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The kinetochore interaction network (KIN) of ascomycetes

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Abstract: Chromosome segregation relies on coordinated activity of a large assembly of proteins, the kinetochore interaction network (KIN). How conserved the underlying mechanisms driving the epigenetic phenomenon of centromere and kinetochore assembly and maintenance are remains unclear, even though various eukaryotic models have been studied. More than 50 different proteins, many in multiple copies, comprise the KIN or are associated with fungal centromeres and kinetochores. Proteins isolated from immune sera recognized centromeric regions on chromosomes and thus were named centromere proteins (CENPs). CENP-A, sometimes called centromere-specific H3 (CenH3), is incorporated into nucleosomes within or near centromeres. The constitutive centromere-associated network (CCAN) assembles on this specialized chromatin, likely based on specific interactions with and requiring presence of CENP-C. The outer kinetochore comprises the Knl1-Mis12-Ndc80 (KMN) protein complexes that connect CCAN to spindles, accomplished by binding and stabilizing microtubules (MTs) and in the process generating load-bearing assemblies for chromatid segregation. In most fungi the Dam1/DASH complex connects the KMN complexes to MTs. Fungi present a rich resource to investigate mechanistic commonalities but also differences in kinetochore architecture. While ascomycetes have sets of CCAN and KMN proteins that are conserved with those of budding yeast or metazoans, searching other major branches of the fungal kingdom revealed that CCAN proteins are poorly conserved at the primary sequence level. Several conserved binding motifs or domains within KMN complexes have been described recently, and these features of ascomycete KIN proteins are shared with most metazoan proteins. In addition, several ascomycete-specific domains have been identified here.

Key words: CCAN, centromere, Dam1, histones, kinetochore, KMN, *Neurospora crassa*, *Zymoseptoria tritici*

Introduction

The faithful transmission of chromosomes to daughter cells relies on a large assembly of proteins, the kinetochore interaction network (KIN), yet after more than 30 y of intense research it is still unclear whether the mechanisms driving this inherently epigenetic phenomenon in various eukaryotes are substantially different. Constricted, specialized sites of spindle attachment on chromosomes during mitosis first were described more than 130 y ago (Flemming 1882). In the 1930s two terms appeared, centromere and kinetochore, to describe these constrictions and attachment point of spindles (Cheeseman and Desai 2008, Fukagawa and Earnshaw 2014). Genetically these regions are associated with reduced meiotic crossing over (Beadle 1932) and over time the centromere, as a genetic locus, became synonymous with a stretch of DNA. This perception intensified after identification and cloning of the budding and fission yeast centromeric DNA (Clarke and Carbon 1980, Clarke et al. 1986) and the first proteins that associated with these specific DNA sequences (Earnshaw and Migeon 1985). Since then centromere has been used regularly to describe DNA-associated features of chromatin while kinetochore is now understood to mean a large protein complex that assembles on the centromere but does not contain DNA (Fukagawa and Earnshaw 2014). How exactly functional centromeres and kinetochores are maintained and inherited remains one of the fundamental questions in cell biology, biochemistry and biophysics.

More than 50 different proteins that are associated more or less stably with fungal centromeres and kinetochores comprise the KIN (Fig. 1). The first centromere proteins (CENPs) were identified in immune serum from individuals with scleroderma because antibodies from these patients specifically recognized centromeric regions (Moroi et al. 1980). Immunoprecipitation performed with these antibodies resulted in the identification of three proteins, CENP-A, CENP-B and CENP-C (Earnshaw and Migeon 1985). CENP-A is incorporated into nucleosomes within centromeres, and the constitutive centromere-associated network (CCAN) builds the foundation or inner kinetochore layer directly contacting centromeric DNA and the specialized CENP-A-containing nucleosomes. In yeasts this network initially was defined by functional genetic studies and divided into several subcomplexes (Meraldi et al. 2006). In metazoans five homologous complexes were identified (CENP-C, CENP-H-I-K, CENP-L-M-N, CENP-O-P-Q-R-U, CENP-T-W-S-X) by extensive biochemistry (Foltz et al. 2006, Okada et al. 2006, Nishino et al. 2012;). The outer kinetochore of metazoans, the Knl1-Mis12-Ndc80 (KMN) network (Cheeseman et al. 2006), contains at

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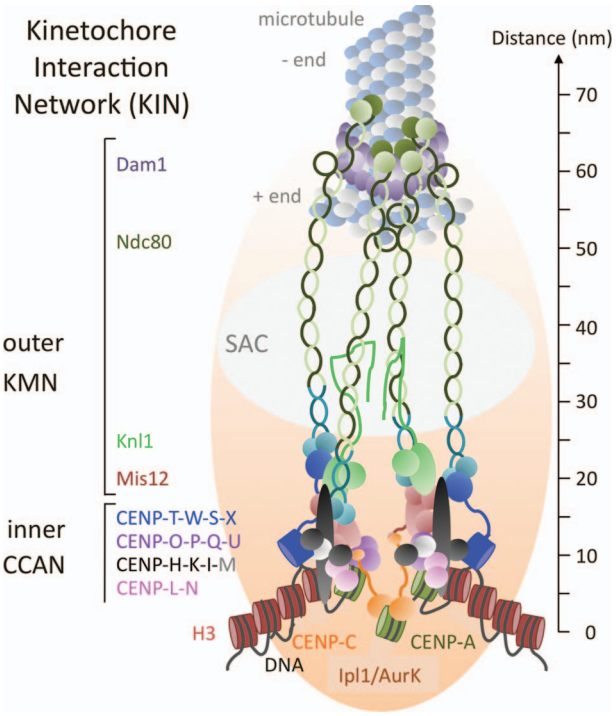

FIG. 1. Working model of the kinetochore interaction network (KIN). KIN components found in most fungi are shown and depicted roughly to scale (based on data compiled from the various sources cited in the text). Complexes are divided into the outer kinetochore (Knl1-Mis12-Ndc80, or KMN network and Dam1 complex) and the inner kinetochore (constitutively centromere-associated network; CCAN). Microtubule (MT) plus (+) ends are bound by the 10-component, fungal-specific Dam1 complex (purple). The Ndc80 and Nuf2 components of the Ndc80 complex (light and dark green) stretch across the Dam1 complex and bind to the MT. Four to 14 Ndc80 complexes are estimated to bind to one MT. Two Ndc80 subunits, Spc24 and Spc25 (light and dark teal) make connections to either the Mis12 or CENP-T complexes, respectively. Mis12 also binds to Knl1, a large protein that serves as an interaction and phosphorylation platform for the spindle assembly checkpoint (SAC). It is unclear whether filamentous ascomycetes have Kre28 or Zwint homologs that bind to Knl1. The rod-shaped Mis12 complex (light brown) integrates binding of CENP-C (orange) and the CENP-O-P-Q-U (purple) complex and thus connects centromeric chromatin, generated by assembly of CENP-A/CenH3 (green) nucleosomes and binding of CENP-L-N (pink), to the Ndc80 complex. CENP-H-K-I (black) form a complex when bound by CENP-M (gray); no homolog of CENP-M has been found in fungi by sequence similarity alone. CENP-H binds to CENP-C and helps to assemble CENP-T-W-S-X (blue) to form a second linkage to the Ndc80 complex by binding of CENP-T to Spc24/Spc25. Assembly of the CENP-C arm is dependent on the activity of Ipl1/aurora B kinase and the gradient it generates from the inner chromatid faces towards the outer kinetochore. Several aspects of this model are speculative and based on testable hypotheses. It is still unclear if CENP-T-W-S-X assembles into

least three well-defined complexes that build the bridge from chromatin to spindles, by stabilizing microtubule (MT) attachment and generating load-bearing assemblies for chromatid segregation. The KMN network also controls the spindle assembly checkpoint (SAC), a signal transduction process that involves cell cycle regulators and prevents premature exit from mitosis when kinetochores are not under tension (Lara-Gonzalez et al. 2012, Foley and Kapoor 2013). Chemical perturbation or mutation of components within this assembly results in chromosome segregation defects. Additional proteins contact KMN complexes in many fungi. The budding yeast Dam1/DASH complex, a large ring-shaped structure encircling the microtubule, increases MT binding and processivity of KMN complexes (Cheeseman et al. 2001a, Westermann et al. 2005). Homologs for this complex apparently are absent from basal fungi (the phyla Microsporidia, Chytridiomycota, Blastocladiomycota, Neocallimastigomycota, Glomeromycota and the unplaced subphyla Kickxellomycotina, Zoopagomycotina, Entomophthoromycotina and Mucoromycotina; Hibbett et al. 2007 FIG. 2A) and it is also not conserved in metazoans, although the three-subunit Ska complex may be a functional counterpart (Welburn et al. 2009).

Arguably fungi present the richest resource to decipher overall similarities but also small mechanistic differences in kinetochore architecture and function within a single kingdom, in that by now more than a thousand fungal genomes have been sequenced, thus yielding information about the kinetochore complement from closely related strains or species as well as vastly different taxa. The first comprehensive appraisal of fungal kinetochore proteins was conducted just before proteomic screens of chicken and human cells substantially increased the number of known vertebrate kinetochore complexes (Meraldi et al. 2006). Sequence similarity searches and hidden Markov

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a tetramer, let alone a nucleosome-like structure, but it is clear that the subunits contact DNA via their histone-fold domain. CENP-T-W binds preferentially to linker DNA. The precise shape of the CENP-H-K-I-M complex is unclear but the CENP-I subunit can stretch across long distances. It is unclear how many CENP-A/CenH3 nucleosomes are involved in generating a MT attachment site (from one in *S. cerevisiae* and *C. albicans* to several in *S. pombe*), and how long these patches are, although all appear to be embedded in canonical H3 (brown) nucleosomes. This model shows three CENP-A/CenH3 nucleosomes and two CENP-T-W-S-X nucleosome-like structures to accommodate CENP-C and CENP-N binding to various domains of CENP-A/CenH3 nucleosomes. The five-nucleosome unit also fits with the phasing observed in ChIP-seq experiments with *N. crassa* CenH3 (Smith et al. 2011, 2012).

