The KMN protein network – chief conductors of the kinetochore orchestra

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Summary
Successful completion of mitosis requires that sister kinetochores become attached end-on to the plus ends of spindle microtubules (MTs) in prometaphase, thereby forming kinetochore microtubules (kMTs) that tether one sister to one spindle pole and the other sister to the opposite pole. Sites for kMT attachment provide at least four key functions: robust and dynamic kMT anchorage; force generation that can be coupled to kMT plus-end dynamics; correction of errors in kMT attachment; and control of the spindle assembly checkpoint (SAC). The SAC typically delays anaphase until chromosomes achieve metaphase alignment with each sister kinetochore acquiring a full complement of kMTs. Although it has been known for over 30 years that MT motor proteins reside at kinetochores, a highly conserved network of protein complexes, called the KMN network, has emerged in recent years as the primary interface between the kinetochore and kMTs. This Commentary will summarize recent advances in our understanding of the role of the KMN network for the key kinetochore functions, with a focus on human cells.

Key words: Checkpoint, Kinetochore, KNL1, MIS12, Mitosis, NDC80

Introduction
Kinetochores are mega-molecular assemblies that are formed at the centromeres of chromosomes at the onset of mitosis (Fig. 1A). Extensive research over the past two decades has identified over 80 different proteins, in ten or more major complexes, that localize to human kinetochores (Fig. 1B). The KMN (named for the Knl1 complex, the Mis12 complex and the Ndc80 complex; see below) network is part of the protein architecture within kinetochores that links centromeric DNA to the plus ends of spindle microtubules (MTs) (Cheesman and Desai, 2008; Santaguida and Musacchio, 2009). The site where kinetochores are assembled is determined by the presence of a modified histone H3, CENP-A in humans (Cse4 in budding yeast), within nucleosomes at the periphery of each sister centromere (Fig. 1B). In addition to CENP-A-containing chromatin, the ‘inner’ domain of the kinetochore (Fig. 1B) contains a complex of peripheral centromeric proteins consisting of CENP-C, -H, -I, -K, -L, -M, -N, -O, -P, -S, -T, -U, -W and -X, called the ‘constitutive centromere-associated network’ (CCAN). A major function of CCAN is to link the MT-binding KMN network in the kinetochore ‘outer’ domain to centromeric DNA within the inner domain (Fig. 1B) (Hori et al., 2008; Hori and Fukagawa, 2012; Perpelescu and Fukagawa, 2011; Nishino et al., 2012; Saitoh et al., 1992; Takeuchi and Fukagawa, 2012). The kinase Aurora B, which localizes to centromeric chromatin before anaphase at high concentrations (Fig. 1B), has a central role in regulating the stability of kMT attachments and attachment error correction in concert with the KMN network (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009; Maresca and Salmon, 2010).

The highly conserved KMN network includes the Knl1 complex, which in vertebrates consists of KNL1 (also known as CASC5 or blinkin in humans, Spc105 in yeast and Spc105R in fly) and ZWINT; the Mis12 complex consisting of the four proteins MIS12 (Mtw1 or MIND in yeast), NSL1 (also called DC31 or MIS14), NNF1 (also known as PMF1) and DSN1 (also known as MIS13 in humans; KNL-3 in Caenorhabditis elegans); and the four-subunit Ndc80 complex comprising NDC80 (also known as HEC1), NUF2, SPC24 and SPC25 (Cheeseman et al., 2006; Cheeseman et al., 2004; DeLuca et al., 2006; Kops et al., 2005). The KMN network associates with kinetochores in prophase and disappears from kinetochores in telophase (Santaguida and Musacchio, 2009). The outer end of the Ndc80 complex is the primary binding site for the plus ends of spindle MTs (DeLuca and Musacchio, 2012; Gascoigne and Cheeseman, 2011; Joglekar et al., 2010; Santaguida and Musacchio, 2009; Tooley and Stukenberg, 2011), whereas the inner end is anchored either to the CCAN protein CENP-T (Hori and Fukagawa, 2012) or to the Mis12 complex (Petrovic et al., 2010). KNL1 also binds, at its outer end, to kMTs and, at its inner end, to the Mis12 complex (Fig. 1B) (Petrovic et al., 2010). The Mis12 complex is linked through CENP-C to CENP-A-containing chromatin (Fig. 1B) (Hori and Fukagawa, 2012). Both the Ndc80 complex and KNL1 have an elongated shape, extending outwards from their junctions with CENP-T or the Mis12 complex, and are oriented along the kMT axis at metaphase (DeLuca et al., 2006; Joglekar et al., 2009; Przewloka et al., 2011; Schittenhelm et al., 2009; Schittenhelm et al., 2007; Wan et al., 2009).

