

Evolutionary Lessons from Species with Unique Kinetochores

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Abstract The kinetochore is the multi-protein complex that drives chromosome segregation in eukaryotes. It assembles onto centromeric DNA and mediates attachment to spindle microtubules. Kinetochore research over the last several decades has been focused on a few animal and fungal model organisms, which revealed a detailed understanding of the composition and organization of their kinetochores. Yet, these traditional model organisms represent only a small fraction of all eukaryotes. To gain insights into the actual degree of kinetochore diversity, it is critical to extend these studies to nontraditional model organisms from evolutionarily distant lineages. In this chapter, we review the current knowledge of kinetochores across diverse eukaryotes with an emphasis on variations that arose in nontraditional model organisms. In addition, we also review the literature on species, in which the subcellular localization of kinetochores has changed from the nucleoplasm to the nuclear membrane. Finally, we speculate on the organization of the chromosome segregation machinery in an early eukaryotic ancestor to gain insights into fundamental principles of the chromosome segregation machinery, which are common to all eukaryotes.

1 Introduction

Mitosis is the process that partitions newly replicated chromosomes from the mother cell into the two emerging daughter cells (McIntosh 2016). Fundamental to this process is the kinetochore, a macromolecular protein complex that assembles onto specialized chromosomal regions called centromeres to mediate the attachment

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of sister chromatids to spindle microtubules (Cheeseman and Desai 2008; Santaguida and Musacchio 2009). Kinetochores also promote the recruitment of cohesin complexes around centromeres to hold duplicated sister chromatids together until anaphase (Nasmyth and Haering 2009). At their DNA-binding interface, kinetochores need to ensure stable attachment to tolerate the pulling forces exerted by kinetochore microtubules (Allshire and Karpen 2008; Fukagawa and Earnshaw 2014; Westhorpe and Straight 2015; McKinley and Cheeseman 2016). In contrast to this more static attachment, the binding to spindle microtubules must be dynamically regulated (Foley and Kapoor 2013; Cheerambathur and Desai 2014; London and Biggins 2014; Etemad and Kops 2016). Faithful chromosome segregation requires that sister kinetochores form bioriented attachments to spindle microtubules emanating from opposite poles (Nicklas 1997). Biorientation is necessary for the accurate distribution of sister chromatids into daughter cells during anaphase.

Research on kinetochores has mainly been performed on a few model organisms, such as fungi, worms, flies, and vertebrates. Their studies have been instrumental in informing us about the basic composition and organization of kinetochores among these species. However, these “traditional” model organisms only represent a small fraction of the entire eukaryotic biodiversity. In fact, both animals and fungi are members of the Opisthokonta, that is only one out of six major supergroups of eukaryotes (Fig. 1) (Walker et al. 2011; Adl et al. 2012). While extensive analyses have not been performed on kinetochores in non-opisthokonts, glances into kinetochores from additional species scattered across the eukaryotic phylogenetic tree have revealed extraordinary levels of variations in kinetochore composition and subcellular location. This stands in sharp contrast to many other cell cycle machines that are highly conserved among diverse eukaryotes (e.g., Cyclin/CDK, cohesin, condensin, the anaphase promoting complex, and proteasomes). In this chapter, we will first discuss the extent of similarity and variation in kinetochore composition among animals and fungi. We will then review kinetochores in select organisms from different supergroups, as well as unique kinetochores that evolved in kinetoplastids. Following up on that, we will highlight membrane-bound kinetochores found in some unicellular organisms. Finally, we will speculate on the organization of the chromosome segregation machinery in early eukaryotes.

2 The Kinetochore Complex in Animals and Fungi

Genetic and biochemical analyses in fungi and vertebrates have led to the identification of more than 80 proteins that are part of the kinetochore (Biggins 2013; Cheeseman 2014). The structural core of the kinetochore consists of an inner and an outer complex. The inner kinetochore complex binds centromeric chromatin. It serves as a platform for the recruitment of the outer kinetochore complex that binds spindle microtubules during mitosis and meiosis. Both complexes are characterized

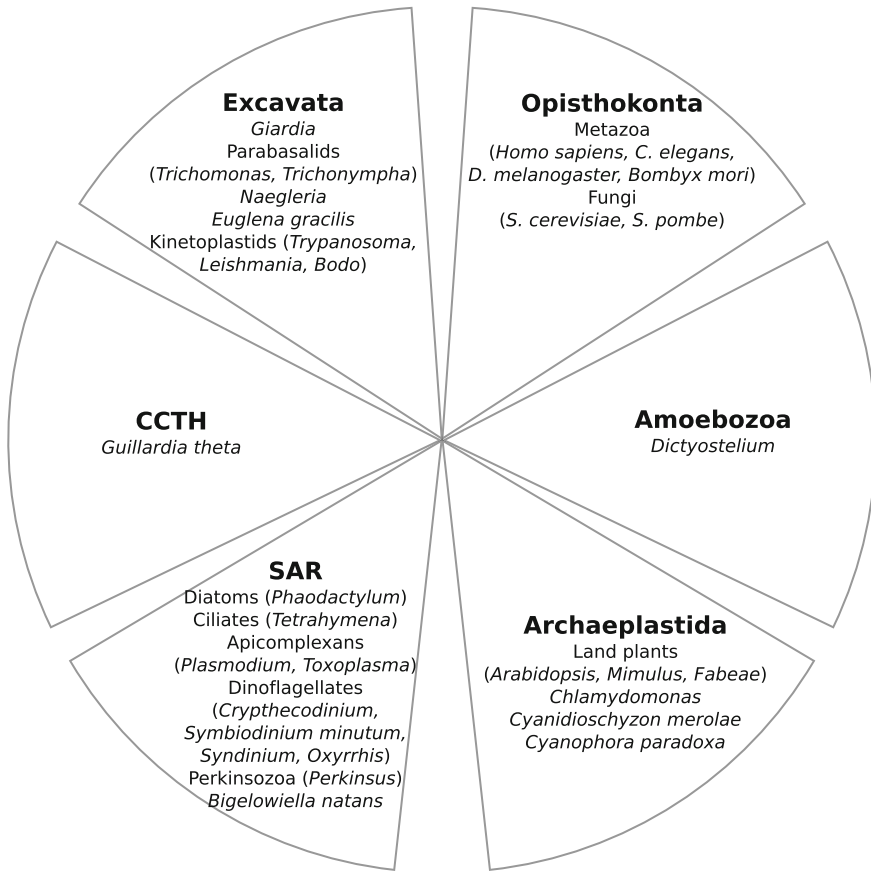


Fig. 1 Six eukaryotic supergroups. Representative organisms from each supergroup are shown as examples

by a network of several protein complexes that work in concert to regulate the proper attachment of kinetochore microtubules to centromeric DNA.