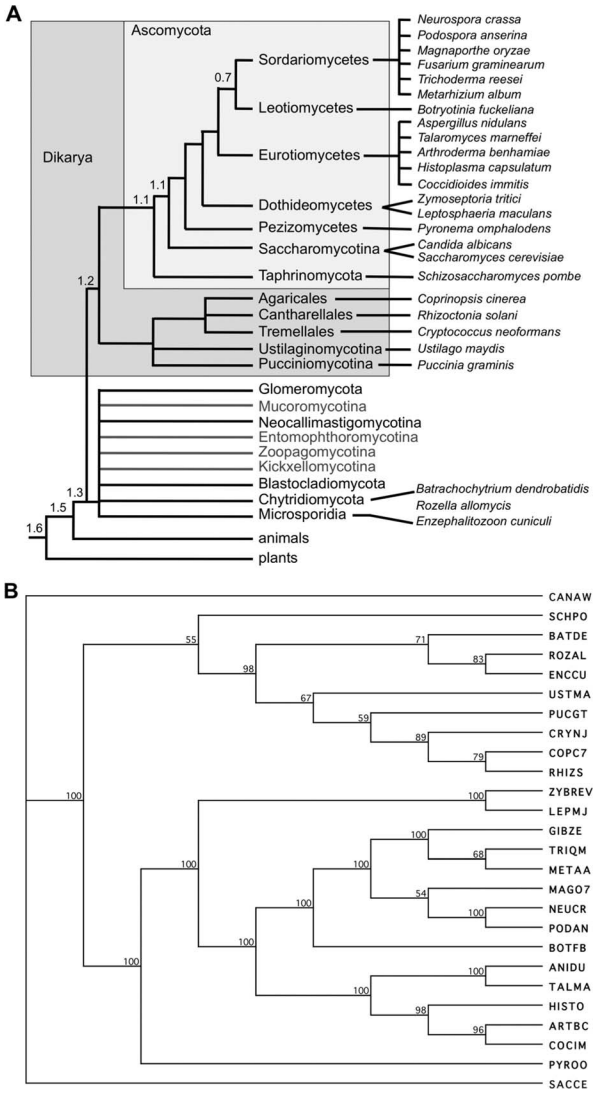

FIG. 2. Predicted phylogenetic trees of the taxa discussed. A. Cartoon of currently accepted relationships of the taxa discussed here; no attempt has been made to resolve basal taxa (Hibbett et al. 2007). Branch lengths do not denote genetic distance. The approximate divergence times of major branches is indicated in billions of years (Hedges 2002). Please note that the selection of taxa used for alignments was driven in part by general current research interests and not by taxonomic placement alone, thus some branches of the Ascomycota are underrepresented. B. Representative phylogenetic tree constructed by combined alignments of the Knl1 and Ndc80 complex (Ndc80, Nuf2, Spc24, Spc25) homologs of all 26 taxa discussed. Taxonomy and abbreviations are defined (SUPPLEMENTARY TABLE I); accession numbers are provided (SUPPLEMENTARY TABLE III). In this tree budding yeasts in the Saccharomycotina (*C. albicans* and *S. cerevisiae*) and the fission yeast *S. pombe* are not placed according to accepted phylogeny, while all other major branches (basal fungi with microsporidia and chytrids, basidiomycetes and ascomycetes) are resolved according to expected relationships

model (HMM)-based modeling showed that fungi more closely related to *S. cerevisiae* contained 11 kinetochore proteins that had not yet been identified in vertebrates and vertebrates appeared to contain proteins absent from budding yeasts (Meraldi et al. 2006). For ascomycete fungi, however, large overlap between protein sets from yeasts and mammals was revealed, suggesting that structural features of kinetochores have been well conserved from yeast to humans, especially within the KMN network (FIG. 2B). More recent bioinformatics and functional studies showed that many yeast proteins are functionally homologous to the CCAN subunits isolated by proteomics approaches in vertebrates (Schleiffer et al. 2012, Westermann and Schleiffer 2013). Most subunits also have been identified in many filamentous fungi (Smith et al. 2012). Only three CCAN subunits (CENP-M, CENP-P, CENP-R) and one KMN protein (Zwint/Kre28) cannot be identified by sequence similarity to either yeasts or metazoan in most filamentous ascomycetes, although functionally similar proteins are expected to exist. Searching other branches of the fungal kingdom reveals that CCAN proteins are poorly conserved at the primary sequence level, because most of them cannot be identified by homology searches alone in basal fungi (microsporidia and the clades formerly called chytrids and zygomycetes) and basidiomycetes (SUPPLEMENTARY TABLE I). Kinetochore proteins of ascomycetes share functionally important features with most metazoan proteins. Considering the large differences on the level of individual protein structure uncovered in early genetic and biochemical studies it is remarkable how similar the overall structures of CCAN and KMN complexes in metazoans and fungi are. The purpose of this review is to illustrate commonalities between metazoans and fungi and to identify proteins and interactions that should be further studied in the fungi.

MICROTUBULE-ASSOCIATED PROTEINS AT THE OUTER KINETOCHORE: THE DAM1/DASH COMPLEX

The most obvious difference between fungal and metazoan kinetochore complexes is the presence of the Dam1/DASH complex in fungi (FIG. 3A). The *Saccharomyces cerevisiae* Dam1 (in *Schizosaccharomyces pombe* DASH) complex is composed of 10 subunits, all of which are essential for survival in budding yeast (Cheeseman et al. 2001b, Janke et al. 2002) and *Candida albicans*

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(see FIG. 1A). Tree was constructed by neighbor joining (bootstrap 1000 replicates; tie breaking systematic; gaps were distributed proportionally).

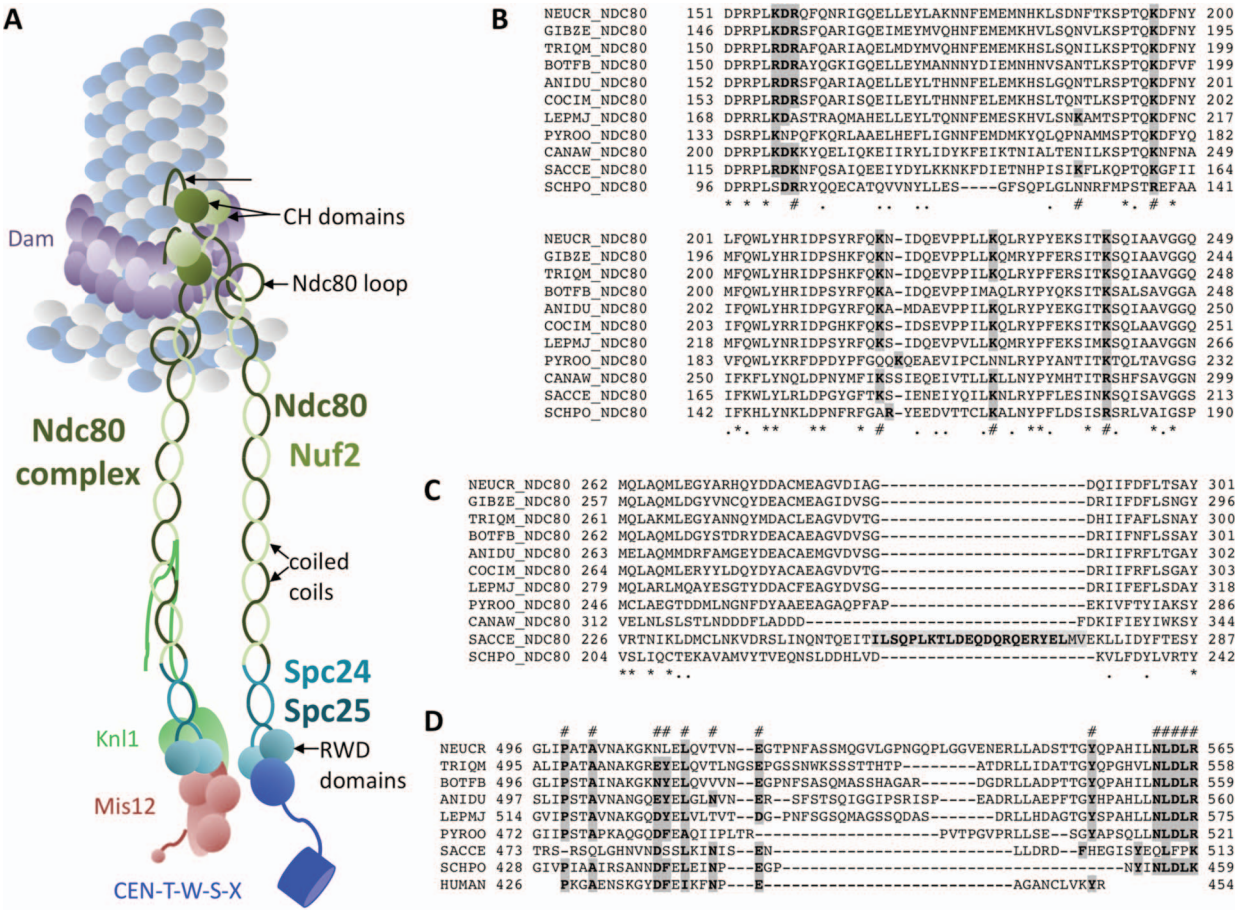

FIG. 3. The Ndc80 complex connects microtubules (MT) to the Mis12 assembly and the CCAN CENP-T complex. A. Structural features of the Ndc80 complex. N-terminal, positively charged patches bind directly to the MT. Calponin-homology (CH) domains are well-conserved and essential for MT binding. The function of the exposed Ndc80 loop is still unclear. All four Ndc80 subunits (Ndc80, Nuf2, Spc24, Spc25) contain regions of coiled-coils that connect the two head units, the CH domains of Ndc80/Nuf2 and the heterodimeric RWD domain of Spc24/Spc25. Binding to the RWD domain by Mis12 (and Knl1) or CENP-T is exclusive; only one of these complexes can bind to one Spc24/Spc25 RWD domain. B. Alignment of a conserved regions within the Ndc80 CH domain, containing lysine (K) residues previously shown to be important for function (#). Completely conserved residues (*) or similar (.) residues are marked. Double mutants for the first (K122E) and last (K204E) lysine in yeast were unable to survive, and mutants with only K204E showed benomyl sensitivity. C. A short loop separating coiled-coil regions of Ndc80 in some yeast species is not found in filamentous ascomycetes. Instead these regions are highly conserved. D. The exposed Ndc80 loop varies in length and primary sequence but several residues are highly conserved (#). Mutational analyses of the features in B–D needs to be carried out for filamentous ascomycetes. Species abbreviations are provided (SUPPLEMENTARY TABLE I). Important residues are shown in boldface and shaded in gray.

(Thakur and Sanyal, 2011) but not in fission yeast (Sanchez-Perez et al. 2005). In *Neurospora crassa* single gene deletions have been generated for all Dam1 complex subunits and not all of them are essential (G. Ekberg and M. Freitag unpubl). These differences may be based on the different numbers of MTs attached per kinetochore (Burrack et al. 2011). In budding yeast and the dimorphic *Candida albicans* a single MT attaches (Joglekar et al. 2006, Burrack et al. 2011, Thakur and Sanyal 2011), but in fission yeast two to four MT attach (Ding et al. 1993), which is likely also true for most filamentous fungi but has not been studied.