A number of other proteins within and at the periphery of the kinetochore outer domain depend on the presence of members of the KMN network for their kinetochore localization. These include MT-associated proteins in the proximity of kinetochore MT (kMT) plus-ends and members of the spindle assembly checkpoint (SAC) (Fig. 1B) (Musacchio and Salmon, 2007; Kops and Shah, 2012). The KMN network is also thought to associate...
with proteins that form the peripheral region of the outer
kinetochore, including fibrous proteins, such as CENP-F, and MT
motor proteins, such as the kinesin CENP-E and the cytoplasmic
dynein–dynein complex (Fig. 1B) (Cheeseman and Desai,
2008). The precise nature of the interactions between KMN
network and outer domain kinetochore proteins are only
just being unraveled. In this Commentary, we will discuss recent
advances in our knowledge of the structure and function of the
proteins associated with KMN network and their potential roles
in achieving the key functions of the kinetochore to ensure
accurate chromosome segregation. We begin with the inner-most
member of the KMN network, the Mis12 complex, then continue
to describe the structure and function of the other two
components, the Knl1 and Ndc80 complexes, and close with a
discussion on other proteins and mechanisms involved in
controlling the function of the KMN network.

The Mis12 complex
The Mis12 complex has been referred to as the kinetochore
‗keystone‘ complex, as it serves as a major platform for outer
kinetochore assembly (Cheeseman and Desai, 2008). The human
Mis12 complex is 22-nm long and rod shaped, with the subunits
linearly tethered to each other in the order NNF1, MIS12, DSN1
and NSL1, from inside to outside, within vertebrate kinetochores
(Fig. 2) (Maskell et al., 2010; Petrovic et al., 2010). Electron
microscopy analysis of the homologous yeast Mtw1 complex
assembled in vitro essentially yields a similar elongated structure,
although the yeast complex has been proposed to have a ‗comma‘
or bi-lobbed shape (Hornung et al., 2011; Maskell et al., 2010).
Both the yeast Dsn1 and Nsl1 subunits have been found to be
elongated, with them spanning much of the length of the entire
yeast complex. On the basis of in vitro protein assembly assays
using purified components of the human KMN network, it has
been found that the C-terminal tail of the NSL1 subunit is able to
attach to both SPC24 and SPC25, which are located at the inner
end of the Ndc80 complex, and to the C-terminus of KNL1
(Fig. 2) (Petrovic et al., 2010). A similar analysis of the yeast
Mtw1 complex suggests that the complex has a direct link to
Spc24 and Spc25 of the Ndc80 complex (Hornung et al., 2011).
Hence, the current evidence suggests that even though the human
and yeast Mis12 complexes might show variations in their
structure, they essentially perform very similar functions.