2.1 Similarities and Variations of the Inner Kinetochore in Animals and Fungi

In vertebrates and fungi, the inner kinetochore consists of ~16 members that are commonly referred to as the Constitutive Centromere Associated Network or CCAN (Cheeseman and Desai 2008; Westermann and Schleiffer 2013; Westhorpe and Straight 2013; Fukagawa and Earnshaw 2014) (Table 1). It is generally agreed that the recruitment of all CCAN members in these species depends on a specialized

centromeric histone H3 variant, CENP-A (also known as CenH3—see the Note on nomenclature at the end of this chapter) (Black and Cleveland 2011; Müller and Almouzni 2014; Earnshaw 2015) and its direct DNA-binding partner CENP-C (Carroll et al. 2010; Basilico et al. 2014). In addition to CENP-A and CENP-C, other CCAN components also make DNA contacts, including the histone-fold proteins CENP-T and CENP-W as well as CENP-U^{Ame1} and CENP-Q^{Okp1} in budding yeast (Hori et al. 2008; Hornung et al. 2014).

Given their central role in kinetochore function, it is surprising that several inner kinetochore components undergo rapid evolution at the amino acid level, which complicates homology-based predictions even in well-sequenced species (Henikoff et al. 2001; Talbert et al. 2009; Malik and Henikoff 2009). While sequence similarity of several CCAN components between vertebrates and budding yeast was revealed early on (Meraldi et al. 2006), the identification of phylogenetic relationship for other CCAN components often required advanced bioinformatics tools due to limited sequence similarities (Schleiffer et al. 2012; Westermann and Schleiffer 2013). For example, the budding yeast CENP-T^{Cnn1} was only identified using a combination of proteomic approaches and remote homology predictions (Schleiffer et al. 2012). Thus, experimental approaches as well as advanced bioinformatics are required to obtain a comprehensive picture of kinetochores.

While most CCAN components are conserved between vertebrates and fungi, CCAN proteins appear to be absent in *Caenorhabditis elegans* and *Drosophila melanogaster* except for CENP-C (Table 1). While it is formally possible that these species have highly divergent CCAN members, the wealth of extensive genetic screens for chromosome segregation defects and biochemical purifications of kinetochore components makes this unlikely (Cheeseman et al. 2004; Goshima et al. 2007; Przewloka et al. 2007, 2011). Therefore, it appears that nematodes and Diptera have “simpler” inner kinetochore complexes that just consist of CENP-C, which connects the CENP-A-containing chromatin to outer kinetochore proteins. The reason for this potential reduction in kinetochore complexity remains unknown. In contrast to *D. melanogaster*, homologous CCAN members have been identified in other insects (see below), showing that the near-complete loss of CCAN is not common to all insects and instead occurred in a dipteran ancestor around 250 Mya (Hedges et al. 2006).

While CENP-A was thought to be essential for kinetochore assembly in all animals and fungi, recent studies showed that a number of insects have recurrently lost CENP-A (Drinnenberg et al. 2014). Intriguingly, all CENP-A-deficient insects analyzed are derived from independent transitions from monocentric chromosomes (where microtubules attach to a single chromosomal region) to holocentric chromosomes (where microtubules attach along the entire length of the chromosome) (Melters et al. 2012; Drinnenberg et al. 2014). This strong correlation between the change in centromeric architecture and the loss of CENP-A supports a causal relationship between the two events in that the transition to holocentromeres facilitated the loss of CENP-A or vice versa. While CENP-A and its binding partner CENP-C are lost, several of the CCAN components continue to be present even in CENP-A-deficient insects (e.g., the silkworm *Bombyx mori* (Table 1)). These findings suggest that the

Table 1 List of putative kinetochore proteins in select eukaryotes

Centromere/kinetochore protein		Opisthokonta					
	<i>Homo sapiens</i>	<i>Saccharomyces cerevisiae</i>	<i>Schizosaccharomyces pombe</i>	<i>Caenorhabditis elegans</i>	<i>Drosophila melanogaster</i>	<i>Bombyx mori</i>	
CENP-A	CENP-A	Cse4	Cnp1	HCP-3	Cid		
CENP-C	CENP-C	Mhf2	Cnp3	HCP-4	CENP-C		
CENP-H	CENP-H	Mcm16	Fta3				
CENP-I	CENP-I	Ctf3	Mis6			XP_004931054.2	
CENP-K	CENP-K	Mcm22	Sim4				
CENP-L	CENP-L	Iml3	Fta1			NP_001266344.1	
CENP-M	CENP-M					XP_004928337.1	
CENP-N	CENP-N	Chl4	Mis15			XP_004922229.1	
CENP-O	CENP-O	Mcm21	Mal2				
CENP-P	CENP-P	Ctf19	Fta2				
CENP-Q	CENP-Q	Okp1	Fta7				
CENP-U	CENP-U	Ame1	Mis17				
CENP-T	CENP-T	Cnn1	Cnp20				
CENP-W	CENP-W	Wip1	New1				
CENP-S	CENP-S	Mhf1	Mhf1	CCD31143		NP_001266386.1	
CENP-X	CENP-X	Mhf2	Mhf2	CCD69638		NP_001268381.1	
Mis12	Mis12	Mtw1	Mis12	Mis12	Mis12	fcaL18J21	
Nnf1	PMF1	Nnf1	Nnf1	KBP-1	Nnf1a, Nnf1b	XP_004928275.1	
Dsn1	Dsn1	Dsn1	Mis13	KNL3		XP_004928630.1	
Nsl1	DC8	Nsl1	Mis14	KBP-2	Kmm1		
Kn11	KNL1	Spe105	Spe7	KNL1	Spe105R	XP_012546088.1	
Ndc80	Hec1	Ndc80	Ndc80	Ndc80	Ndc80	XP_012545504.1	
Nuf2	Nuf2	Nuf2	Nuf2	Nuf2	Nuf2	XP_004929441.1	
Spe24	Spe24	Spe24	Spe24	KBP-4	Kmm2	XP_004925111.1	
Spe25	Spe25	Spe25	Spe25	KBP-3	Spe25	NP_001040214.1	

Table 1 (continued)