In vitro and with purified kinetochore particles, the budding yeast Dam1 complex forms heterodecamers that assemble into 16-unit rings encircling the MT plus end (Miranda et al. 2005, Westermann et al. 2005, Gonen et al. 2012). In vivo forming Dam1 complex rings around the MT may not be the rule for all fungi, however, as Dam1 speckles or patches have been described in fission yeast (Gao et al. 2010). The function of this large assembly may be to couple force generated by depolymerization of MTs with chromatid movement (McIntosh et al. 2013). Deletion of the Hsk3 subunit splits the complex into the

oligomerization-deficient Dam1^{OD} complex (Dam1, Duo1, Spc34, Spc19, Dad1, Dad3), which binds MTs in vitro, and a smaller Ask1/Dad2/Dad4 complex, which does not bind MTs (Miranda et al. 2007). Oligomerization is required in vitro for the Dam1 complex to form microtubule attachments. Thus, the in vivo Dam1 complex also may form a ring that prevents detachment from the MT, especially under tension during mitosis. When Hsk3 was depleted in vivo the Dam1^{OD} complex formed and bipolar centromere pairs collapsed to monopolar attachments (Umbreit et al. 2014). Failure of MT attachment at one kinetochore was explained by the inability of the Dam1^{OD} complex to mediate stable coupling to MTs under tension.

Thus the Dam1 complex appears to be an integral part of the outer kinetochore in many fungi, although it assembles only when the kinetochore meets MTs head-on (Malvezzi and Westermann 2014) and in presence of Ndc80 (Janke et al. 2002, Lampert et al. 2013). Coordinated action of the Dam1 and Ndc80 complexes is required for correcting monopolar attachments by the Ipl1/Aurora B kinase in yeast. Also arguing for inclusion of the Dam complex as a bona fide kinetochore component are experiments showing that artificially tethering Dam1 complexes to DNA proved sufficient to segregate DNA in budding yeast (Kiermaier et al. 2009, Lacefield et al. 2009) and to assemble Cse4/CENP-A-containing kinetochores (Ho et al. 2014). Outside the yeasts no mechanistic work has been done on Dam1/DASH complexes in the fungi and it is still not clear if some basal taxa (FIG. 2A; SUPPLEMENTARY TABLE II) have this complex.

NDC80 COMPLEX

The Ndc80 complex is required to connect the Mis12 assembly platform as well as the inner kinetochore CENP-T-W-S-X complex (FIG. 3A) to generate load-bearing MT attachments to centromeric nucleosomes (Powers et al. 2009). Together with CENP-T it is the kinetochore component with the highest ability to stretch. The length of the Ndc80 complex was estimated by electron microscopy to be ~57 nm (Wei et al. 2005, Alushin et al. 2010). In yeast strains in which the components of the Ndc80 complex were tagged with fluorescent proteins, the overall length of the complex changed from 55 to 34 nm, mostly due to changes within the Ndc80/Nuf2 dimer (Aravamudhan et al. 2014). The dumbbell-shaped complex comprises two heterodimers, Ndc80-Nuf2 and Spc24-Spc25, respectively. Both dimers have globular head domains and interact via their long coiled-coil regions (Wigge and Kilmartin 2001, Bharadwaj et al. 2004, McCleland et al. 2004, Wei et al. 2005). On average four Ndc80 complexes assemble on MTs in vitro (Alushin et al. 2010) and 14 in vivo (Suzuki et al. 2015), although the arrangement in fungi (attached ring-like around the MT and associated with the Dam1 complex) may be different from that in humans (as longitudinal oligomers). In vivo estimates from *S. cerevisiae*, *S. pombe* and *C. albicans* obtained by fluorescence microscopy also suggest that for each CENP-A about eight Ndc80 proteins are present, even though *S. pombe* has two to four MTs per kinetochore instead of the single MT of *S. cerevisiae* and *C. albicans* (Joglekar et al. 2008). In yeast the Ndc80 complex appears to stretch across the top surface of the Dam1 complex ring (Aravamudhan et al. 2014).

A positively charged patch in the Ndc80 N-terminal tail is important for MT binding, potentially by interacting with acidic patches on tubulin and by oligomerizing Ndc80 (Guimaraes et al. 2008, Miller et al. 2008). In yeast this tail can be deleted as long as the Dam1 complex is present (Demirel et al. 2012), but it is not yet known whether this is true for other fungi as well. The N-terminal tail contains phosphorylation sites for Ipl1/Aurora B kinase, which is required to resolve erroneous kinetochore to MT connections (DeLuca et al. 2011); there are kinase binding motifs in all fungal Ndc80 proteins in this region. This conserved pathway, the spindle assembly checkpoint (SAC), monitors kinetochore to MT attachment status, including presence of MTs and tension between sister kinetochores. In the absence of attachment or tension the SAC is activated, relaying the signal to master regulatory kinases that drive cell cycle progression (Musacchio and Ciliberto 2012). A phosphorylation gradient generated by Ipl1/Aurora B kinase that emanates from the centromere core has been suggested to reach outer kinetochore components when they are not under tension (Liu et al. 2009, Welburn et al. 2010). Phosphorylation of the very N-terminal tail thus may control clustering of Ndc80 and prevent interactions with tubulin.

The globular heads of Ndc80 and Nuf2 interact tightly through their calponin homology (CH) domains and have MT-binding activity (Wei et al. 2007; Alushin et al. 2010), which is reduced or lost in CH domain mutants (Ciferri et al. 2008, Sundin et al. 2011, Tooley et al. 2011). In reconstitution experiments Dam1 enhanced Ndc80 binding to the MT plus end, suggesting that Dam1 might strengthen Ndc80-dependent MT attachment in vivo (Lampert et al. 2010, Tien et al. 2010). The CH domains of fungal Ndc80 have conserved lysine- or arginine-rich toe patches (FIG. 3B), which are predicted to bind to the MT surface, as shown with Ndc80 homologs (Alushin et al. 2010). The toes are proposed to act as sensors for depolymerization, thereby

helping to track the MT ends (Alushin et al. 2010). In yeast positions K122, K152, K160, K181, K192 and K204 (indicated by # in FIG. 3B) have been mutated (Lampert et al. 2013). The combination of K122E and K204E was lethal in yeast, matching mutation of human Ndc80 K89 and K166, which crippled Ndc80 to MT interactions in vitro (Ciferri et al. 2008). Lysine 122 is not truly conserved in other ascomycetes, instead either arginine is found in the same position and/or there are lysines in the same context, changing an RDK into a KDR or RDR motif (FIG. 3B). By itself mutation of yeast Ndc80 K204 yielded benomyl-sensitive strains. This position is conserved, although sometimes the lysine is replaced by arginine. Overall MT binding by the Ndc80 CH domain is essential and conserved, based on the primary amino acid structure of all fungal Ndc80 proteins examined.

In yeast the Ndc80 CH domain is connected via a short disordered segment to a helical region preceding the coiled-coil shaft of the protein (Lampert et al. 2013); a shorter connector also is present in human Ndc80 (aa 203–211). While several *Saccharomyces* species contain large insertions in this region, all of which are enriched for polar residues that may yield an interface for protein-protein interactions, this region is missing from Ndc80 of other ascomycetes (FIG. 3C). In yeast deletion of this stretch (aa 256–273) made Ndc80 insensitive to the presence of the Dam1 complex, although MT binding of the Ndc80 complex was not changed (Lampert et al. 2013). Similar experiments on the Ndc80 tail of other ascomycetes need to be conducted to assess whether the situation in yeast is conserved.

Much debate has centered on a loop (FIG. 3D) that breaks the coiled-coil region and contributes to the attachment of kinetochores to MTs, presumably by recruiting regulatory proteins (Ciferri et al. 2008, Wang et al. 2008). Whether this loop directly contacts Dam1 (Maure et al. 2011) and human Ska 1 (Zhang et al. 2012) or indirectly contributes to Ndc80-Dam1 interactions (Lampert et al. 2013) is controversial. Yeast strains with short deletions (Ndc80 D490–510) or point mutations showed temperature-sensitive phenotypes (Maure et al. 2011). Depletion of Ska complex activity resembles the phenotype obtained when the loop region is removed in Ndc80 (Guimaraes and Deluca 2009), and the loop is also required in fission yeast (Hsu and Toda 2011). Mutations within the loop independently may affect Dam1 binding in vivo by changing intramolecular Ndc80 flexibility (Maiolica et al. 2007, Ciferri et al. 2008, Wang et al. 2008, Joglekar et al. 2009). Separating defects in Ndc80 loop mutants caused by structural changes versus direct effects on binding of regulatory proteins will be important. Replacement of the loop region with alternative loop sequences from closely related species of ascomycetes followed by assays for sequence specificity may help to resolve this question. To address changes in Ndc80 flexibility mixed Ndc80 complexes could be assembled. Length requirements for Ndc80 loop mutants and other KMN network components need to be assessed, in that shortened proteins may not be able to escape the Ipl1/Aurora B phosphorylation gradient. It is unclear whether loop sizes are similar or vary significantly between taxa (FIG. 3D). It also is possible that loops themselves, just like the N-terminal tail, are phosphorylation targets.

On the opposite end of the dumbbell-shaped Ndc80 complex the globular domain of the conserved Spc24–Spc25 heterodimer connects to the Mis12 complex and to CCAN by direct interaction with CENP-T (Petrovic et al. 2010, Gascoigne et al. 2011). Interactions between Spc24/Spc25 and the CENP-T Ndc binding motif or the Mis12/Nsl1 component are exclusive so that an Ndc80 complex bound to the Mis12 complex cannot interact with CENP-T at the same time (Bock et al. 2012, Malvezzi et al. 2013, Nishino et al. 2013). Spc24/Spc25 contain N-terminal coiled-coil motifs and C-terminal RWD (RING finger, WD repeat, DEAD-box-like helicase) domains, used to form the heterodimer (Schmitzberger and Harrison 2012). Within ascomycetes Spc24 and Spc25 primary sequences are conserved, even in the N-terminal and central coiled-coil domain, which carry some hallmark motifs that can serve to differentiate SPC24 proteins from fungal families into subclasses. RWD domains have emerged as hallmark interaction domains in kinetochores, forming numerous attachments, especially to the Mis12 complex (Petrovic 2014). Structurally highly conserved RWD domains are organized as α+β sandwich fold (FIG. 4). The Spc24/Spc25 heterodimeric RWD domain binds to sequence motifs at the C-termini of the Mis12 complex subunits Nsl1 (at PVIHL), and they can bind to conserved Dsn1 and CENP-T helical motifs (Malvezzi et al. 2013, Petrovic et al. 2010).