Although it has been shown that the inner end of the Mis12
complex interacts directly with CENP-C, it is not clear which of the
Mis12 complex subunits are involved in these interactions in
vertebrate cells (Screpanti et al., 2011). In Drosophila melanogaster,
it has been shown that the centromere-proximal Nnf1 subunit is
instrumental in the interaction between the Mis12 complex and
CENP-C (Przewloka et al., 2011). The yeast Mtw1 complex has also
been shown to associate directly with the yeast functional
homologue of CCAN, a Ctf19-containing complex called COMA
(Hornung et al., 2011). As CCAN in turn directly associates with
CENP-A-containing chromatin (Foltz et al., 2006; Okada et al.,
2006), the Mis12 complex is an integral part of the machinery that
connects the outer kinetochore to the inner centromeric DNA.
Initially isolated as a key component of the core MT attachment site,
Mis12 has not been shown to bind to purified MTs, even though it
has been reported to enhance the MT-binding affinities of the Ndc80
complex and of KNL1 (Cheeseman et al., 2006), and recent
evidence indicates that it interacts with the microtubule-associated
protein (MAP) complex Ska (Chan et al., 2012).
accurate chromosome segregation, sister centromere biorientation and separation (Goshima et al., 1999; Goshima and Yanagida, 2000). *Drosophila* embryos with mutations in Mis12 complex subunits also exhibit similar defects in chromosome segregation (Venkei et al., 2011). Interestingly, extensive investigations have failed to identify a Dsn1 homologue in *Drosophila*, and it has been proposed that this subunit has been functionally replaced in part by the fly Knl1 homologue, Spc105R, on which the other three subunits of the Mis12 complex depend for their localization to the kinetochore (Przewloka and Glover, 2009). In budding yeast, the V-shaped heterotetrameric monopolin complex, consisting of Mam1, Csm1, Lrs4 and Hrr25, has been shown to bridge the Dsn1 subunit of Mis12 with centromere-localized CENP-C and influence normal meiotic chromosome segregation (Corbett et al.,
2010), but it is not known if a similar complex exists in higher vertebrates. In addition to centromere-proximal CENP-A and CCAN, Mis12 has also been shown to require the chaperone complex Hsp90–Sgt1 for its appropriate targeting to kinetochores (Davies and Kaplan, 2010). Several studies have found that the Ns1 subunit interacts with the heterochromatin protein HP1, and that this association is important for the formation of the inner kinetochore (Kiyomitsu et al., 2010; Kiyomitsu et al., 2007; Obuse et al., 2004). However, many of the mitotic defects observed by the perturbation of HP1 could be attributed to improper kinetochore recruitment of the Ndc80 complex, as both HP1 and the Spc24 or Spc25 subunits of the Ndc80 complex have been shown to bind to the same site in the C-terminal of Ns1 and that their binding to Ns1 is competitive in nature (Petrovic et al., 2010). A recent study has also shown that the Dsn1 subunit of the Mis12 complex and Knl1 are targets for Aurora B kinase phosphorylation which reduces the MT-binding affinity of the KMN network (Welburn et al., 2010). This study provides evidence that the phosphorylation of Dsn1 sensitizes the KMN network towards dramatic changes in MT-binding activity.

In summary, the Mis12 complex is a key component of the KMN network that is required to properly target the other two MT-binding components, the Ndc80 complex and Knl1 to kinetochores. The main function of this complex is to connect the outer kinetochore region to the inner centromeric DNA in association with the proteins of the CCAN network.

The Knl1 complex

The Knl1 complex is a heterodimer of Knl1 (also known as CASC5 or blinkin in humans, Spc105 in yeast and Spc105R in fly) with ZWINT (Petrovic et al., 2010). Human Knl1 is a large 300-kDa protein that is recruited to kinetochores by the Mis12 complex (Fig. 2).

Knl1

Knl1 has been shown to have a role in the recruitment of several outer-domain kinetochore proteins, including its binding partner ZWINT, CENP-F and the mitotic checkpoint proteins BUB1 and BUBR1 (also known as BUB1B) (Cheeseman et al., 2008; Kiyomitsu et al., 2007). X-ray crystallographic and other studies have revealed that helical motifs, called the KI motifs (~10–12 amino acids long), in the N-terminal region of Knl1 interact with the TPR domains of BUBR1 and BUB1 and are important for maintaining normal SAC activity in human cells (Bolanos-Garcia et al., 2011; Kiyomitsu et al., 2011; Krenn et al., 2012). One of these studies has also demonstrated that the interaction between the KI motif of Knl1 and the TPR motif of BUB1 does not control the targeting of BUB1 to the kinetochore, but instead might influence the ability of BUB1 to interact with BUBR1, suggesting that Knl1 serves as a scaffold that brings together BUB1 and BUBR1 to help generate the kinetochore SAC signal (assembly of the mitotic checkpoint complex, Cdc20, Mad1, BubR1 and Bub3; Krenn et al., 2012).