Centromere/kinetochore protein	Amoebozoa	Archaeplastida	<i>Chlamydomonas reinhardtii</i>	<i>Cyanidioschyzon merolae</i>	<i>Cyanophora paradoxa</i>
CENP-A	<i>Dictyostelium discoideum</i> Q54F38.1	<i>Arabidopsis thaliana</i> HTR12	EDP07681.1 and/or EDO99752.1	BAM7932.1.1	Contig11189-snap-gene-0.0
CENP-C		CENP-C	XP_001701669.1	BAM80204.1	
CENP-H					
CENP-I	XP_635116.1				
CENP-K					
CENP-L	XP_635393.1				
CENP-M					
CENP-N					
CENP-O		NP_001190285.1			
CENP-P					
CENP-Q					
CENP-U					
CENP-T					
CENP-W					
CENP-S	XP_638695.1	NP_199906.1			
CENP-X	XP_003290871.1	NP_178000.2		BAM78991.1	
Mis12	XP_638733.1	Mis12		BAM81376.1	Contig39033-abinit-gene-0.2
Nnf1	XP_641683.1	AAO39938.1	XP_001697791.1	BAM78899.1	Contig54317-snap-gene-0.0
Dsn1					Contig25943-abinit-gene-0.0
Nsl1			XP_001703375.1		
Kn1l	XP_639120.1	NP_671774.4	XP_001690815.1	BAM81078.1	
Ndc80	XP_638830.1	NP_191024.1	XP_001699834.1	BAM79598.1	Contig10691-abinit-gene-0.1
Nuf2	XP_641696.1	NP_176296.2	XP_001700269.1	BAM78839.1	Contig53383-abinit-gene-0.8
Spe24	XP_638781.1	NP_187500.2	XP_001697066.1	BAM79682.1	Contig54569-abinit-gene-0.4
Spe25	XP_638784.1	NP_566900.1	XP_001697914.1	BAM79992.1	Contig26077-abinit-gene-0.7

Table 1 (continued)

Centromere/ kinetochore protein	SAR									
	<i>Phaeoactylum tricornutum</i>	<i>Terrahymena thermophila</i>	<i>Plasmodium falci-parum</i>	<i>Toxoplasma gondii</i>	<i>Symbiodinium minutum</i>	<i>Bigeloviella nautans</i>				
CENP-A	EEC43222.1	Q2N2K6.1	CAD52476.1	EPT27047.1	symbB1.v1.2.034671.tl	aug1.3_g1557				
CENP-C	XP_002184047.1		CZT98473.1			fgenesH_pg.119_#_34				
CENP-H	XP_002177997.1									
CENP-I										
CENP-K										
CENP-L	XP_002185884.1									
CENP-M										
CENP-N										
CENP-O										
CENP-P										
CENP-Q										
CENP-U										
CENP-T										
CENP-W										
CENP-S	XP_002180057.1					aug1.3_g1673				
CENP-X						aug1.96_g19929				
Mis12	XP_002179310.1					fgenesH_pg.45_#_162				
Nnf1	XP_002180232.1				symbB.v1.2.039506.tl					
Dsn1										
Ns11										
Kn11						fgenesH_pg.8_#_205				
Ndc80	XP_002177760.1	XP_001024017.1	XP_966148.1	XP_002365215.1	symbB.v1.2.002582.tl	estExt_fgenesH_pg.C_190200				
Nuf2	XP_002185912.1		XP_001351235.1	XP_002364205.1	symbB.v1.2.033878.tl	e_gw1.128.32.1				
Spe24	XP_002182127.1					estExt_fgenesH_pg.C_680067				
Spe25	XP_002178778.1	XP_001030098.2	XP_002809072.1	XP_002368113.1	symbB.v1.2.035905.tl	aug1.38_g12030				

Table 1 (continued)

Centromere/ kinetochore protein	CCTH		Excavata		<i>Euglena gracilis</i>
	<i>Guillardia theta</i>		<i>Giardia intestinalis</i>	<i>Naegleria gruberi</i>	
CENP-A	EKX34792.1 (nucleus) AAK39657.1? (nucleomorph)		EFO62248.1	EAY15370.1	dark_m.11114 and/or dark_m.83792
CENP-C	EKX50218.1			XP_001311235.1	EFC46326.1
CENP-H					
CENP-I					
CENP-K	XP_005834533.1				
CENP-L					
CENP-M					
CENP-N					
CENP-O					
CENP-P					
CENP-Q					
CENP-U					
CENP-T					
CENP-W					
CENP-S				XP_001306373.1	
CENP-X				XP_001579665.1	
Mis12	XP_005839738.1			XP_001308419.1	EFC50450.1
Nnfl	XP_005820862.1			XP_001307740.1	
Dsn1					
Nsl1	XP_005822661.1				
Knll	XP_005832827.1				EFC50203.1
Ndc80	EKX45170.1			XP_0013111732.1	dark_m.19013
Nuf2	CAC27079.1		EFO61302.1		dark_m.38633
Spe24	EKX52051.1		EDO78311.1		
Spe25	EKX40188.1		EFO63967.1	XP_001323375.1	light_m.34007

The composition of the outer kinetochore, not inner kinetochore, is widely conserved. In those cases where multiple homologous proteins are identified, only one is listed unless otherwise specified. Note that kinetochore localization has yet to be established for many of the proteins listed here. CENP-S and CENP-X have independent roles in DNA repair and may not be involved in CCAN function in some organisms. KNLL1 homologs were predicted in Tromer et al. (2015)

assembly of the inner kinetochore has been altered in CENP-A-deficient insects, allowing CENP-A-independent kinetochore formation. Whether or not new kinetochore components have evolved to compensate for the loss of CENP-A is an open question. It is important to note that other holocentric organisms including nematodes (e.g., *C. elegans*) have retained CENP-A^{HCP-3}. Thus, despite the usage of the generic term “holocentromere”, the basic architecture and regulation of holocentromeres is likely to be diverse among different species.

2.2 The Composition of the Outer Kinetochore Is Highly Conserved in Animals and Fungi

The outer kinetochore complex is recruited to centromeres upon the onset of mitosis to connect to spindle microtubules. This interaction is accomplished by the ~10-subunit KMN network that consists of the Knl1, Mis12, and Ndc80 complexes (Cheeseman et al. 2006; Petrovic et al. 2014). In contrast to the inner kinetochore, the composition of the outer kinetochore is widely conserved across animals and fungi (Meraldi et al. 2006; Tromer et al. 2015). Even CENP-A-deficient insects encode the same repertoire of outer kinetochore components, implying similar means of attaching to microtubules while utilizing alternate inner kinetochore assembly pathways (Drinnenberg et al. 2016). A notable exception to the otherwise conserved composition of the KMN network is found in Diptera. *D. melanogaster* has lost Dsn1, a subunit of the Mis12 complex (Przewloka and Glover 2009). In addition, the Nnf1 subunit of the Mis12 complex underwent a duplication event giving rise to two paralogs, Nnf1a and Nnf1b, that are part of two distinct Mis12 complexes with similar biochemical behaviors (Przewloka et al. 2007; Schittenhelm et al. 2007; Liu et al. 2016; Richter et al. 2016; Blattner et al. 2016). The loss of Dsn1 could have been compensated by the C-terminal part of the *Drosophila* Knl1 homolog (Przewloka et al. 2009). Indeed, the overall organization of this complex appears to resemble the human and yeast counterparts (Hornung et al. 2011; Przewloka et al. 2011; Screpanti et al. 2011). Whether these changes have any functional consequences on the *Drosophila* KMN complex is currently unclear.