KNL1/SPC105/SPC7

Mammalian Knl1 (budding yeast Spc105, fission yeast Spc7) functions as scaffold during early mitosis, contributing to formation of proper attachment between kinetochore and microtubule (see Ghongane et al. 2014 for a recent perspective and references therein). Knl1 is the largest single subunit of the KMN network, essential for accurate chromosome segregation during mitosis and required for both activation and inactivation of the SAC via Bub proteins.

Overall the primary sequence of Knl1 proteins is poorly conserved, but a number of motifs and domains can be recognized in all ascomycetes. The N-terminal

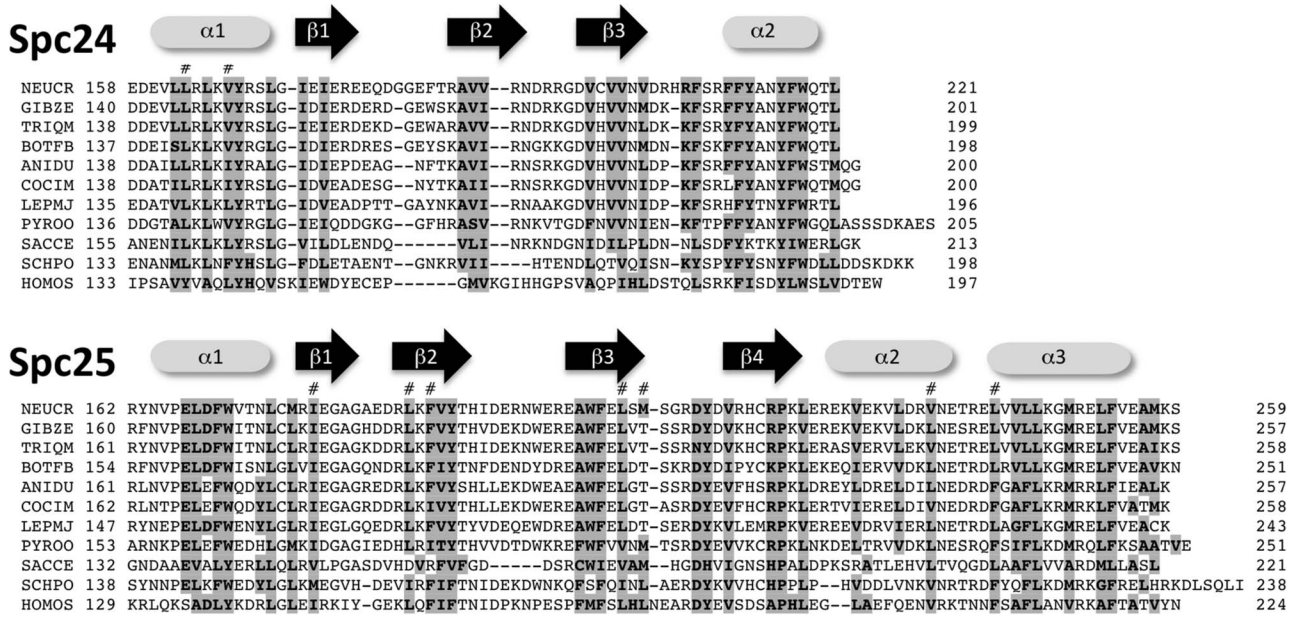

FIG. 4. The heterodimeric RWD domain of Spc24/Spc25 is conserved at the primary sequence level. Each half of the RWD domain is at the very C-terminus of the proteins. Spc24 lacks the β4 and α3 feature, but SPC24/Spc25 form α + β sandwich RWD domain (Malvezzi et al. 2013). Identical or conserved residues are in boldface. Residues that are predicted to contact the CENP-T N-terminal helix are indicated (#). Conserved residues are in boldface and shaded in gray.

region contains patches of basic residues that are required for MT binding in Knl1 orthologs. Knl1 also is integrating the activity of Ipl1/Aurora B kinase and PP1 phosphatase via the highly conserved G/SILK and RVFS motifs (Welburn et al. 2010), which are embedded in this basic N-terminus (FIG. 5A). The RVFS motif is conserved in all fungal sequences examined, while the G/SILK sequence varies. In *S. cerevisiae* and *S. pombe* it is GILK, in *Zymoseptoria* and *Leptosphaeria* it is SILS and in all other taxa examined it is SILK. Aurora B inhibits the interaction between PP1 and Knl1 by phosphorylation of the RVSF serine, while PP1 requires KNL1 for its localization to kinetochores to inactivate the SAC. In mammals PP1 antagonizes Aurora B phosphorylation of Ndc80, resulting in stabilization of the kinetochore to MT attachment. The mitotic checkpoint kinase Mps1 phosphorylates Knl1 of budding yeast, fission yeast and humans (Yamagishi et al. 2014) at the threonine of the more centrally located MELT or MELT-like motifs. While the exact number of these motifs varies, their presence is conserved in the species that have Knl1. Phosphorylation by Mps1 is required for the recruitment of the SAC subunits Bub1 and Bub3, and the number and especially the type of MELT motifs available for phosphorylation may allow tuning of the SAC activity (Vleugel et al. 2015). Examining predicted Knl1 sequences from selected species (SUPPLEMENTARY TABLE III) showed that five motifs (MDMT, MDIT, MDVT, MEFT, MELT) made up two-thirds of all conserved MELT sites. Searching Knl1 sequences with all potential previously recognized motifs (i.e. M/I/L/V-E/D-L/M/I/V-T/S) showed that $> 95\%$ of all motifs found begin with a methionine, followed by equal likelihood of aspartic and glutamic acid (FIG. 5B). In the third position phenylalanine was found in a quarter of all motifs, somewhat unusual when compared to yeasts, where MELT and MD(L/I/V)T were most common. Based on activity assays the consensus sequence for human MELT sites has a DKT motif and an E/D-rich patch in the 15 residues preceding the MELT motif (M-E/D-L/M/I-T), followed by a K/S/R-S-H/Y-T motif. This region is quite different in ascomycetes, although the acidic patch is conserved with many E/D residues in the 10 residues before the M-E/D-M/F/L/I/V-T motif. The immediately following residues are conserved in fungi (AxGGI motif, found by WebLogo; Crooks et al. 2004). Specific patterns of motifs are conserved within taxa in respective orders, suggesting some adaptive role of specific MELT motifs, although this needs to be examined by deletion and swapping experiments.

In mammalian Knl1 KI motifs help to recruit Bub proteins, but these short motifs appear to be missing from all fungal Knl1 homologs, suggesting that the KI motif has evolved more recently (Ghongane et al. 2014). In fungal Knl1 proteins the longest conserved domain is the Spc7 region, located ~ 200 aa from the C-terminus (FIG. 5A); this domain is not conserved in human Knl1. This domain extends for ~ 300 aa in both yeasts and the filamentous ascomycetes, in which