Human mitotic cells that have been depleted of Knl1 are severely compromised in forming stable kMTs, and cells that enter anaphase often exhibit sister chromosome missegregation (Cheeseman et al., 2008; Kiyomitsu et al., 2007; Schittenhelm et al., 2009). In agreement with a role for Knl1 in the recruitment of BUB1 and BUBR1, both proteins are found to be displaced from kinetochores after Knl1 depletion (Kiyomitsu et al., 2007). Live imaging has revealed that these cells undergo accelerated mitosis and premature chromosome decondensation prior to the onset of anaphase (Kiyomitsu et al., 2007). Apart from recruiting BUB1 and BUBR1 to the kinetochore, Knl1 has also been shown to have a role in recruiting the Ndc80 complex and the RZZ checkpoint complex in C. elegans (Cheeseman et al., 2008; Essex et al., 2009; Gassmann et al., 2008). However, in human cells, NDC80 recruitment to kinetochores does not appear to require the function of Knl1 (Cheeseman et al., 2008; Kiyomitsu et al., 2007). Knl1 has also been shown to have MT-binding activity at its extreme N-terminus (Cheeseman et al., 2006; Espeut et al., 2012), and it has been shown in C. elegans that this is necessary for SAC activity but dispensable for the formation of proper kMT attachments and other aspects of chromosome segregation (Espeut et al., 2012). In that study, the authors expressed a mutant KNL-1 that lacked the MT-binding activity in C. elegans embryos, and found that they exhibit delays in anaphase onset and persistent checkpoint activation. Even though the amino acids at positions 1–500 are required for MT binding, the crucial sequences were narrowed down to a basic stretch of nine amino acid residues at the extreme N-terminus of the protein (Espeut et al., 2012).

The extreme N-terminus of Knl1 has also been shown to be important for the recruitment of protein phosphatase 1 (PP1) to outer kinetochores. PP1 activity counteracts the activity of the kinase Aurora B, which is involved in the destabilization of kMTs (by phosphorylating multiple sites within the KMN network), and consequently assists in accurate chromosome biorientation and alignment (Liu et al., 2010; Meadows et al., 2011; Rosenberg et al., 2011; Welburn et al., 2010). A recent study has suggested that the recruitment of PP1 to the kinetochore by Knl1 also contributes to SAC activity in an independent and possibly additive manner to that of its MT-binding activity (Espeut et al., 2012). This study further proposes that the MT-binding activity of Knl1 acts as a sensor of kMT attachment and relays this information to the checkpoint machinery for silencing of the SAC. More recently, three separate studies have shown that the yeast Knl1 homologue Spc105 is a target for the Mps1 (Mph1) checkpoint kinase and that this activity in turn is responsible for the recruitment of the Bub1–Bub3 checkpoint complex to kinetochores (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). One of these studies also suggests that this phosphorylation activity of Mps1 is opposed by the phosphatase activity of Knl1-recruited PP1 (London et al., 2012).

In summary, Knl1 is a large multi-domain and multi-functional scaffold protein that is required for kinetochore targeting of several other outer-domain kinetochore proteins, including its binding partner ZWINT, the SAC proteins BUB1 and BUBR1, CENP-F and possibly, the RZZ complex. In concert with the other constituents of the KMN network, Knl1 also serves important roles in kMT attachment and silencing the SAC.

ZWINT

ZWINT (KBP-5 in C. elegans) is a 277-amino-acid kinetochore protein that initially has been implicated in the targeting of ZW10 to kinetochores (Starr et al., 2000) (Fig. 2). ZW10 is part of another outer kinetochore complex, the RZZ complex, which also contains the two proteins ROD (also known as KNTC1) and ZWILCH (Karess, 2005). The RZZ complex in turn recruits the adaptor protein Spindly that serves to attach the dynein–dynactin motor complexes to checkpoint proteins such as the MAD1–MAD2
complex (Gassmann et al., 2008; Grifﬁs et al., 2007). A role for ZWINT in the recruitment of the RZZ complex to kinetochores has been conﬁrmed in subsequent studies (Kops et al., 2005; Lin et al., 2006; Wang et al., 2004). Surprisingly, ZWINT was found to co-puriﬁed with the components of the KMN network, but not with the RZZ complex (Cheeseman et al., 2006; Cheeseman et al., 2004; Kops et al., 2005). Recently, ZWINT, whose structure has coiled-coil motives, has been demonstrated to form a tight complex with the C-terminal coiled-coil domain of KNL1 (Petrovic et al., 2010), and this might explain reports of interactions between ZWINT and MIS12 (Obuse et al., 2004) and the NDC80 (HEC1) subunit of the Ndc80 complex (Lin et al., 2006; Vos et al., 2011). Similar to the other constituents of the KMN network (Santaguida and Musacchio, 2009), it has also been shown that ZWINT is a stable component of the kinetochore, whereas the RZZ complex is more dynamic in its residency at metaphase kinetochores (Fanmuls et al., 2008).