3 Glimpses into Kinetochore Compositions in Diverse Eukaryotes

While research on kinetochores in fungi and animals has revealed a paradigm for the basic organization of kinetochores, it remains unclear whether other eukaryotes have similar kinetochores. Comparative studies in additional eukaryotic lineages are a key to revealing the degree of conservation and divergence of kinetochores among

eukaryotes. Although bioinformatic analyses have identified some homologous kinetochore proteins in diverse eukaryotes (Table 1), very few studies have characterized the function of individual kinetochore proteins. Furthermore, extensive proteomic screens have not been carried out in most organisms, leaving open the possibility of lineage-specific evolution of additional kinetochore proteins. Below we summarize the current knowledge of kinetochores in select eukaryotes from different supergroups to highlight their peculiarities.

3.1 *Supergroup Amoebozoa*

The only kinetochore protein that has been characterized in the supergroup Amoebozoa is the centromere-specific histone H3 variant in *Dictyostelium discoideum* (Dubin et al. 2010). In contrast to nearly all other characterized CENP-A proteins that have at least one extra amino acid in the loop 1 region within the histone fold compared to histone H3 (Malik and Henikoff 2003), *D. discoideum* CENP-A^{CenH3} does not have a longer loop 1. While alterations or shortening of residues in loop 1 in other species can impair centromere targeting (Vermaak et al. 2002), cytological studies of *D. discoideum* CENP-A^{CenH3} revealed incorporation into centromeric DNA (Dubin et al. 2010). Therefore, the insertion of extra amino acids in loop 1 is not an obligatory feature of CENP-A.

3.2 *Supergroup Archaeplastida*

Several kinetochore proteins have been characterized in land plants (e.g., *Arabidopsis*, maize, and barley) (Dawe et al. 1999; ten Hoopen et al. 2000; Sato et al. 2005). For example, homologous kinetochore proteins (such as CENP-C and Mis12) identified by bioinformatics searches were analyzed by means of cytological and mutational studies, confirming their importance for chromosome segregation in mitosis and meiosis. Although most eukaryotes have a single CENP-A protein, multiple CENP-A^{CENH3} variants are found in *Arabidopsis halleri*, *A. lyrata* (Kawabe et al. 2006), *Brassica* sp. (Wang et al. 2011), *Mimulus* monkeyflowers (Finseth et al. 2015), barley (Ishii et al. 2015), and *Fabaeae* sp. (Neumann et al. 2012; Neumann et al. 2015). While it is currently unclear whether the individual CENP-A^{CENH3} variants are functionally distinct, it has been hypothesized that CENP-A^{CENH3} duplications occurred to counteract the evolutionary force from centromere drive (Finseth et al. 2015) (centromere drive is discussed in the chapter “Cell Biology of Cheating—Transmission of Centromeres and Other Selfish Elements Through Asymmetric Meiosis” by Chmátal et al.).

Compared to land plants, much less is known about kinetochores in other Archaeplastida species. *Cyanidioschyzon merolae* is a thermoacidiphilic red alga that is thought to be one of the most primitive photosynthetic eukaryotes. Its simple cellular architecture and reduced genome make it an attractive organism for cell

biological study (Matsuzaki et al. 2004). Among several homologous kinetochore proteins identified (Table 1), only CENP-A^{CENH3} has been experimentally characterized to date (Maruyama et al. 2007; Kanasaki et al. 2015). Given its hot and acidic living habitats, it will be interesting to test for potential adaptations of kinetochore components that evolved to cope with such extreme environments.

3.3 *Supergroup SAR*

The supergroup SAR (Stramenopiles, Alveolates, and Rhizaria; also referred to as Harosa) includes diatoms, ciliates, apicomplexans, and dinoflagellates (discussed later). Ciliates have a somatic macronucleus with highly amplified genes for RNA synthesis as well as several germline micronuclei for genome maintenance. The number of chromosomes in the somatic macronucleus can be as high as 16,000 in some species (Swart et al. 2013). While the germline micronucleus has CENP-A^{CNA1} and segregates its chromosomes accurately, the somatic macronucleus does not have CENP-A^{CNA1} and segregates its chromosomes randomly (Cervantes et al. 2006; Cui and Gorovsky 2006).

Apicomplexans include a number of important human pathogens, including *Plasmodium* and *Toxoplasma* (Francia and Striepen 2014). Several kinetochore proteins have been identified and functionally characterized in *Plasmodium falciparum* and *Toxoplasma gondii* including CENP-A^{CENH3}, CENP-C, and members of the Ndc80 complex (Brooks et al. 2011; Verma and Surolia 2013; Farrell and Gubbels 2014). While the domain architecture appears to be conserved, the *T. gondii* Nuf2 homolog contains a conserved amino acid motif that appears specific to apicomplexan (Farrell and Gubbels 2014). The functional relevance of this motif, however, remains unclear.

3.4 *Supergroup CCTH*

Very little is known about kinetochores in the supergroup CCTH (Cryptophytes, Centrohelids, Telonemids, and Haptophytes; also called Hacrobia). Cryptophyte algae are thought to have evolved by engulfing a red alga that contained a primary plastid (Tanifuji and Archibald 2014). In the cryptomonad *Guillardia theta*, the secondary plastid has retained the red algal-derived relict nucleus (called nucleomorph) (Curtis et al. 2012). How the nucleomorph genome is maintained during cell division remains unknown. While the nucleomorph genome encodes for a putative CENP-A homolog (Douglas et al. 2001) (Table 1), this protein lacks the hallmark of an extended loop 1 region. It will therefore be necessary to experimentally confirm whether it indeed functions as the centromeric histone variant for the segregation of the nucleomorph genome.

3.5 Supergroup Excavata

Excavata is a group of predominantly flagellated species (Walker et al. 2011; Adl et al. 2012). It is divided into Metamonads and Discoba. A number of human parasites belong to this supergroup, such as *Giardia*, *Trichomonas vaginalis*, *Naegleria fowleri*, and *Trypanosoma brucei*.