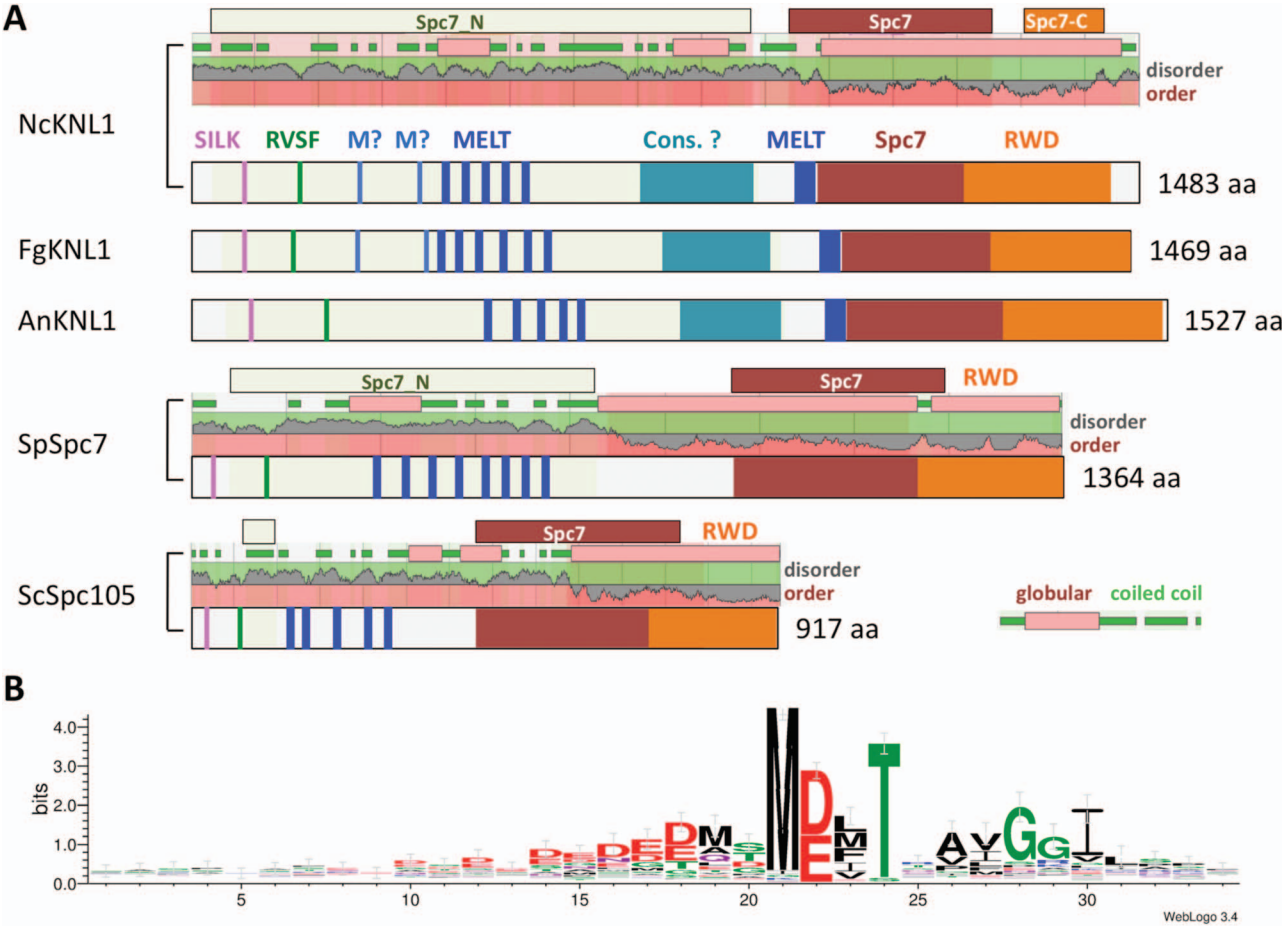

FIG. 5. Structural features of fungal Knl1/Spc7/Spc105 proteins. A. Cartoons showing important features of Knl1 homologs from fungi. Extent of Pfam domains (Spc7, Spc_N, Spc-C) are above plots depicting predicted disorder or order of proteins, and coiled-coil (green) or globular domains (pink). Knl1 homologs from *N. crassa* (NcKnl1), *F. graminearum* (FgKnl1) and *A. nidulans* (AnKnl1) are shown. Overall distribution of hallmark motifs (SILK, RVSF, MELT, Spc7 and tandem RWD domain) is similar in the three examples. The number and type of MELT motifs (MELT and M?) is different, however, and compared to the *S. cerevisiae* (ScSpc105) and *S. pombe* (SpSpc7), as well as the metazoan homologs, a potential additional MELT motif immediately before the Spc7 domain was identified. Also unique to filamentous ascomycetes is a conserved globular domain (Cons.?) of unknown function. The sequence of this motif is family or order-specific. B. Logo plot of 106 MELT motifs identified in 18 species (*Neurospora crassa, Podospora anserina, Magnaporthe oryzae, Fusarium graminearum, Trichoderma reesei, Metarhizium album, Botryotinia fuckeliana, Aspergillus nidulans, Talaromyces marneffei, Arthroderma benhamiae, Histoplasma capsulatum, Coccidioides immitis, Zymoseptoria brevis, Leptosphaeria maculans, Pyronema omphalodens, Candida albicans, Saccharomyces cerevisiae, Schizosaccharomyces pombe*) identified M-D/E-L/M/F-T as the preferred motif. Preceding residues are often D/E, the immediately following residues show preference for xAYGGI. Logo was generated by WebLogo 3.4 (Crooks et al. 2004).

we found a highly conserved additional MELT-like motif at the N-terminal end that is not conserved in mammals or yeasts. In some filamentous ascomycetes a C-terminal Spc7-C2 domain is found, which is part of a conserved tandem RWD domain (FIG. 5A). Like in human Knl1 (Petrovic et al. 2014), all fungi inspected have the standard arrangement of α+β sandwich folds (α-β-β-β-α-α) in both RWD domains, which have been strongly implicated in binding to the Mis12 complex subunit Nsl1.

Yeast Kre28 and mammalian Zwint together with the Mis12 complex are required for normal kinetochore assembly (Pagliuca et al. 2009, Caldas et al. 2013). Kre28 forms heterotrimers with Spc105, the budding yeast Knl1 homolog, and human Knl1 binds to Zwint likely via a coiled coil region N-terminal of the tandem RWD domains (Ghongane et al. 2014). Homology searches failed to yield obvious Zwint or Kre28 counterparts in most ascomycete fungi, suggesting that the homolog is different in primary structure from both Kre28 and Zwint. We found a previously unrecognized globular domain of unknown function between the conserved MELT motifs and the tandem RWD domains of Knl1 that is highly conserved in Sordariomycetes,

Leotiomycetes and Eurotiomycetes but not Dothideomycetes and Pezizomycetes (FIG. 5A). This domain may serve as interaction domain with a predicted Zwint homolog.

MIS12 COMPLEX

Four subunits (Mis12, Mis13/Dsn1, Mis14/Nsl1, Nnf1) constitute the rod-like Mis12 complex, which acts as a platform for the formation of the KMN network (Cheeseman et al. 2006). In budding yeast the centromere-specific H3, Cse4 (CENP-A) and Ndc10 help to recruit the Mis12 complex, while in fission yeast Knl1 is required for Mis12 complex recruitment (Kerres et al. 2007). The Mis12 complex forms one connection between MTs/Ndc80 complex and the CCAN by direct binding to CENP-C (Przewloka et al. 2011, Screpanti et al. 2011) and CENP-U (Hornung et al. 2014). Once recruited to kinetochores the Mis12 complex serves as a platform for Ndc80 and Knl1/Zwint binding (Petrovic et al. 2014).

Several key interactions of the Mis12 complex are made via RWD domains on the interacting proteins. These recent findings suggest that the RWD domain has evolved as an adaptor motif in kinetochore interactions (Schmitzberger and Harrison 2012, Petrovic et al. 2014). The C-terminus of Mis13/Dsn1 interacts directly with the Spc24/Spc25 heterodimeric RWD domain, while the Knl1 tandem RWD domain directly interacts with the C-terminal PVIHL motif of Mis14/Nsl1, which also contacts Spc24/Spc25. The CCAN subcomplex containing homologs of CENP-P-Q-O-U in yeast, COMA (Ctf19, Okp1, Mcm21, Ame1), is also predicted to have a heterodimeric RWD domain (between Ctf19/CENP-P and Mcm21/CENP-O). How this subcomplex interacts with Mis12 has been addressed only recently (Hornung et al. 2014). An additional yeast complex, monopolin, is recruited by Mis12/Dsn1 during meiosis via its Csm1 RWD domain (Sarkar et al. 2013). It is unknown whether the recruitment of CENP-P-Q-O-U and monopolin are conserved in other fungi.

CENTROMERE IDENTITY IS CONVEYED BY CENP-A-DEPENDENT ASSEMBLY OF THE CONSTITUTIVE CENTROMERE-ASSOCIATED NETWORK (CCAN)

The epigenetic nature of centromeres has been firmly established, because specific DNA sequences alone are insufficient, let alone essential, to generate normal centromeres or aberrant neocentromeres (Cleveland et al. 2003, Black and Cleveland 2011), perhaps even in budding yeast (Ho et al. 2014). Replacement of canonical H3 at centromeric nucleosomes with an H3 variant called CENP-A in mammals (Palmer et al. 1987), Cid in Drosophila (James and Elgin 1986), $Cnp1^{CenpA}$ in *S. pombe* (Takahashi et al. 2000), Cse4 in *S. cerevisiae* (Stoler et al. 1995) and *Candida albicans* (Sanyal and Carbon 2002) and CenH3 in *N. crassa* (Smith et al. 2011) has been found in most systems studied, although there are exceptions in insects and trypanosomes (Drinnenberg et al. 2014).

How CENP-A is targeted and maintained in centromeric regions has been subject of intensive studies over the past decade (Perpelescu and Fukagawa 2011, Fukagawa and Earnshaw 2014, Scott and Sullivan 2014). CenH3 sequences of yeasts are highly variable both in length and sequence of the N-terminal tail and loop I region within the histone fold domain (Baker and Rogers 2006). The CENP-A targeting domain (CATD), embedded in the otherwise conserved histone fold domain (HFD), proved sufficient to target CenH3 (or heterologous H3 with a CATD) into centromeric nucleosomes (Black et al. 2007) but not in *Arabidopsis thaliana* (Ravi et al. 2010). The CATD is conserved in filamentous fungi because CenH3 proteins from several filamentous fungi yield the expected chromocenter localization when the native Neurospora CenH3 gene is replaced with heterologous genes (Phatale, Smith, Connolly, Freitag unpubl).

CENP-A is deposited by its chaperone HJURP (Dunleavy et al. 2009, Foltz et al. 2009, Sanchez-Pulido et al. 2009), called Scm3 in the fungi (Camahort et al. 2007, Mizuguchi et al. 2007, Stoler et al. 2007), which now also has been shown to interact with CENP-C (Tachiwana et al. 2015). The dynamics of deposition of CENP-A is an intensely studied aspect of centromere biology, but apart from trailblazing work in budding and fission yeast (Camahort et al. 2007, Mizuguchi et al. 2007, Stoler et al. 2007, Pidoux et al. 2009, Mishra et al. 2011) there is little known about these processes in fungi; thus these processes will not be further discussed here. Similarly five proteins that are required for proper chromosomes segregation in fission yeast (named Mis14 to Mis18; Hayashi et al. 2004, Shiroiwa et al. 2011) have not been studied in filamentous fungi. Several of these proteins had known counterparts (Mis14 is CENP-I, Mis15 is CENP-N, and Mis16 is the small subunit of chromatin assembly factor 1, CAF1-3 or RbAp46/48) in mammals. A four-protein Mis18 complex was found in humans, although Mis18 showed little sequence identity (Fujita et al. 2007, Barnhart et al. 2011, Moree et al. 2011). Among other fungi similar proteins can be identified only in the Ustilagomycotina and Mis17, which previously had been proposed to be a CENP-M homolog, remains unknown from other fungi.

Conserved mechanism for CENP-A recognition by CENP-C/Mif2 (Carroll et al. 2010) and CENP-T (Fachinetti et al. 2013, Folco et al. 2015) have been

proposed. **S**hort motifs in the N-terminal tail and CATD were found to be required for recruitment of CENP-C and CENP-T by targeting chimeric proteins consisting of mammalian H3 and CENP-A that were tagged with a LacI domain to genomic LacO sequences (Logsdon et al. 2015). The authors separated features of CENP-A necessary for centromere establishment from those required for centromere maintenance. After formation of centromeres the CENP-A N-terminal tail seems no longer necessary to recruit normal levels of CENP-C and CENP-T and the CENP-A C-terminal tail is no longer essential to recruit normal levels of CENP-T (Logsdon et al. 2015). The model emerging from these studies is that it is difficult to gain neocentromeres and that redundant connections to CCAN complexes must be broken to lose a functional centromere (Logsdon et al. 2015). Even if CENP-A is mislocalized or available in concentrations higher than required for maintenance of centromeres (Heun et al. 2006, Smith et al. 2011), neocentromeres do not arise frequently by spreading of CENP-A. One approach to cast more light on the differences between assembly and maintenance pathways is by in vitro reconstitution experiments using CENP-A nucleosome arrays and purified CCAN complexes (Logsdon et al. 2015). Another way would be to use the information available from ascomycete fungi to assemble centromeres and kinetochores in vivo in a heterologous system to determine which motif or domains of the CCAN are required for assembly or maintenance of centromeres. *Zymoseptoria tritici* may become a facile system for this approach, in that its centromeric regions are short, composed of unique, non-repetitive sequences and resemble those of neocentromeres in other organisms (Schotanus et al. 2015). At the same time many CCAN components cannot be found by sequence homology alone (SUPPLEMENTARY TABLE IV).

