DASH complex may be to maintain kinetochore–microtubule attachment, as even a single detached kinetochore will lead to chromosome missegregation. *S. pombe* kinetochores attach to small bundles of two to four microtubules; therefore, DASH-deficient kinetochores may completely detach from the spindle less frequently than in *S. cerevisiae*. Accordingly, DASH is not essential in *S. pombe*, but DASH-deficient cells display mitotic checkpoint delay, a slower anaphase A and high rates of chromosome missegregation (Sanchez-Perez et al. 2005; Saitoh et al. 2008). Mammalian kinetochores attach to larger bundles of 20 or more microtubules, and in this case, a single detachment event will have little consequence, so a direct DASH analogue may be unnecessary. Interestingly, a recent study shows that the Ska complex is not essential for survival in DT40 cells (Ohta et al. 2010).

Differences between fungal and metazoan Ndc80 complexes may account for the requirement for DASH at fungal kinetochores. Purified metazoan Ndc80 complexes bind cooperatively to microtubules in vitro (for a detailed review, see Tooley and Stukenberg, this issue) forming oligomeric ‘clusters’. Cooperative microtubule binding requires the N-terminus of the Ndc80 protein and is abrogated by mutations that mimic Aurora B phosphorylation (Alushin et al. 2010). The role of cooperative microtubule binding by Ndc80 has not been established. In their recent paper, Alushin et al. suggest that Ndc80 oligomers could track depolymerising microtubule ends by biased diffusion. Metazoan Ndc80 could therefore create a processive, Aurora B-regulated microtubule attachment in the absence of DASH. Presumably, bead-bound Ndc80 complexes do not exhibit this cooperativity due to steric hinderance by the beads.

Whilst the N-terminus of Ndc80 is essential for kinetochore–microtubule interactions in human cells, mutations in the N-terminus of *S. cerevisiae* Ndc80 have comparatively mild phenotypes (Miller et al. 2008; Akiyoshi et al. 2009; Kemmler et al. 2009). Alushin et al. assert that the fungal Ndc80 complex has diverged to require DASH to enhance cooperativity. They identified a region close to the proposed Ndc80 oligomerisation site that is highly conserved in metazoans, but less so in fungi. They suggest that this site may be required for microtubule-dependent oligomerisation. In addition, they identified a second site that is conserved in fungi, but not metazoans. They propose this site as a candidate for the interaction of Ndc80 with DASH (Alushin et al. 2010).

If fungal Ndc80 complexes lack the capacity to self-organise, then the essential role of DASH in *S. cerevisiae* could be to organise the microtubule attachment site once sister chromatid biorientation has been achieved. This is consistent with the observation that DNA-tethered DASH is sufficient to organise a ‘minimal’ kinetochore competent for chromosome segregation (Kiermaier et al. 2009; Lacefield et al. 2009). Since *S. pombe* DASH is non-essential, this hypothesis implies that *S. pombe* Ndc80 complexes may have retained some limited capacity to self-organise beyond that of *S. cerevisiae*. Alternatively, there could be additional factors required to facilitate Ndc80 oligomerisation in these cells.

Conclusion

Recent insights into DASH function have brought a fuller understanding of the complex interactions between kinetochores and microtubules in yeast. In particular, a recent work suggests that DASH functions in concert with the Ndc80 complex to create a highly regulated, processive link between chromosomes and the spindle in fungi. Only by understanding how different kinetochore subunits act together in this way can we fully appreciate how the dynamic interface between the spindle and chromosomes acts to ensure chromosomal stability.

Despite the recent leaps made in our understanding of DASH function, there are still many questions left unanswered. For technical reasons, the individual roles of different DASH subunits are largely unresolved, and so it remains something of a mystery why DASH is made of so many small individual subunits. Similarly, DASH is essential in *S. cerevisiae*, but in the only other species with a well-studied DASH complex *S. pombe*, DASH appears to have a less prominent role. As *S. pombe* DASH has not been reconstituted in vitro, the extent to which this represents two functionally different versions of the same complex remains unclear.

Arguably, the major question raised by recent insights into DASH function is how non-fungal eukaryotes maintain regulated interactions between the kinetochore and microtubule in the absence of DASH. This review has mentioned two possible candidates for aiding this connection, although there are many more. A major focus of future studies will undoubtedly be to determine if any of the proposed candidates can fulfil this role in higher eukaryotes, or if fungal kinetochores are fundamentally diverged from those of other eukaryotes. Doing so will greatly enhance our understanding of how chromosomal stability is maintained in human cells.

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