Giardia intestinalis (Metamonads) has two histone H3-like molecules that have a longer loop 1. Cytological studies have revealed that only one H3 variant incorporates into centromeres, while the other variant localizes to pericentric heterochromatin (Dawson et al. 2007), underlining the need for experimental approaches to corroborate the identity of the centromeric histone H3 variant. As in *Dictyostelium* (see above), another metamonad *Trichomonas vaginalis* has a CENP-A^{CenH3} protein that does not have a longer loop 1, but its localization pattern is suggestive of centromeric incorporation (Zubáková et al. 2012).

Discoba (also called JEH for Jakobids, Euglenozoa, Heterolobosea) includes *Naegleria*, *Euglena*, and kinetoplastids. Although canonical kinetochore proteins have been identified in *Naegleria gruberi* and *Euglena gracilis* (Table 1), none has been identified in the genome of kinetoplastids.

4 Unconventional Kinetoplastid Kinetochores

Identification of at least a fraction of canonical kinetochore proteins (especially CENP-A and the Ndc80 complex) in diverse eukaryotes led to a notion that all eukaryotes may build the structural core of the kinetochore using a conserved set of kinetochore proteins (Meraldi et al. 2006). However, none of the canonical kinetochore proteins were identified in the genome of kinetoplastids (Lowell and Cross 2004; Berriman et al. 2005), a group of unicellular eukaryotes defined by the presence of kinetoplast (a large structure in the mitochondrion that contains mitochondrial DNA) (Vickerman 1962). They belong to the supergroup Excavata, Discoba group, Euglenozoa. Euglenozoa is a diverse group of flagellates that include euglenids, diplomonads, symbionts, and kinetoplastids (Walker et al. 2011; Cavalier-Smith 2016).

To uncover the repertoire of kinetoplastid kinetochores, recent studies utilized proteomic and functional approaches and identified 20 kinetochore proteins in *Trypanosoma brucei*, named KKT1–20 (Akiyoshi and Gull 2014; Nerusheva and Akiyoshi 2016). The majority of these proteins are conserved among kinetoplastids, including the free-living *Bodo saltans*. However, obvious orthologs of KKT proteins were not found even in euglenids, which instead have canonical kinetochore proteins (Akiyoshi 2016). The unique KKT-based kinetochores are therefore not conserved across Euglenozoa but are apparently restricted to kinetoplastids. It remains unclear why kinetoplastids possess a unique set of kinetochore proteins (discussed below).

4.1 Domain Architectures of Kinetoplastid Kinetochores Proteins

Sequence analyses of kinetoplastid kinetochores proteins have revealed the following conserved domains: a BRCT (BRCA1 C terminus) domain in KKT4, FHA (Forkhead-associated) domain in KKT13, WD40-like domain in KKT15, divergent polo boxes (DPB) in KKT2, KKT3 and KKT20, unique protein kinase domain in KKT2 and KKT3, and CLK (cdc2-like kinase) kinase domain in KKT10 and KKT19. While orthologs of any of the KKT proteins have not been identified in non-kinetoplastid species, the domain architecture and sequence similarity of KKT2, KKT3, and KKT20 suggest that these proteins may share common ancestry with a Polo-like kinase (PLK) (Nerusheva and Akiyoshi 2016). Consistent with this possibility, although the kinase domain of KKT2/3 is apparently unique (Parsons et al. 2005), the next closest kinase domain is that of PLK (Akiyoshi 2016). Furthermore, putative DNA-binding motifs are present in KKT2 and KKT3, suggesting that these proteins likely bind DNA and play a critical role in establishing unique kinetochores in kinetoplastids. Although PLK localizes at the kinetochores in some species, it is not considered to be a structural kinetochores protein in any eukaryote. Substrates of these KKT kinases have yet to be identified.

BRCT, FHA, or CLK-like kinase domains are not present in canonical kinetochores proteins. Domains found in canonical kinetochores proteins such as CH (calponin homology) and RWD (RING finger, WD repeat, DEAD-like helicases) domains have not been identified in KKT proteins. Although KKT proteins do not have similarity to canonical kinetochores proteins at the primary sequence level, high-resolution structural data are necessary to reveal if there is any similarity at the tertiary level.

4.2 Common Features

Although components of the core kinetoplastid kinetochores appear to be distinct from canonical kinetochores proteins present in other eukaryotes, various regulatory proteins that are known to be important for chromosome segregation are conserved, including Aurora B, Cyclin/CDK, cohesin, condensin, separase, and the anaphase promoting complex (Berriman et al. 2005; Akiyoshi and Gull 2013). Aurora B apparently localizes at the kinetochores during prometaphase and metaphase in *Trypanosoma brucei* (Li et al. 2008), suggesting that its kinetochores regulatory function may be conserved. It is known that the kinase-phosphatase balance is important for regulating kinetochores functions in other eukaryotes. For example, the KNL1 outer kinetochores protein recruits the PP1 phosphatase (Liu et al. 2010; Rosenberg et al. 2011; Meadows et al. 2011; Espeut et al. 2012). Interestingly, a conserved PP1-binding motif is present in KKT7, suggesting that PP1 may regulate kinetochores functions in kinetoplastids. It is therefore possible that kinetoplastid

kinetochores, while being structurally distinct, may still utilize a conserved mechanism for the regulation of kinetochore functions.

4.3 Implications from Kinetoplastid Kinetochores

The discovery of KKT-based kinetochores in kinetoplastids challenged a widely held assumption that the core of the kinetochore would be composed of proteins conserved throughout eukaryotes (e.g., CENP-A and Ndc80). A corollary is that eukaryotic chromosome segregation can be achieved using proteins distinct from CENP-A or Ndc80. Understanding how KKT proteins carry out the conserved kinetochore functions will likely provide important insights into fundamental principles of the kinetochore. It also raises a possibility that there might be as yet different types of kinetochores to be discovered in eukaryotes.