CENP-C

While the identity of centromeres seems to be determined by the placement of CENP-A into nucleosomes, the way in which the CCAN assembles is topic of intense research and debate. Studies suggest that CENP-C acts as the major recruiter of CCAN complexes (Basilico et al. 2014, Kim and Yu 2015, Klare et al. 2015, Krizaic et al. 2015, Nagpal et al. 2015, Tachiwana et al. 2015). CENP-C is the most widely conserved CCAN subunit, containing a central CENP-C motif and a C-terminal dimerization motif (Perpelescu and Fukagawa 2011). The N-terminal domain appears to be dispensable for centromere localization but is required for the localization of many other KIN proteins (Perpelescu and Fukagawa 2011) and interaction with the Mis12 subunit Nnf1 (Przewloka et al. 2011). CENP-C binds directly to CENP-A in vivo (Carroll et al. 2010, Logsdon et al. 2015), and by this interaction reshapes and stabilizes the CENP-A nucleosome (Falk et al. 2015). On synthetic centromeres, CENP-C recruitment requires the CENP-C binding region and the CATD of CENP-A (Tachiwana et al. 2015). CENP-C also binds to the CENP-H-I-K complex by direct interaction of CENP-H with specific residues in the PEST domain of CENP-C (Klare et al. 2015). Abrogating this interaction by mutation of conserved LFL residues also resulted in reduction of CENP-T-W recruitment via CENP-H interactions. CENP-C binding to CCAN proteins appears dependent on cell cycle stage, which has been studied in detail in chicken cells. During interphase CENP-L-N bind to CENP-C but interactions with CENP-A nucleosomes may be weaker (Nagpal et al. 2015). In contrast, during mitosis CENP-C binds CENP-A by its C-terminal dimerization domain but the middle region, which binds to CENP-N and CENP-H shows less association with centromeric nucleosomes (Nagpal et al. 2015). Whether this is also true in mammals or fungi remains to be seen but the possibility of CENP-C shifting between CENP-L-N, CENP-H-I-K and CENP-A provides an excellent model for cell cycle regulation of CENP-A.

In fungi CENP-C is the only CCAN protein that can be detected in basidiomycetes by homology searches. In *Z. tritici* only CENP-A, CENP-C and CENP-S-X were found by homology, all other CCAN members seem to be absent (SUPPLEMENTARY TABLE IV). An earlier study suggested that CENP-C has domains that are under negative or positive selection and that these domains vary depending on the taxa inspected (Talbert et al. 2004). Limited phylogenetic analysis to define CENP-C regions under positive or negative selection has been carried out (Smith et al. 2012). Signatures of positive selection were found in the central region (245–258 and 288–318 aa in *Neurospora* CENP-C) but overall CENP-C of filamentous fungi show evidence of negative selection. Detailed experimental work on this is still lacking in fungi other than budding and fission yeast.

CENP-N-L COMPLEX

CENP-N-L and CENP-M were co-purified with CENP-H-I-K (Foltz et al. 2006, Okada et al. 2006), but detailed relationships between these subunits remained obscure (Perpelescu and Fukagawa 2011) until recent work revealed the importance of the mammalian CENP-M subunit (Basilico et al. 2014). CENP-N interacts with CENP-A nucleosomes in vitro, independently of DNA sequence context, but dependent on the CENP-A CATD (Carroll et al. 2009). The heterodimer assumes a structure that is reminiscent of TATA-binding protein,

and it contacts CENP-C directly. Deletion of the conserved C-terminus abrogated CENP-N targeting and interactions with CENP-A, CENP-H, CENP-K and CENP-L, suggesting that CENP-N might serve to integrate information carried by CENP-A to recruit the other CCAN components (Carroll et al. 2009, Klare et al. 2015). Indeed recent findings suggest that CENP-N is recognized by the exposed RG loop in the CENP-A CATD. The RG loop helps to arrange CENP-A nucleosomes in more compact ladder-like structures in which the CATD loop is buried and thus unable to contact CENP-N (Fang et al. 2015). During the transition to S phase, the chromatin changes to a more open conformation, exposing the RG residues and allowing recruitment of CENP-N. These results demonstrated how structural transitions of chromatin allow for stage-specific recruitment of CCAN components. Future studies will address how these interactions are integrated with the known direct CENP-C and CENP-H interactions with CENP-A (Carroll et al. 2010, Fachinetti et al. 2013, Falk et al. 2015, Folco et al. 2015, Klare et al. 2015, Logsdon et al. 2015).

Fungal homologs for CENP-N and CENP-L have been studied only in budding and fission yeast so far, where they are called Chl4 and Iml3 and Mis15 and Fta1, respectively (Meraldi et al. 2006). Chl4 and Iml3 form a heterodimer and interact with Sgo1, a protein involved in sister chromatid cohesion (Hinshaw and Harrison 2013). All three proteins were found necessary in a screen for genes required for centromeric cohesion (Marston et al. 2004), but their roles in chromosome segregation were known before (Mythreye and Bloom 2003, Pot et al. 2003). De novo centromere assembly required Chl4 both on introduced plasmids and conditionally active centromeric DNA on chromosomes, but Chl4 was not required for the maintenance of centromeric chromatin (Mythreye and Bloom 2003). Fission yeast CENP-N (Mis15) forms a complex with CENP-I (Mis6) and a proposed functional equivalent of CENP-M (Mis17; Hayashi et al. 2004). Overall both CENP-L and CENP-N from filamentous ascomycetes are conserved; phylogenetic trees constructed by several different methods reveal relationships that mirror generally accepted phylogeny (data not shown).

CENP-H-K-I-M COMPLEX

This complex co-purified as a CCAN unit (Foltz et al. 2006, Okada et al. 2006) and previous extensive genetic and biochemical studies had established all subunits as CCAN components (Perpelescu and Fukagawa 2011). Mutation or depletion results in severe kinetochore defects and CENP-H overexpression has become a diagnostic tool in many forms of cancers (Tomonaga et al. 2005, Liao et al. 2007). CENP-I/Mis6 affects centromeric localization of CENP-A in fission but not in budding yeast and vertebrates (Perpelescu and Fukagawa 2011), and it can directly interact with the Ndc80 complex (Kim and Yu 2015). CENP-H-K-I are conserved in budding yeast and interact (Measday et al. 2002). All subunits are present in most ascomycetes, and *Aspergillus* CENP-H has been shown to interact with the CENP-E kinesin (Herrero et al. 2011). Depletion of CENP-H coupled to Aurora B kinase inhibition resulted in SAC defects (Matson et al. 2012, Kim and Yu 2015), and CENP-T attachment to the Ndc80 complex depends on CENP-H-K-I. CENP-H-K-I alone, however, is insufficient to establish KMN-MT interactions (Basilico et al. 2014, Kim and Yu 2015).

CENP-H-K-I and CENP-T-W-S-X complexes mutually recruit each other in a CENP-M-dependent manner to mediate one of two partially redundant pathways to fulfill the SAC function of the KMN network (Basilico et al. 2014, Kim and Yu 2015). So far CENP-M homologs have been studied in detail only in animals. CENP-I bridges the CENP-H-K heterodimer and CENP-M, CENP-I forming a fold similar to that of importin-β and CENP-M fitting into the fold similar to Ran proteins (Basilico et al. 2014). CENP-M originally was isolated as proliferation associated nuclear element 1 (PANE1) in that it is highly expressed in proliferating cells but later also was found by proteomic approaches (Foltz et al. 2006, Okada et al. 2006) and it is required for chromosome alignment and mitotic progression (Basilico et al. 2014). It influences localization of CENP-N-L, as well as CENP-H-K-I and CENP-O-P-Q-U, and has been shown to be a noncatalytic GTPase (Basilico et al. 2014). Similar "pseudo GTPases" have not been found in most fungal genomes by homology searches. A study confirmed this (Basilico et al. 2014), yet the CENP-M phylogenetic tree presenting a potential homolog from *Mucor* was included. Indeed this protein has some similarity to proteins from other zygomycetes and seems to contain a CENP-M domain with a conserved arrangement of β sheets and α helices (FIG. 6). Whether this is a true homolog of mammalian CENP-M remains to be determined. It is possible that fungal CENP-M-like proteins are very different in structure or that the *Mucor* pseudo GTPase is involved in other processes. Fission yeast Mis17, for example, was identified as potential regulator of the CENP-I/Mis6 complex (Perpelescu and Fukagawa 2011), yet based on structural studies it seems unlikely that this is a true functional homolog of CENP-M (Basilico et al. 2014); it also lacks homologs in other fungi. It also is possible that a functional GTPase has taken the place of CENP-M in fungi. This question needs to be investigated.

