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Abstract	<p>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.</p>	



Evolutionary Lessons from Species with Unique Kinetochores

Ines A. Drinnenberg and Bungo Akiyoshi

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, the macromolecular protein complex that assembles onto specialized chromosomal regions called centromeres to mediate the attachment of sister chromatids to spindle microtubules (Cheeseman and Desai 2008;

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Santaguida and Musacchio 2009). Kinetochores also promote 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 attachment 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 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 microtubules during mitosis and meiosis. Both complexes are characterized by a

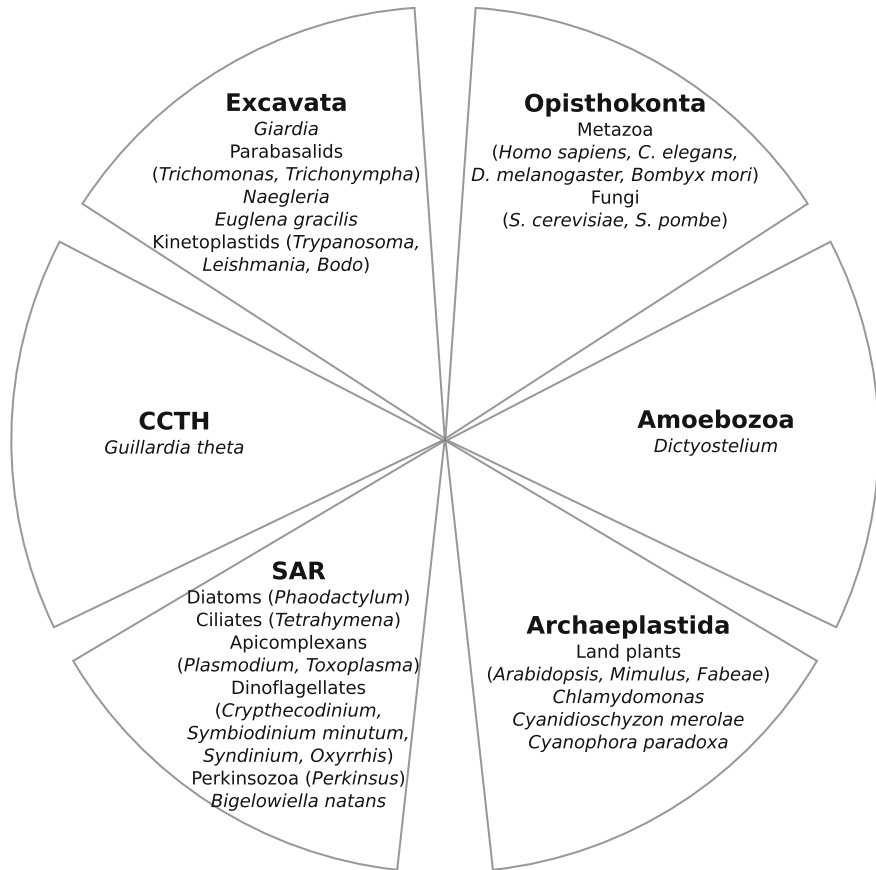


Fig. 1 Six eukaryotic supergroups. Representative organisms from each supergroup are shown as examples

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

69 **2.1 Similarities and Variations of the Inner Kinetochore** 70 **in Animals and Fungi**

71 In fungi and vertebrates, the inner kinetochore consists of ~ 16 members that are
 72 commonly referred to as the Constitutive Centromere Associated Network or
 73 CCAN (Cheeseman and Desai 2008; Westermann and Schleiffer 2013; Westhorpe
 74 and Straight 2013; Fukagawa and Earnshaw 2014) (Table 1). It is generally agreed
 75 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

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	Mif2	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	Kmn1	
Kn11	KNL1	Spc105	Spc7	KNL1	Spc105R	XP_012546088.1
Ndc80	Hec1	Ndc80	Ndc80	Ndc80	Ndc80	XP_012545504.1

(continued)

Table 1 (continued)

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>
Nuf2	Nuf2	Nuf2	Nuf2	Nuf2	Nuf2	XP_004929441.1
Spc24	Spc24	Spc24	Spc24	KBP-4	Kmn2	XP_004925111.1
Spc25	Spc25	Spc25	Spc25	KBP-3	Spc25	NP_001040214.1
Centromere/kinetochore protein	Amoebozoa		Archaeplastida			
	<i>Dictyostelium discoideum</i>		<i>Arabidopsis thaliana</i>	<i>Chlamydomonas reinhardtii</i>	<i>Cyanidioschyzon merolae</i>	<i>Cyanophora paradoxa</i>
CENP-A	Q54F38.1		HTR12	EDP07681.1 and/or EDO99752.1	BAM79321.1	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