ZWINT is also known to be a substrate for phosphorylation by the Aurora B kinase. It has been proposed that this phosphorylation is required for recruitment of the RZZ complex and the dynein motor to kinetochores, which in turn is important for chromosome motility and SAC signaling (Fanmuls and Chan, 2007; Kasuboski et al., 2011). Very recently, a potential functional homologue of ZWINT has been identiﬁed in ﬁssion yeast as a binding partner for the yeast KNL1 homologue Spc105 (Jakopec et al., 2012).

The Ndc80 complex

A number of extensive reviews have discussed the structure and function of the Ndc80 complex (see Alushin and Nogales, 2011; DeLuca and Musacchio, 2012; Joglekar et al., 2010; Lampert and Westermann, 2011; Takeuchi and Fukagawa, 2012), and this Commentary will thus only attempt to summarize the recent advances in our understanding of this complex. The Ndc80 complex is a 57-nm-long heterotetrameric complex consisting of two dimers, NDC80 (HEC1) and NUF2, and SPCC4 and SPCC25, that are held together by overlapping α-helical coiled coil domains located at the C-terminus of the NDC80–NUF2 dimer and the N-terminus of the SPCC4–SPCC25 dimer (Cheeseman and Desai, 2008; Ciferri et al., 2008; Wei et al., 2007). The C-terminus of SPCC4–SPCC25 interacts with both CENP-T in humans (Gascoigne et al., 2011) and budding yeast (Bock et al., 2012; Schleiff er et al., 2012), or with the NSL1 and DSN1 components of the Mis12 complex in humans and Dro sophila (see above).

Studies that have analyzed the various predicted MT-binding domains within the Ndc80 complex during mitosis suggest that both the NDC80 CH domain and its charged N-terminal tail are important for the formation of stable kMT attachments and for chromosome alignment, whereas the CH domain of NUF2, although it is non-essential for these functions, is required for producing normal MT-dependent kinetochore force and timely mitotic progression (Sundin et al., 2011; Tooley et al., 2011). Furthermore, it has been demonstrated that the N-terminal tail of NDC80 is required to interact with the negatively charged C-terminal tails (the so-called E-hooks) of tubulin monomers (Alushin et al., 2010; Ciferri et al., 2008; Tooley et al., 2011). These interactions are weakened by phosphorylation of the nine Aurora B target sites within the N-terminal tail of NDC80 (Cheeseman et al., 2006; DeLuca et al., 2006; Santaguida and Musacchio, 2009; Wei et al., 2007). It has been demonstrated in vivo that Aurora B kinase activity is important for destabilizing kMT attachment and correction of MT-attachment errors, which is highly relevant for the fidelity of mitotic chromosome segregation (Cheeseman et al., 2006; Cimini et al., 2006; DeLuca et al., 2006; Sandall et al., 2006; Welburn et al., 2010). Studies with reconstituted protein preparations in vitro show that dephosphorylated NDC80 bound to beads can track, stabilize and promote rescue of the ends of depolymerizing MTs, whereas Aurora B phosphomimetic versions of the complex are unable to do so, further emphasizing the importance of Aurora B phosphorylation for the function of the Ndc80 complex (Umbreit et al., 2012).

Structural studies have provided important details of MT binding by the Ndc80 complex (Wilson-Kubalek et al., 2008; Alushin et al., 2010). A key MT-binding region is located within the CH domain of NDC80 at a site termed ‘toe print’. The ‘toe’ serves as a sensor of the tubulin conformation that enables the Ndc80 complex to bind to straight MT protoﬁlaments with high afﬁnity and to curled protoﬁlaments at the ends of depolymerizing MTs with low afﬁnity. The Alushin et al. study and two other studies have found that the Ndc80 subunit NUF2, which also contains a CH domain, is not involved in the interaction with MTs (Sundin et al., 2011; Alushin et al., 2010; Wilson-Kubalek et al., 2008), despite a previous report showing that mutations within the NUF2 CH domain interfere with the binding of the Ndc80 complex to MTs (Ciferri et al., 2008). Additionally, Alushin et al. provide evidence that the unstructured N-terminal tail of NDC80 aids in tethering together adjacent Ndc80 complexes upon MT binding, an idea that has been supported by several other studies (Cheeseman et al., 2006; Ciferri et al., 2008; Powers et al., 2009; Tooley et al., 2011). Another recent study dissected the role of the tail domain in MT binding and Ndc80 clustering in even more detail (Alushin et al., 2012). This study shows that Aurora B sites are organized as two separate segments or zones in the tail region, one at the tubulin-binding interface and the other at the interface with an adjacent MT-bound Ndc80 complex; phosphorylation at the different sites serves to fine tune the interaction of the Ndc80 complex with tubulin monomers and with neighboring Ndc80 complexes.