5 Membrane-Embedded Kinetochores

In addition to compositional variations, the subcellular location of kinetochores has also been altered in some lineages. In all eukaryotes, chromosomes are enclosed inside the nuclear envelope during most of the cell cycle. This keeps chromosome-based activities physically separated from the cytoplasm where protein synthesis and metabolic processes take place (Martin and Koonin 2006; Koumandou et al. 2013). This separation necessitates proper nuclear remodeling to be coordinated with the chromosome segregation apparatus. There are mainly three types of mitoses depending on the extent of nuclear envelope breakdown: open, semi-open, and closed (Sazer et al. 2014; Makarova and Oliferenko 2016). In open mitosis, the nuclear envelope breaks down completely during mitosis, facilitating access for cytoplasmic spindle microtubules to chromosomes. Semi-open mitosis involves a partial breakdown of the nuclear envelope, allowing transport of material while keeping chromosomes inside the nucleus. In this case, the spindle assembles either inside or outside of the nucleus. In the latter case, spindle microtubules appear to fenestrate through the nuclear envelope and capture chromosomes that are located inside the nucleus. Finally, in closed mitosis, the nuclear envelope does not break down. To enable capturing of sister chromatids, most eukaryotes with closed mitosis assemble an intranuclear spindle. Some eukaryotes, however, assemble an extranuclear spindle where spindle microtubules are located outside of the nucleus. This type of mitosis, though not very common, is found in some Alveolata (dinoflagellates and Perkinsozoa) and Parabasalids (Trichomonads and Hypermastigia), suggesting that it arose independently. To enable attachments between spindle microtubules and kinetochores, these organisms embed their kinetochores in the nuclear envelope. Below we will summarize the current

literature on these organisms as well as their sister species and then discuss potential adaptations and implications from such kinetochores.

5.1 *Dinoflagellates*

Dinoflagellates are a highly diverse group of flagellates, including photosynthetic free-living and parasitic species (Taylor et al. 2007). They belong to the supergroup SAR, Alveolata group, and their sister groups include Perkinsozoa and Apicomplexa (Saldarriaga et al. 2004) (Fig. 2). Dinoflagellates are characterized by large genome sizes in the range of 1,500 Mbp to 185,000 Mbp (Wisecaver and Hackett 2011). Despite having all core histone genes, histones are not involved in packaging the majority of nuclear DNA (Hackett et al. 2005; Marinov and Lynch 2015). In addition, other basic nuclear proteins including Dinoflagellates/Viral NucleoProteins (DVNPs) and HU-like proteins might substitute major histone functions in some of these organisms (Sala-Rovira et al. 1991; Chan and Wong 2007; Gornik et al. 2012; Talbert and Henikoff 2012; Bachvaroff et al. 2014). Their chromosomes are permanently condensed, showing a characteristic liquid crystalline state even in interphase. Interestingly, some, but not all, dinoflagellates have kinetochores embedded in the nuclear envelope with an extranuclear spindle (Leadbeater and Dodge 1967; Kubai and Ris 1969; Spector and Triemer 1981).

Dinoflagellates are divided into core dinoflagellates, Syndiniales, and early diverging Oxyrrhinales (Fig. 2) (Wisecaver and Hackett 2011). Electron microscopy revealed that kinetochores are embedded in the nuclear envelope in core dinoflagellates [e.g., *Amphidinium* (Oakley and Dodge 1974) and *Cryptocodinium cohnii* (Bhaud et al. 2000) (Fig. 3)] as well as in Syndiniales (e.g., *Syndinium* sp. (Ris and Kubai 1974)). Due to their large genome sizes, genome sequence data are limited in dinoflagellates. In fact, the only dinoflagellate genome sequence available to date is for *Symbiodinium minutum* (Shoguchi et al. 2013), which revealed putative CENP-A and outer kinetochore components (Table 1) as well as a spindle assembly checkpoint protein (Mad3/BubR1: symbB.v1.2.026514.t1). These findings suggest that this organism still utilizes canonical kinetochore components and the spindle checkpoint. Indeed, a microtubule inhibitor nocodazole delayed mitotic exit in *Cryptocodinium cohnii*, showing that the spindle checkpoint is functional in core dinoflagellates (Yeung et al. 2000).

In contrast, a member of the early diverging Oxyrrhinales, *Oxyrrhis marina*, has an intranuclear spindle, and its chromosomes are not attached to the nuclear envelope (Triemer 1982; Gao and Li 1986; Kato et al. 2000). These studies show that the extranuclear spindle is not a ubiquitous feature of dinoflagellates.

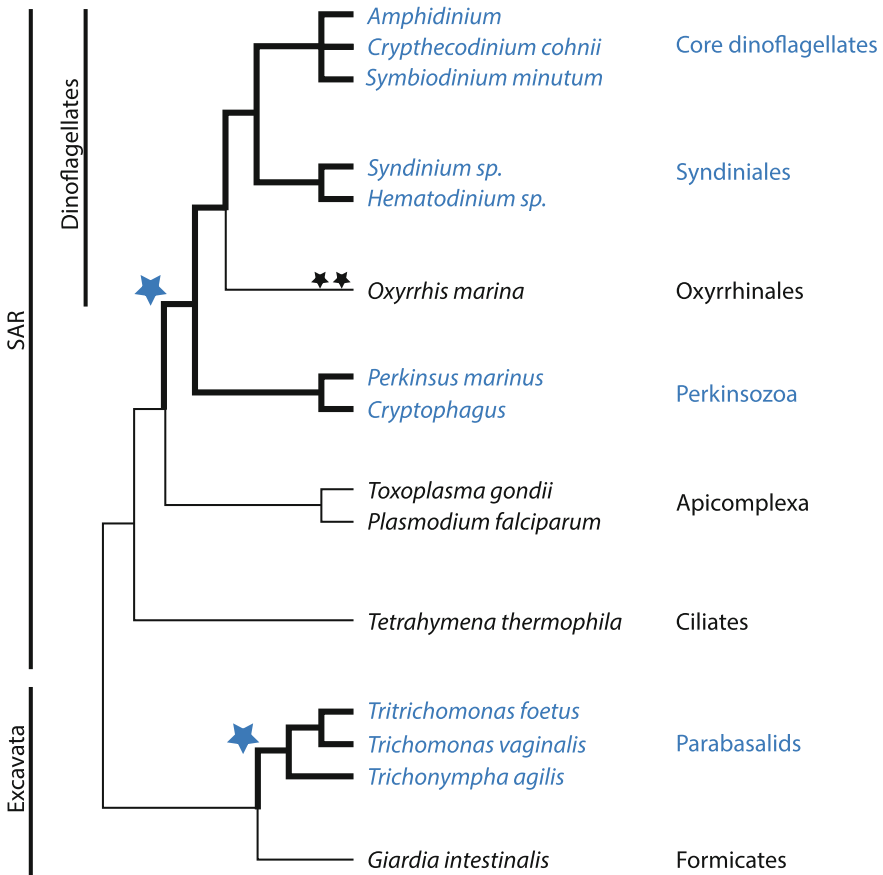


Fig. 2 Membrane-bound kinetochores have independently evolved at least twice. The diagram shows the evolutionary transition to membrane-bound kinetochores in Perkinsozoa and Parabasalids indicated by the blue star and thick branches. Two black stars indicate the reversion to non-membrane-bound kinetochores