(continued)

Table 1 (continued)

Centromere/kinetochore protein	Amoebozoa	Archaeplastida				
	<i>Dictyostelium discoideum</i>	<i>Arabidopsis thaliana</i>	<i>Chlamydomonas reinhardtii</i>	<i>Cyanidioschyzon merolae</i>	<i>Cyanophora paradoxa</i>	
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			
Kn11	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	
Spc24	XP_638781.1	NP_187500.2	XP_001697066.1	BAM79682.1	Contig54569-abinit-gene-0.4	
Spc25	XP_638784.1	NP_566900.1	XP_001697914.1	BAM79992.1	Contig26077-abinit-gene-0.7	
Centromere/kinetochore protein	SAR					
	<i>Phaeodactylum tricorutum</i>	<i>Tetrahymena thermophila</i>	<i>Plasmodium falciparum</i>	<i>Toxoplasma gondii</i>	<i>Symbiodinium minutum</i>	<i>Bigelowiella natans</i>
CENP-A	EEC43222.1	Q2N2K6.1	CAD52476.1	EPT27047.1	symbB1.v1.2.034671.t1	aug1.3_g1557
CENP-C	XP_002184047.1		CZT98473.1			fgenes1_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						

(continued)

Table 1 (continued)

Centromere/kinetochore protein	SAR					
	<i>Phaeodactylum tricornutum</i>	<i>Tetrahymena thermophila</i>	<i>Plasmodium falciparum</i>	<i>Toxoplasma gondii</i>	<i>Symbiodinium minutum</i>	<i>Bigelowiella natans</i>
CENP-U						
CENP-T						
CENP-W						
CENP-S	XP_002180057.1					aug1.3_g1673
CENP-X						aug1.96_g19929
Mis12	XP_002179310.1				symbB. v1.2.039506.t1	fgenesH1_pg.45_#_162
Nnf1	XP_002180232.1					
Dsn1						
Nsl1						
Knl1						fgenesH1_pg.8_#_205
Ndc80	XP_002177760.1	XP_001024017.1	XP_966148.1	XP_002365215.1	symbB. v1.2.002582.t1	estExt_fgenesH1_pg. C_190200
Nuf2	XP_002185912.1		XP_001351235.1	XP_002364205.1	symbB. v1.2.033878.t1	e_gw1.128.32.1
Spc24	XP_002182127.1					estExt_fgenesH1_pg. C_680067
Spc25	XP_002178778.1	XP_001030098.2	XP_002809072.1	XP_002368113.1	symbB. v1.2.035905.t1	aug1.38_g12030

(continued)

Table 1 (continued)

Centromere/kinetochore protein	CCTH	Excavata			
	<i>Guillardia theta</i>	<i>Giardia intestinalis</i>	<i>Trichomonas vaginalis</i>	<i>Naegleria gruberi</i>	<i>Euglena gracilis</i>
Centromere/kinetochore protein	CCTH	Excavata			
CENP-A	<i>Guillardia theta</i> EKX34792.1 (nucleus) AAK39657.1? (nucleomorph)	<i>Giardia intestinalis</i> EFO62248.1	<i>Trichomonas vaginalis</i> EAY15370.1	<i>Naegleria gruberi</i> EFC49963.1	<i>Euglena gracilis</i> 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	
Nnf1	XP_005820862.1		XP_001307740.1		
Dsn1					
Nsl1	XP_005822661.1				

(continued)

Table 1 (continued)

Centromere/kinetochore protein	CCTH	Excavata			
	<i>Guillardia theta</i>	<i>Giardia intestinalis</i>	<i>Trichomonas vaginalis</i>	<i>Naegleria gruberi</i>	<i>Euglena gracilis</i>
Knl1	XP_005832827.1			EFC50203.1	
Ndc80	EKX45170.1		XP_001311732.1		dark_m.19013
Nuf2	CAC27079.1	EFO61302.1			dark_m.38633
Spc24	EKX52051.1	EDO78311.1			
Spc25	EKX40188.1	EFO63967.1	XP_001323375.1	EFC47967.1	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. KNL1 homologs were predicted in Tromer et al. (2015)



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

133 2.2 The Composition of the Outer Kinetochore Is Highly 134 Conserved in Animals and Fungi