The CH domain of NDC80 is also important for recruiting spindle checkpoint proteins such as the MAD1–MAD2 complex (Guimaraes et al., 2008, Martin-Lluesma et al., 2002, Miller et al., 2008). In addition, serine 165 within the NDC80 CH domain is a target for the kinase NEK2, and this phosphorylation is important for chromosome alignment and the SAC (Wei et al., 2011). Cells expressing a non-phosphorylatable mutation of NDC80 at serine 165 are unable to recruit MAD1–MAD2 to kinetochores and exhibit premature anaphase onset with erroneous chromosome segregation. Phosphorylation of NDC80 and the Ndc80 complex by Aurora B have also been shown to be important for the recruitment of the checkpoint kinase MPS1 (also known as TTK in vertebrates) to kinetochores, leading to its activation, which is essential for the SAC to prevent anaphase (Vigneron et al., 2004; Hewitt et al., 2010; Santaguida et al., 2010; Maciejowski et al., 2010).

Taken together, the heterotetrameric Ndc80 complex emerges as the major MT-binding interface at kinetochores and uses both the N-terminal CH domain and tail domains of its Ndc80 subunit to bind MTs. Phosphorylation of key amino acid residues at the N-terminal tail by Aurora B kinase negatively influences MT-binding
affinity and serves as an effective mechanism to regulate the strength of kMT attachment and the process of attachment error correction. The N-terminal CH domain also plays a role in recruiting MAD1, MAD2 and Mps1 to kinetochores.

**Further insights into KMN network function**

**Dam/Dash and Ska MAP complexes**

Another active area of research has been to address the pivotal question of how the activity of the KMN network is coupled to the polymerization and depolymerization dynamics of kMT plus-ends by MAPs that potentially interact with the Ndc80 complex or the KMN network and are required for effective kMT attachments and force generation (Joglekar et al., 2010). In yeast, the Dam complex (also known as the Dash complex) is a MT-binding protein that depends on the kinetochore-bound Ndc80 complex for its localization to kinetochores. The Dam/Dash complex is composed of 10 subunits that assemble into oligomers and form either closed stable ring-like structures or open spirals around the MT lattice (Lampert and Westermann, 2011). Dam/Dash has been shown to increase the processivity of the Ndc80 complex when it is attached to depolymerizing MTs, and is also a prominent target for the yeast homologue of Aurora B kinase, Ip1 (Alushin and Nogales, 2011, Grischuk et al., 2008; Lampert and Westermann, 2011). Structural homologs of the Dam/Dash complex have not been discovered in higher eukaryotes, but recent studies are beginning to shed light on other MAPs that interact with the Ndc80 complex or the KMN network. In human cells, the kinetochore-localized Ska complex has emerged as one of the major modes of these regulatory functions as discussed below.

The Ska complex was initially identified in human cells as a complex comprising two proteins SKA1 and SKA2. SKA1 and SKA2 were found to localize to spindle MTs and to concentrate at kinetochores in a manner that is dependent on kinetochore-bound NDC80 (Hanisch et al., 2006). Subsequently, a third subunit of the complex, SKA3, also called RAMA1, was discovered that localizes to kinetochores and spindle MTs, and knockdown of this protein results in identical phenotypes to those upon knockdowns of SKA1 and SKA2 (Daum et al., 2009; Gaitanos et al., 2009; Raaijmakers et al., 2009; Theis et al., 2009; Welburn et al., 2009). SKA3 is required for the proper stability and functioning of the Ska complex as a whole (Gaitanos et al., 2009). In human cells, the Ska complex is reported to be important for normal kMT formation, metaphase alignment, SAC silencing and anaphase chromosome segregation (Daum et al., 2009; Gaitanos et al., 2009; Hanisch et al., 2006; Raaijmakers et al., 2009; Theis et al., 2009; Welburn et al., 2009). The human Ska complex has been reconstituted in vitro and shown to bind MTs in a cooperative manner (Welburn et al., 2009). Moreover, that study, as well as more recent data (Schmidt et al., 2012), have shown that the Ska complex can form oligomeric assemblies that diffuse along MTs and that it binds to...
depolymerizing MT plus-ends along both straight and curved MT protofilaments (the Ndc80 complex binds only straight protofilaments near MT ends). The purified Ska complex exhibits processive motion at the ends of depolymerizing MTs and strengthens plus-end tracking of the purified Ndc80 complex. Moreover, the Ska complex enhances the capacity of the Ndc80 complex to bind MTs in a cooperative manner. These properties of the Ska complex, together with the requirement of MTs and the Ndc80 complex for its localization to kinetochores, suggests that the Ska complex could be a functional (but possibly not structural) homologue in vertebrates of the Dam/Dash complex in yeast (Gaitanos et al., 2009; Hanisch et al., 2006; Welburn et al., 2009; Schmidt et al., 2012).