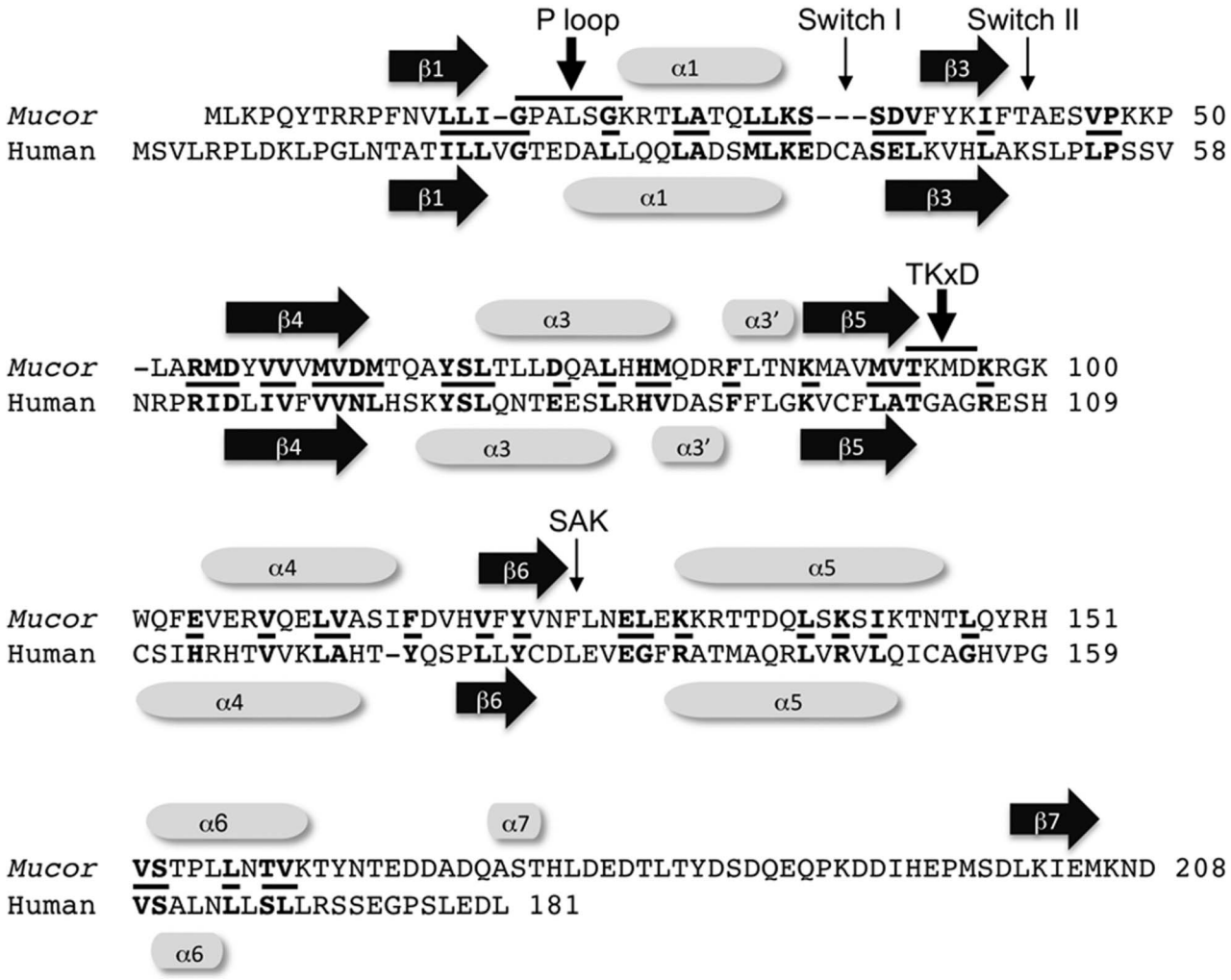

FIG. 6. Alignment of human CENP-M and a potential fungal CENP-M homolog. CENP-M from human (Q9NSP4.1) and *Mucor circinelloides f. circinelloides* (EPB82532.1) share overall similar domain structure. Similar proteins can be found in several other Mucoromycotina but not in other fungi. CENP-M is predicted to be a nonfunctional pseudo GTPase (Basilico et al. 2014). Several features of functional GTPases are no longer present in CENP-M, for example, the P-loop (GxxxxGKS/T) involved in GTP binding, Switch I and Switch II motifs required for conformational changes, the TKxD and the SAK motifs. A conserved catalytic glutamine residue right after Switch II is present in GTPases and is changed into a leucine in human and glutamic acid in *Mucor* CENP-M. Based on structural similarity to CENP-M and small GTPases the *Mucor* protein is predicted to assume a pseudo-GTPase fold but if it acts as a true CENP-M homolog will need to be addressed experimentally. Conserved residues are in boldface and short lines between the sequences.

CENP-T-W-S-X COMPLEX

Homologs for all four CENP-T-W-S-X subunits have been found in most ascomycetes (Smith et al. 2011, Schleiffer et al. 2012, Smith et al. 2012, Westermann and Schleiffer 2013) but they are more difficult to identify in basidiomycetes. Relationships of all four proteins across taxa resemble established phylogenies (Smith et al. 2012). Studies with the *Neurospora* proteins revealed that only CENP-T and CENP-W are essential, while deletion of CENP-S and CENP-X resulted in only minor growth defects (J. Galazka and M. Freitag unpubl). The fission yeast CENP-T, Cnp20, also is essential (Tanaka et al. 2009), while the budding yeast CENP-T, Cnn1, is not (Bock et al. 2012).

CENP-T has a C-terminal HFD, which can bind DNA and is necessary for CENP-T centromere localization, while CENP-W is a small protein composed entirely by a HFD (Hori et al. 2008a). CENP-T and CENP-W are tightly associated and form a heterodimer (Nishino et al. 2012), which also has been found in MS analyses of *Neurospora* extracts (J. Galazka and M. Freitag unpubl). CENP-S was identified by proteomic approaches (Foltz et al. 2006), and CENP-X subsequently was described as its binding partner (Amano et al. 2009). Both proteins have potential HFDs that

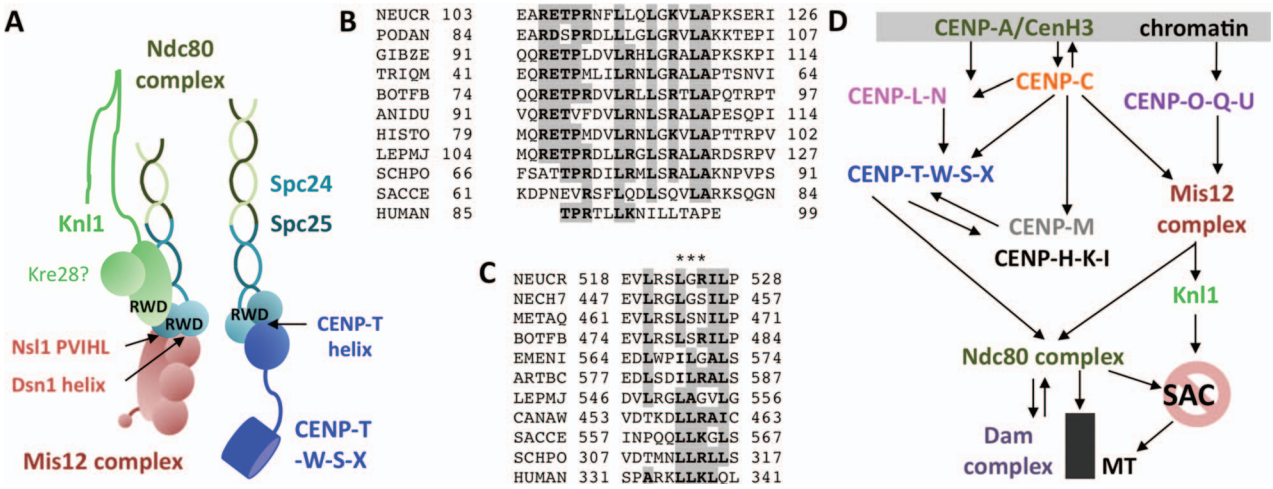

FIG. 7. Interactions between Ndc80, Mis12 and CENP-T and assembly of the KIN. A. The Spc24/Spc25 heterodimeric RWD domain interacts either with a helical patch on the CENP-T N-terminus (CENP-T helix) or a similar region on the Mis12 subunit Dsn1 (Dsn1 helix). This latter interaction also involves the Mis12 subunit Nsl1 PVIHL motif, which brings the Knl1 tandem RWD domain closer for binding to Spc24/Spc25. Kre28/Zwint homologs have not been identified in filamentous fungi by homology searches. B. Conservation of the CENP-T helix motif from human and 10 different fungi (abbreviations in SUPPLEMENTARY TABLE I). Conserved residues are in boldface and shaded in gray. C. Conservation of the Dsn1 helix motif from human and ten different fungi (abbreviations in SUPPLEMENTARY TABLE I) and aligned with the motif in B. Conserved residues are in boldface and shaded in gray and residues that resulted in loss of function in yeast (Malvezzi et al. 2013) are indicated (***). D. Proposed hierarchy of CCAN and KMN assembly in fungi and mammals. See text for details. SAC = spindle assembly checkpoint.

are conserved within the ascomycetes. Localization of CENP-S and CENP-X is interdependent and also dependent on CENP-H-K-I (Amano et al. 2009). CENP-S-X are identical to MHF1/MHF2, binding partners of FANCM (a member of the Fanconi anemia nuclear core complex), but centromeric localization of FANCM has not been reported (Perpelescu and Fukagawa 2011). Studies with metazoan CENP-T-W-S-X suggested that these proteins can form a tetrameric complex that may mimic canonical nucleosomes (Nishino et al. 2012). In both yeast (Schleiffer et al. 2012) and in *Neurospora* (J. Galazka and M. Freitag unpubl), however, direct interactions of all four proteins were not found by in vivo proteomic approaches with tagged CENP-S or CENP-X, while analyses with tagged CENP-T at low stringency revealed interactions with most CCAN subunits (Schleiffer et al. 2012).

Tethering experiments suggested that the CENP-T-W-S-X complex, in the presence of CENP-C, is sufficient to nucleate at least partially functional kinetochores, even in the absence of CENP-A (Gascoigne et al. 2011). These interactions rely on direct binding of the CENP-T N-terminal region to the Ndc80 complex via a conserved motif (Schleiffer et al. 2012). The conserved Ndc80 receptor motif of CENP-T/Cnn1 (FIG. 7) is bound in a hydrophobic cleft between Spc24/Spc25 (Malvezzi et al. 2013). Mutations in Spc24/Spc25 disrupting this binding are lethal in yeast. Residues that are important for the interaction in yeast are mostly conserved in other fungi, although some critical residues are different in several species. The precise interactions therefore should be tested by mutational analyses. A similar motif in the Mis12 complex subunit Mis12/Dsn1/Mtw1 (FIG. 7) also was found to be necessary for Ndc80 binding and mutation was lethal in yeast, but replacement with the motif from CENP-T/Cnn1 restored growth, suggesting another modular motif in CCAN and KMN interactions, similar to RWD domains (Malvezzi et al. 2013).

The CENP-T N-terminal domain is phosphorylated by CDK and Mps1 for mitotic progression, coordinating binding of the different partners to the Mis12 complex (Malvezzi et al. 2013). Yeast CENP-T/Cnn1 localizes to the kinetochore from G1 through metaphase and mutation of either the HFD or Spc24/Spc25 interaction domain results in abnormal CENP-T levels at the kinetochore (Thapa et al. 2015). When Mps1 activity decreases (at the onset of anaphase) CENP-T is enriched mostly through its N-terminal Spc24/Spc25 interaction sequence; thus this study provided in vivo evidence of CENP-T enrichment during anaphase.

That CENP-T/Cnn1 relied on CENP-A and CENP-C for targeting to the kinetochore had been established in budding yeast (Bock et al. 2012) and in mammals (Basilico et al. 2014). Studies in fission yeast dissected the importance of the CENP-A N-terminal tail for centromere functions (Folco et al. 2015). An altered

CENP-A tail reduced recruitment of CENP-T and CENP-I but not CENP-C. Overexpression of CENP-T suppressed defects caused by mutation of the CENP-A N-terminal tail mutant. Ndc80 complex localization, however, was not altered, perhaps because CENP-T mutants also do not change Ndc80 recruitment (Tanaka et al. 2009). Thus effects of mutation, deletion and overexpression of CENP-T in different fungi result in different phenotypes. In *Neurospora*, recruitment of CENP-T in CENP-A point mutants or strains with heterologous CENP-A was not altered by cytology and ChIP-seq (P. Phatale, J. Galazka, S. Friedman and M. Freitag unpubl), but this must be addressed further by examining the situation in additional species.