5.2 Perkinsozoa

Perkinsozoa is one of the closest relatives of dinoflagellates (Fig. 2). Like core dinoflagellates and Syndiniales, Perkinsozoa undergoes a closed mitosis with an extranuclear spindle, suggesting that its kinetochores are embedded in the nuclear envelope (e.g., *Perkinsus marinus* (Perkins 1996) and *Cryptophagus* (Brugerolle 2002)). Unlike dinoflagellates, however, Perkinsozoa has a smaller genome size that is packaged into nucleosomes (58 Mbp in *Perkinsus marinus* (Gornik et al. 2012)), and its chromosomes are not permanently condensed. Taken together, the observations in Perkinsozoa suggest that extranuclear spindles and membrane-embedded

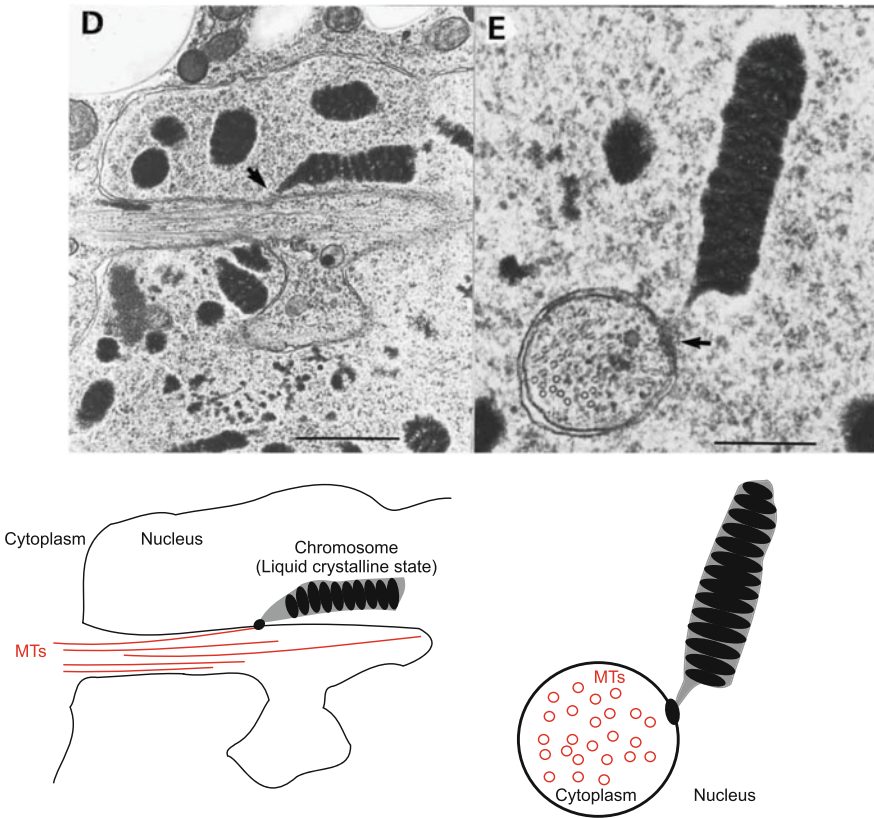


Fig. 3 Membrane-embedded kinetochores in dinoflagellates. *Top* Electron microscopy micrograph of mitotic *Crypthecodinium cohnii* cells. Note that the kinetochore-like structure embedded in the nuclear membrane makes contact with extranuclear spindle microtubules (arrows). Bars 0.8 μm (left), 0.3 μm (right). Reproduced from Bhaud et al. (2000) with permission from the Company of Biologists Limited. *Bottom* Simplified schematic of images on top

kinetochores are not necessarily the consequence of an expanded genome or the diminution of packaging histones.

In contrast to Perkinsozoa, its sister group Apicomplexa (e.g., *Plasmodium* and *Toxoplasma gondii*) undergoes a closed mitosis with an intranuclear spindle as is the case for many other species in the SAR supergroup (Francia and Striepen 2014). These observations suggest that the nuclear envelop-embedded kinetochores and the extranuclear spindle appeared at or before the emergence of Perkinsozoa (Cavalier-Smith and Chao 2004) (Fig. 2). Therefore, the intranuclear spindle in *Oxyrrhis* is most likely a derived feature, i.e., back to a more canonical state. The driving forces underlying the switch to the extranuclear spindle or back, however, remain unclear.

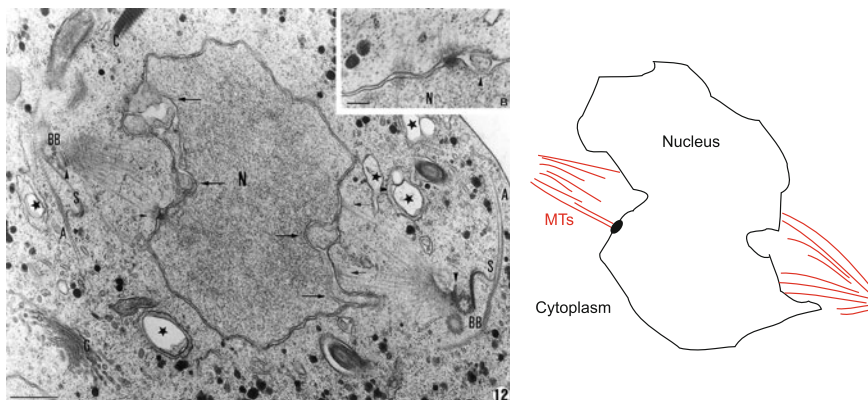


Fig. 4 Bipolar organization of an extranuclear mitotic spindle in Parabasalids. *Left* Electron microscopy image of *Tritrichomonas foetus*. Note that some extranuclear spindle microtubules terminate outside the nuclear membrane. Bars 560 and 320 nm (*inset*). Reproduced from Ribeiro et al. (2002) with permission from John Wiley and Sons. *Right* Simplified schematic of the images on *left*

5.3 Parabasalids

Membrane-bound kinetochores have also evolved in Parabasalids that belong to the supergroup Excavata, Metamonads group. They are characterized by a unique parabasal apparatus (Honigberg 1963). Electron microscopy studies showed that Trichomonads (*Tritrichomonas foetus* and *Trichomonas vaginalis*) and Hypermastigia (*Trichonympha agilis*) undergo a closed mitosis with an extranuclear spindle, and have kinetochores embedded in the nuclear envelope (Kubai 1973; Ribeiro et al. 2002) (Fig. 4). As in dinoflagellates and Perkinsozoa, canonical kinetochore proteins are found in the genome of *Trichomonas vaginalis* (Carlton et al. 2007; Zubáková et al. 2012) (Table 1). Because other members of metamonads such as *Giardia* have intranuclear spindles (Sagolla et al. 2006), membrane-embedded kinetochores and extranuclear spindles in Parabasalids appear to be a derived feature that independently evolved in this lineage.