There is also evidence that the Ska complex interacts with KNL1 and the Mis12 complex, in addition to the Ndc80 complex, and that the recruitment of Ska to kinetochores depends on all three members of the KMN network (Chan et al., 2012). That study also showed that SKA1 and SKA3 are targets for phosphorylation by Aurora B and that their phosphorylation negatively regulates the association of the Ska complex with the KMN network, which in turn inhibits the recruitment of the Ska complex to kinetochores and impairs its role in stabilizing kMT attachments.

Taken together, these studies show that the Ska complex works in close concert with the components of the KMN network, and the Ndc80 complex in particular, to provide stability to kMT attachments unless it is phosphorylated by Aurora B. Recent evidence also indicates that Ska is indeed a vertebrate functional homologue of the yeast Dam/Dash complex, but the finer details of how Ska associates with the KMN network and the MT lattice to fulfill this function are far from being resolved. Also unresolved is whether there are additional activities for the Ska complex in controlling mitotic progression, such as has been proposed by Daum et al. (Daum et al., 2009), for preventing the premature loss of sister chromatid cohesion. Further insights that can be gained from the Ska structure are presented in Box 1.

The role of the NDC80 loop domain

There is a prominent kink or bend in the structure of the Ndc80 complex ~16 nm away from the MT-binding CH domain of NDC80. The location of the kink coincides with a break in registry of the central coiled-coil region within NDC80, which likely produces a loop in the secondary structure at this site (Wang et al., 2008). The sequence encompassing the loop was found to be evolutionarily conserved from yeast to human

Box 2. Function of the human NDC80 loop domain

Recent studies in human cells on the role of the KMN network in kMT attachment have found that the loop domain of NDC80 (HEC1) is instrumental in stabilizing kMTs attachments (see figure) (Zhang et al., 2012; Varma et al., 2012; Matson and Stukenberg, 2012). Several lines of evidence suggest that the Ndc80 complex is slightly bent with a centrally located loop region that provides flexibility in complex confirmation. Both the CH domain and the N-terminal tail of NDC80 (see figure, shown in red) and the extreme N-terminus of KNL1 bind to MTs. In addition, the central loop region of NDC80 provides a third MT-binding site within the Ndc80 complex, albeit indirectly. Linkage from the loop domain to kMTs requires MAPs, such as Dis1 and Dam1 (fission yeast), the Dam/Dash complex (budding yeast), or possibly the Ska complex (human cells). In human cells, the DNA replication licensing protein CDT1 also binds to the loop region and is required for robust kMT attachment (see below). We only have limited knowledge about the exact location and orientation of these proteins relative to kMTs, and their relative positions within the kinetochore are not depicted accurately in the figure.