CENP-P-Q-O-U-(R) COMPLEX

This complex was identified as interacting with CENP-H-K-I and as part of the inner centromere complexes (Foltz et al. 2006, Izuta et al. 2006, Okada et al. 2006), and is important in the recovery from spindle damage (Hori et al. 2008b). CENP-R appears to be located most downstream in all assays and is not conserved in fungi, thus may not be a bona fide member of this complex. All other components are interdependent on each other for centromere localization and require the CENP-H-K-I complex (Okada et al. 2006, Hori et al. 2008b). The homologous complex, called COMA, had been isolated earlier from budding yeast, where the CENP-U/Ame1 and CENP-Q/Okp1 subunits are essential (Ortiz et al. 1999). CENP-U mutants are viable in certain mammalian cell lines but essential in mouse embryos and embryonic stem cells (Kagawa et al. 2014). CENP-U is required for recovery from spindle damage, preventing premature sister chromatid separation, a process controlled by CENP-U phosphorylation (Foltz et al. 2006, Hori et al. 2008b). The leucine-zipper domain of CENP-U and the coiled-coil domain of CENP-Q are necessary for interaction (Kang et al. 2011).

Recent evidence from studies with yeast strongly suggests that the COMA (Ctf19, Okp1, Mcm21, Ame1) complex, equivalent to CENP-P-Q-O-U in metazoans respectively, constitutes a third independent linkage between centromeric chromatin and the KMN network, acting via the Mis12 complex (Corbett and Desai 2014, Hornung et al. 2014). Detailed mass spectrometry showed that CENP-U/Ame1 associates with other CCAN proteins as well as KMN complexes, and reconstituted CENP-U-Q/Ame1–Okp1 binds directly to a reconstituted Mis12 complex in vitro. Binding is mediated by a short motif in the Ame1 amino terminus; mutations in this motif abolish Mis12 interactions and are lethal in vivo, causing chromosome missegregation. Thus Ame1 to Mis12 complex interactions are critical for inner-outer kinetochore linkage in budding yeast. Deletion of the Ame1 N-terminal motif reduced both Mis12 and COMA complex localization, suggesting interdependence for assembly, but because Ame1-Okp1 can bind directly to CENP-C/Mif2 and to DNA, at least in vitro, and it was suggested that it forms a link between the CDEIII-binding CBF3 complex and the KMN (Ortiz et al. 1999), it remains possible that there are secondary effects. These interactions have not been studied in any great detail, however. The Mis12-binding motif of Ame1 seems conserved in the CENP-U/Ame1 proteins of other ascomycetes but cannot be found by homology searches in basidiomycetes, where CENP-U is difficult to identify, or in the metazoan CENP-U sequences. In the region of the Mis12-binding motif of Ame1, however, mammalian CENP-U shows similar predicted secondary structure (Hornung et al. 2014).

Identification of the RWD domain, important for many interactions between KMN proteins and the Mis12 complex in particular, suggest that there may be another way that the CENP-U complex can interact with Mis12, in that CENP-P/Ctf19 and CENP-O/Mcm21 can form a heterodimeric RWD domain (Petrovic et al. 2014). While putative CENP-O homologs can be identified from many filamentous ascomycetes CENP-P or Ctf19 homologs have not been identified by homology searches.

ASSEMBLY OF THE INNER KINETOCHORE: WHO'S ON FIRST?

Much recent work has been dedicated to understanding the interactions between CENP-A and the innermost CCAN subunits, including CENP-C, CENP-T (and its potential binding partners, CENP-W-S-X) and the yeast COMA or mammalian CENP-O-P-Q-U complexes (FIG. 7). While ChIP-seq showed that CENP-T and CENP-C are localized to the same regions as CENP-A (Smith et al. 2011, Thakur et al. 2015), they appeared to interact primarily with centromeric H3 oligonucleosomes within CENP-A chromatin (Hori et al. 2008a), which also was confirmed by super-resolution microscopy for CENP-T (Ribeiro et al. 2010). Ordering the recruitment pathway in chicken DT40 cells by analyses of mutants placed CENP-T-W upstream of CENP-H-K-I and the CENP-U complex but parallel with CENP-C (Hori and Fukagawa 2012, Nishino et al. 2012). Thus one model proposes that CENP-C, together with CENP-U and CENP-T, forms a three-legged platform on which the Mis12 and Ndc80 complexes assemble (Gascoigne et al. 2011, Screpanti et al. 2011, Hori and Fukagawa 2012, Hornung et al. 2014). This view is not unchallenged; studies showed dependence of CENP-T assembly on CENP-C (Basilico et al. 2014, Klare et al., Suzuki et al. 2015, Tachiwana

et al. 2015), thus placing CENP-C at the root of CCAN assembly as proposed based on RNAi experiments in human cells (Carroll et al. 2010). Depletion of CENP-C reduces both CENP-T and CENP-H-K-I levels (Carroll et al. 2010, Gascoigne et al. 2011), but depletion of CENP-M did not affect CENP-C or Nsl1 levels yet resulted in reduced CENP-T and Ndc80 levels, where interactions between CENP-M and CENP-T-W seem to be mediated by the HFD (Basilico et al. 2014). Recruitment of Ndc80 complexes by CENP-C and CENP-T was tested by quantitative immunofluorescence of kinetochore proteins in HeLa cells (Suzuki et al. 2015). In these studies a chimeric N-terminal CENP-T with CENP-C DNA-binding domain under CENP-C depletion conditions was used to measure CENP-T-only assemblies. On average there were 244 Ndc80 complexes, 215 CENP-C, 72 CENP-T and 151 Mis12 at a HeLa kinetochore (Susuki et al. 2015). This translates to 13 CENP-C and four CENP-T molecules per MT (nine Mis12 and 14 Ndc80 molecules). Each CENP-T recruits one Mis12/KMN network and one Ndc80 but only ~ 38% of CENP-C proteins recruit a KMN. The CENP-T-only chimeric linkage to the KMN resulted in functional kinetochores with the expected numbers of Ndc80 and Mis12 proteins. It is still unclear why both CENP-C and CENP-T are needed, but the authors suggested that it is the total number of Ndc80 complexes recruited that determines functionality of the kinetochore (Suzuki et al. 2015).

What emerges from the studies discussed above is a partial feedback loop of assembly (FIG. 7D) that is subject to cell cycle control. CENP-C and CENP-L-N interact directly with the CENP-A nucleosome; CENP-L-N requires CENP-C for recruitment. CENP-C also appears to be essential for the recruitment of both CENP-T-W-S-X and CENP-H-K-I bound to CENP-M, and these two complexes are interdependently recruiting each other (Basilico et al. 2014). However, there are conflicting studies available on this point, in that recruitment pathways to artificially tethered subcomplexes were in parallel and not hierarchical. CENP-C-driven KMN assembly controlled by Aurora B and CENP-T-driven KMN assembly controlled by CDK (Rago et al. 2015) and multiple depletion studies also suggested parallel pathways (Kim and Yu 2015, Susuki et al. 2015). In parallel to the CENP-T and CENP-C axes, CENP-U binds to the Mis12 complex, thus connecting DNA- or chromatin-binding activities of CBF3 or the other CENP-O-P-Q-U subunits (Hornung et al. 2014), but this needs to be established in other organisms as well. Thus while CENP-T-W-S-X directly interacts with the Ndc80 complex Mis12 integrates the binding of the other CCAN complexes. It remains to be seen whether CENP-T, CENP-U and CENP-C (the latter via Mis12) are connected to Ndc80 complexes at the same time. It is already clear that binding to one heterodimeric Spc24/Spc25 RWD domain is exclusive: either Mis12 or CENP-T are bound. It is likely that Ndc80 complexes in one kinetochore use both CENP-T and Mis12 as binding partners, however, and that CENP-T binding is dependent on its phosphorylation by CDK during mitosis (Malvezzi et al. 2013, Nishino et al. 2013).

CONCLUSION

Centromeres and kinetochores of filamentous fungi appear to span the gap between those of *S. cerevisiae* and *S. pombe* to those of animals. Filamentous fungi offer now almost the same experimental advantages for the analyses of kinetochore proteins and their interactions as the yeasts. The availability of hundreds of genome sequences allows in-depth studies on the relatedness of centromere and kinetochore proteins and will help to decipher truly conserved versus specialized mechanisms of kinetochore function. That some specialization has occurred and that KIN components may be useful to study adaptive evolution is exemplified by CCAN complements of Dothideomycetes. While homologs can be found for most known components in *Leptosphaeria maculans* and *Pyrenophora* species, these proteins (save for CENP-A and CENP-C) cannot be found in all known *Zymoseptoria* species when relying on homology searches.

Thus the composition of CCAN and even some KMN complexes within all fungi is still unresolved (SUPPLEMENTARY TABLES I–IV). This is not a unique situation in that some well-studied model organisms (e.g. *Drosophila melanogaster* and *Caenorhabditis elegans*) also have incomplete sets of kinetochore proteins. For example, most CCAN subunits conserved in ascomycetes and mammals cannot be found in either species by homology searches. For the fungi it seems logical to use proteomic approaches that have been applied with such success in yeast, chicken and human cells to identify more components of centromeres and kinetochores in the basal lineages and the basidiomycetes. Because there are well known and intensely studied pathogens present in all these lineages there should be sufficient impetus to conduct these studies. Related to this, because filamentous fungi are exceedingly successful pathogens and few dedicated antifungals currently are available there seems to be ample opportunity to screen for or design small-molecule drugs that will interfere with CCAN and KMN function in specific taxa only. That competition between proteins can occur has been shown in studies with viruses, where HPV18 protein E7 can compete with CENP-C binding (Yaginuma et al. 2015).

This application-oriented goal will need to rely on a complete understanding of interactions within KIN subcomplexes. Over the past four y interaction domains between the Ndc80, Mis12 and CENP-T complexes have emerged and these will need to be characterized further. RWD domains are clearly conserved in the ascomycetes, but inspection of 26 taxa from various groups of fungi also revealed that multiple additional conserved regions are present, most of which are lacking in yeasts or animals. Swapping domains of CCAN or KMN proteins among closely or distantly related fungi may help decipher mechanistic details that will not be uncovered when the budding yeast protein is introduced into human cells or vice versa. Thus comparative biology on single domains will help to distinguish between generally applicable mechanisms and interactions that are taxon-specific. Specific examples of interactions that need to be examined in more detail are those between Dam1 and Ndc80, the function of the exposed Ndc80 loop, interactions between Knl1, Mis12 and the Spc24/Spc25 heterodimeric RWD domain, the true nature of the CENP-T complex and potential adaptive nature of all CCAN complexes.

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