135 The outer kinetochore complex is recruited to centromeres upon the onset of mitosis
136 to connect to spindle microtubules. This interaction is accomplished by
137 the ~10-subunit KMN network that consists of the Kn11, Mis12, and Ndc80
138 complexes (Cheeseman et al. 2006; Petrovic et al. 2014). In contrast to the inner
139 kinetochore, the composition of the outer kinetochore is widely conserved across
140 animals and fungi (Meraldi et al. 2006; Tromer et al. 2015). Even
141 CENP-A-deficient insects encode the same repertoire of outer kinetochore com-
142 ponents, implying similar means of attaching to microtubules while utilizing
143 alternate inner kinetochore assembly pathways (Drinnenberg et al. 2016). A notable
144 exception to the otherwise conserved composition of the KMN network is found in
145 Diptera. *D. melanogaster* has lost Dsn1, a subunit of the Mis12 complex
146 (Przewloka and Glover 2009). In addition, the Nnf1 subunit of the Mis12 complex
147 underwent a duplication event giving rise to two paralogs, Nnf1a and Nnf1b, that
148 are part of two distinct Mis12 complexes with similar biochemical behaviors
149 (Przewloka et al. 2007; Schittenhelm et al. 2007; Liu et al. 2016; Richter et al.
150 2016; Blattner et al. 2016). The loss of Dsn1 could have been compensated by the
151 C-terminal part of the *Drosophila* Kn11 homolog (Przewloka et al. 2009). Indeed,
152 the overall organization of this complex appears to resemble the human and yeast
153 counterparts (Hornung et al. 2011; Przewloka et al. 2011; Screpanti et al. 2011).
154 Whether these changes have any functional consequences on the *Drosophila* KMN
155 complex is currently unclear.

156 3 Glimpses into Kinetochore Compositions in Diverse 157 Eukaryotes

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

eukaryotes. Although bioinformatics 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 *Fabae* 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 by Lampson and Black).

Compared to land plants, much less is known about kinetochores in other Archaeplastida species. *Cyanidioschyzon merolae* is a thermoacidophilic 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

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

204 3.3 *Supergroup SAR*

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

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

223 3.4 *Supergroup CCTH*

224 Very little is known about kinetochores in the supergroup CCTH (Cryptophytes,
225 Centrohelids, Telonemids, and Haptophytes; also called Hacrobia). Cryptophyte
226 algae are thought to have evolved by engulfing a red alga that contained a primary
227 plastid (Tanifuji and Archibald 2014). In cryptomonad *Guillardia theta*, the sec-
228 ondary plastid has retained the red algal-derived relict nucleus (called nucleomorph)
229 (Curtis et al. 2012). How the nucleomorph genome is maintained during cell
230 division remains unknown. While the nucleomorph genome encodes for a putative
231 CENP-A homolog (Douglas et al. 2001) (Table 1), this protein lacks the hallmark
232 of an extended loop 1 region. It will therefore be necessary to experimentally
233 confirm whether it indeed functions as the centromeric histone variant for the
234 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 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, diplomonids, symbiontids, 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 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 distinct from canonical kinetochores proteins present in other eukaryotes, various regulatory proteins that are known to be important in 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

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

315 **4.3 Implications from Kinetoplastid Kinetochores**

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

324 **5 Membrane-Embedded Kinetochores**

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

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

351 5.1 *Dinoflagellates*

352 Dinoflagellates are a highly diverse group of flagellates, including photosynthetic
 353 free-living and parasitic species (Taylor et al. 2007). They belong to the supergroup
 354 SAR, Alveolata group, and their sister groups include Perkinsozoa and
 355 Apicomplexa (Saldarriaga et al. 2004) (Fig. 2). Dinoflagellates are characterized by
 356 large genome sizes in the range of 1,500 Mbp to 185,000 Mbp (Wisecaver and
 357 Hackett 2011). Despite having all core histone genes, histones are not involved in
 358 packaging the majority of nuclear DNA (Hackett et al. 2005; Marinov and Lynch
 359 2015). In addition, other basic nuclear proteins including Dinoflagellates/Viral
 360 NucleoProteins (DVNPs) and HU-like proteins might substitute major histone
 361 functions in some of these organisms (Sala-Rovira et al. 1991; Chan and Wong
 362 2007; Gornik et al. 2012; Talbert and Henikoff 2012; Bachvaroff et al. 2014). Their
 363 chromosomes are permanently condensed, showing a characteristic liquid crys-
 364 talline state even in interphase. Interestingly, some, but not all, dinoflagellates have
 365 kinetochores embedded in the nuclear envelope with an extranuclear spindle
 366 (Leadbeater and Dodge 1967; Kubai and Ris 1969; Spector and Triemer 1981).