Sequence modifications in the loop region have shown that this region is important for the recruitment of other proteins that are required for normal kMT attachments. Zhang et al. found that the Ndc80 complexes assembled in the absence of the loop region are unable to associate properly with the Ska complex (Zhang et al., 2012). They proposed that the loop domain directly recruits the Ska complex to kinetochores, and that the absence of the loop-recruited Ska complex led to the observed defects in MT attachments to kinetochores in mitotic cells with loop mutations. A second study, by our group, presented a new mechanism for the stabilization of kMT attachments (Varma et al., 2012). We identified the DNA replication licensing protein CDT1 as a novel kinetochore protein that interacts with the Ndc80 complex. Inhibition of CDT1 function specifically during late prometaphase arrest with severe defects in kMT attachments (Varma et al., 2012). The localization of CDT1 to kinetochores is dependent on the loop region of NDC80, and scrambling the loop region sequence induces a mitotic arrest that is similar to that observed when the loop is missing (Zhang et al., 2012) or when CDT1 is inhibited (Varma et al., 2012). Detailed analysis of the structure of the Ndc80 complex suggests that the binding of CDT1 to the loop region of kinetochore-bound NDC80 helps to maintain an extended confirmation of the Ndc80 complex in the presence of kMTs, which aids in more robust kMT attachments. Intriguingly, our study did not observe a significant decrease in the concentration of the Ska complex at kinetochores when the NDC80 loop region was mutated.
(Wang et al., 2008), but until very recently its function was not clear.

Two studies in yeast determined that the loop region is important for stable kMT attachment. The first study, in *Saccharomyces cerevisiae*, showed that the loop region is important for the recruitment of the Dam/Dash complex to kinetochores and possibly for its loading on to kMTs (Maure et al., 2011). Those authors observed that, when the loop region is deleted or mutated, there is a severe defect in the conversion of lateral to load-bearing (i.e. end-on) kMT attachments and kinetochore bi-orientation. The other study in the fission yeast *Saccharomyces pombe*, which also using deletion or directed mutation of key residues or sequences within the loop region, showed that this region is important for the recruitment of the MAP Dis1 (TOG or XMAP215) to kinetochores with stable end-on attachments to spindle MT ends (Hsu and Toda, 2011). Consequently, the SAC remains persistently activated in cells with mutated loop domains and they undergo prolonged mitotic arrest. Deletion of the Dis1 gene phenocopies the defects seen with loop mutations and was rescued by the artificial targeting of Dis1 to the Ndc80 complex.

These studies, along with recent studies in human cells (see Box 2), support the notion that in addition to the CH domain and N-terminal tail of NDC80, its loop domain also constitutes a MT-binding interface, thus providing a total of three different MT-binding domains within the Ndc80 complex.

**Concluding remarks and future directions**

Future work will help to delineate the connections between the different components of the KMN network and the SAC (including the Mads, the Bubs, and the RZZ). We already know that the CH domain of NDC80 is involved in recruiting MAD1, and that KNL1 is required for recruiting ZWINT, BUBR1 and BUB1, as well as the RZZ complex in *C. elegans* (Gassmann et al., 2008). Both ZWINT and the Ndc80 complex recruit the RZZ complex, which in turn appears to be a prerequisite for the recruitment of MAD1 and MAD2 (Fig. 3). However, we still lack precise knowledge of how MT attachments trigger dynein-mediated removal of SAC components (‘stripping’) and silencing of the checkpoint, or how the checkpoint feeds back into the attachment machinery of the KMN network. Part of the problem lies in the fact that the association of checkpoint proteins with the KMN network are possibly highly transient, which makes their biochemical analysis exceedingly difficult. We are slowly beginning to understand some of these mechanisms as shown by recent work that suggests that MT-binding by Knl1 is likely to be a key sensor for controlling checkpoint activity (Espeut et al., 2012), but more work is mandatory to elucidate the intricacies of the interplay between MT attachment and SAC and control mechanisms governed by Aurora B, MPS1 and PP1. As Knl1 is a large multi-domain protein, progress in our understanding of its structure and function has been relatively slow and more research in this direction is likely to be worthwhile. Although we know to some extent the functions of the N- and the C-termini of Knl1, its central region remains a ‘black box’ and analysis of this region is likely to yield valuable information of its role in the SAC and kMT attachment. An area, in which there is a high chance for immediate progress is the further study of the function of the loop in the Ndc80 complex and the proteins that interact with it. Research efforts in this direction will help us to understand if the Ska complex is indeed an orthologue of Dam/Dash and how exactly the Ska complex and the KMN network coordinate kMT attachment, force generation, attachment error correction and control of the SAC. Future studies should also shed light on the exact molecular mechanism by which the replication licensing protein CDT1 is able to influence the conformational change of Ndc80 complex in the presence of kMTs (see Box 2), and also elucidate whether different Ndc80 loop-binding proteins act in concert to accomplish stable kMT attachments.

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**Note added in proof**

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