5.4 Implications from Membrane-Bound Kinetochores

The findings of nuclear envelope-embedded kinetochores raise several questions.

It is likely that the change in the location required adaptations of the kinetochore due to the change in biophysical environment. What modifications are necessary to allow kinetochores to be embedded in the nuclear envelope and what are possible consequences? Although the exact position of kinetochores/centromeres within the lipid bilayer of nuclear membranes remains unclear, electron microscopy data indicate that microtubules likely interact with kinetochores in the cytoplasm rather

than in the nuclear envelope. This implies that the microtubule-binding domain of the Ndc80 complex is located outside of the nuclear envelope. Other kinetochore proteins that bridge between the Ndc80 complex and CENP-A-containing centromeric chromatin within the nucleus must therefore be embedded within the nuclear envelope. Transmembrane domains have so far not been identified in any of *Symbiodinium minutum* and *Trichomonas vaginalis* kinetochore proteins. It is possible that their kinetochores insert into the lipid bilayer by interacting with other nuclear envelope-embedded components such as the nuclear pore complex as previously suggested (Kubai 1973; Ris and Kubai 1974; Cachon and Cachon 1977; Drechsler and McAinsh 2012).

Another question is how membrane-embedded kinetochores form biorientation and regulate cell cycle progression. Can kinetochores move freely in the nuclear envelope or do they require new membrane synthesis? And once biorientation is achieved, how do nuclear and cytoplasmic environments communicate to promote the transition to anaphase, activating the anaphase promoting complex to disrupt cohesion (in the nucleus) and Cyclin B (in the nucleus or cytoplasm), while coordinating the elongation of spindle microtubules (in the cytoplasm)? Finally, what was the evolutionary driving force that underlies the assembly of kinetochores within the nuclear envelope? To address these unknowns, new tools and model systems need to be developed. Importantly, genetic manipulations have already been established in some dinoflagellates (Te and Lohuis 1998; Radakovits et al. 2010) and *Trichomonas vaginalis* (Delgadillo et al. 1997). Studies on these membrane-embedded kinetochores will likely shed new light onto the diverse mechanism of kinetochore assembly and chromosome segregation in eukaryotes.

6 Speculation of Kinetochores in Early Eukaryotes

Chromosome segregation in the last eukaryotic common ancestor (LECA) was likely driven by tubulin-based polymers because microtubules are a universal feature of the chromosome segregation machinery in all known eukaryotes (McIntosh et al. 2010; Yutin and Koonin 2012; Findeisen et al. 2014). In addition, the LECA likely used condensins to compact chromosomes and cohesins to connect duplicated sister chromatids until anaphase (Nasmyth and Haering 2009; Hirano 2016). Furthermore, the presumed presence of cyclin-dependent kinases and the anaphase promoting complex suggests that chromosome segregation was probably already regulated in the cell cycle dependent manner (Nasmyth 1995; Cavalier-Smith 2010a; Garg and Martin 2016).

In contrast to these components, no obvious ortholog for any of the kinetochore proteins has been identified in prokaryotes, including Lokiarchaeota that is considered to be the closest sister group to eukaryotes (Spang et al. 2015). Therefore, it is unclear whether the LECA utilized canonical kinetochore components, such as CENP-A and Ndc80 that are found in nearly all extant species. It is formally possible that the LECA utilized a KKT-based complex that has later been replaced

by canonical kinetochore components in most eukaryotic lineages. This model is consistent with the controversial hypothesis that kinetoplastids might represent the earliest branching eukaryotes (Cavalier-Smith 2010b; Cavalier-Smith 2013; Akiyoshi and Gull 2014). Alternatively, the KKT-based kinetochore may be a derived feature that replaced early eukaryotic kinetochores at some point during the kinetoplastid evolution. A third possibility is that either canonical kinetochores or the KKT-based kinetochores had not yet evolved in the LECA. In this case, chromosome segregation in early eukaryotes might have been similar to the plasmid-partitioning systems found in Bacteria (Gerdes et al. 2010; Reyes-Lamothe et al. 2012) and Archaea (Barillà 2016) where specific DNA elements are recognized by DNA-binding proteins that connect to filament-forming proteins to drive segregation. In such a system, chromosome movement and DNA attachment in the LECA could have been mediated by kinesin or dynein motor proteins. In fact, motor proteins that transport cargo along microtubules and chromokinesins that are capable of connecting chromosomes to microtubules were likely already present in the LECA (Wickstead and Gull 2007; Wickstead et al. 2010).

Kinetochores in all extant eukaryotes are highly complex and consist of many components. Gene duplication likely played an important role in increasing the structural complexity in both canonical and kinetoplastid kinetochores, as evident by the presence of multiple kinetochore proteins that apparently share common ancestry (Schmitzberger and Harrison 2012; Nerusheva and Akiyoshi 2016; Dimitrova et al. 2016; Petrovic et al. 2016). To ensure proper assembly and biorientation of kinetochores, the invention of the Aurora kinase could have been a key evolutionary step that likely had occurred before the emergence of the LECA (Lampson and Cheeseman 2011; Carmena et al. 2012; Hochegger et al. 2013). Error correction by Aurora and direct stabilization of kinetochore-microtubule attachment by tension likely increased the fidelity of chromosome segregation (Akiyoshi et al. 2010; Miller et al. 2016).

7 Conclusions

Most cell biological research over the last several decades has focused on a limited number of model organisms that were selected largely based on historical, not necessarily biological, reasons. Although these studies revealed insights into basic principles of kinetochore organization, a number of differences have been noted even among traditional animal and fungal model organisms. In addition, the unconventional kinetochore in kinetoplastids, the absence of CENP-A in holocentric insects, and nuclear envelope-embedded kinetochores in some eukaryotic lineages all suggest that kinetochores are more plastic than previously thought. The advance of sequencing and genome editing techniques combined with experimental approaches should enable researchers to characterize kinetochores in nontraditional model organisms in a relatively short space of time (Warren 2015; Kobayashi et al. 2015; Gladfelter 2015; Goldstein and King 2016). Insights into kinetochores from

diverse species outside of our current catalog of model organisms have a potential to reveal fundamental design and working principles of the eukaryotic segregation machines.

Note to Nomenclature

In different organisms, the centromeric histone H3 variant is referred to with different names (Earnshaw et al. 2013; Talbert and Henikoff 2013). To be consistent with other chapters, we generally refer to the centromeric histone as CENP-A across species. To account for the differences in nomenclature in specific organisms, we donate the superscript of the original name wherever appropriate (for example, CENP-A^{HCP-3} for *C. elegans*).

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