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

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

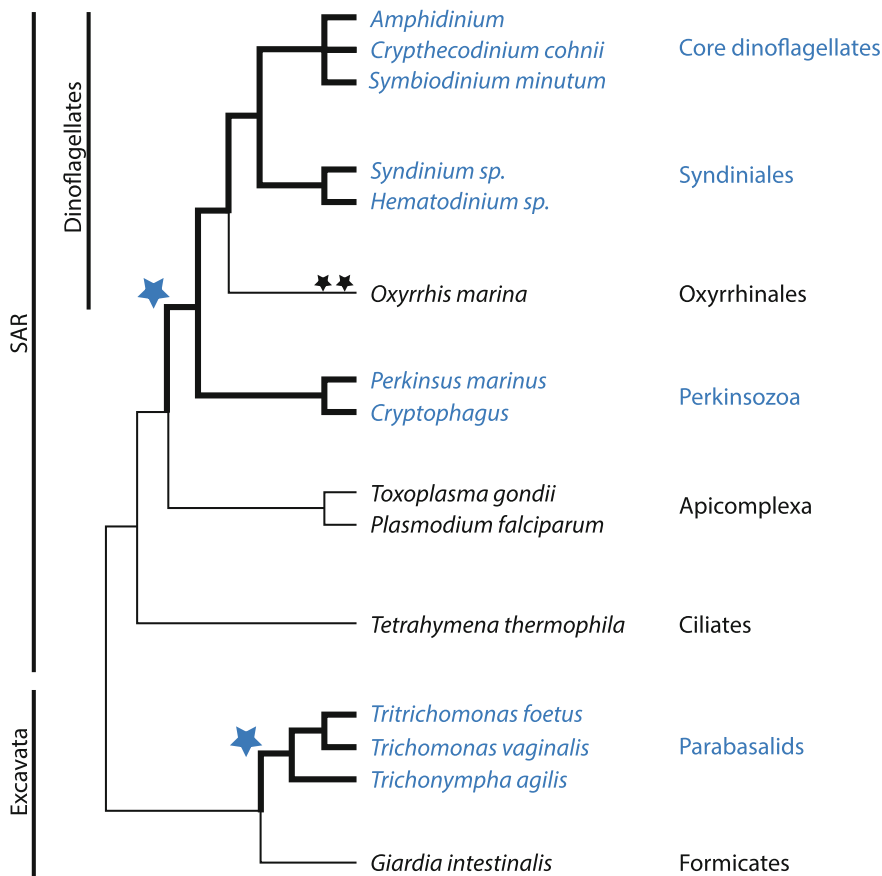


Fig. 2 Membrane-bound kinetochores have independently evolved at least twice. The diagram shows the evolutionary transition to membrane-bound kinetochores in Perkinsozoa and Parabasalids indicated by the blue star and thick branches. Two black stars indicate the reversion to non-membrane-bound kinetochores

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

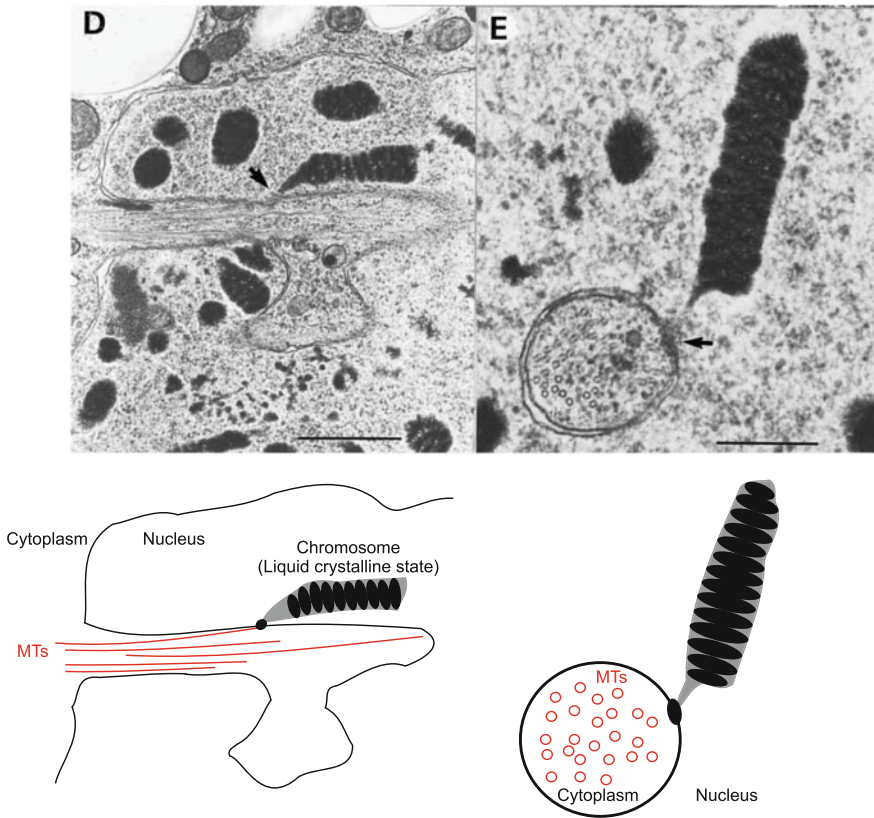


Fig. 3 Membrane-embedded kinetochores in dinoflagellates. *Top* Electron microscopy micrograph of mitotic *Cryptothecodinium cohnii* cells. Note that the kinetochore-like structure embedded in the nuclear membrane makes contact with extranuclear spindle microtubules (arrows). Bars 0.8 μm (left), 0.3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

394 membrane-embedded kinetochores are not necessarily the consequence of expanded
 395 genome or diminution of packaging histones.

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

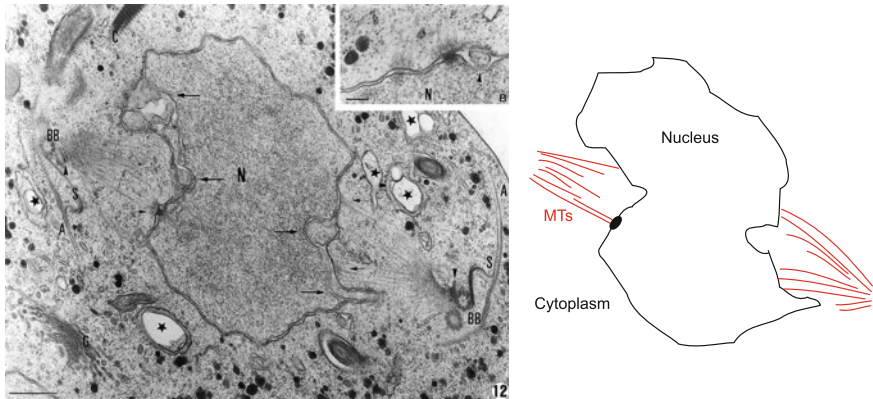


Fig. 4 Bipolar organization of an extranuclear mitotic spindle in Parabasalids. *Left* Electron microscopy image of *Tritrichomonas foetus*. Note that some extranuclear spindle microtubules terminate outside the nuclear membrane. Bars 560 and 320 nm (*inset*). Reproduced from Ribeiro et al. (2002) with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

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

436 Another question is how membrane-embedded kinetochores form biorientation
437 and regulate cell cycle progression. Can kinetochores move freely in the nuclear
438 envelope or do they require new membrane synthesis? And once biorientation is
439 achieved, how do nuclear and cytoplasmic environments communicate to promote
440 the transition to anaphase, activating the anaphase promoting complex to disrupt
441 cohesion (in the nucleus) and Cyclin B (in the nucleus or cytoplasm), while
442 coordinating the elongation of spindle microtubules (in the cytoplasm)? Finally,
443 what was the evolutionary driving force that underlies the assembly of kinetochores
444 within the nuclear envelope? To address these unknowns, new tools and model
445 systems need to be developed. Importantly, genetic manipulations have already
446 been established in some dinoflagellates (Te and Lohuis 1998; Radakovits et al.
447 2010) and *Trichomonas vaginalis* (Delgadillo et al. 1997). Studies on these
448 membrane-embedded kinetochores will likely shed new light onto the diverse
449 mechanism of kinetochore assembly and chromosome segregation in eukaryotes.

450 6 Speculation of Kinetochores in Early Eukaryotes

451 Chromosome segregation in the last eukaryotic common ancestor (LECA) was
452 likely driven by tubulin-based polymers because microtubules are a universal
453 feature of the chromosome segregation machinery in all known eukaryotes
454 (McIntosh et al. 2010; Yufin and Koonin 2012; Findeisen et al. 2014). In addition,
455 the LECA likely used condensins to compact chromosomes and cohesins to connect
456 duplicated sister chromatids until anaphase (Nasmyth and Haering 2009; Hirano
457 2016). Furthermore, the presumed presence of cyclin-dependent kinases and the
458 anaphase promoting complex suggests that chromosome segregation was probably
459 already regulated in the cell cycle dependent manner (Nasmyth 1995;
460 Cavalier-Smith 2010a; Garg and Martin 2016).

461 In contrast to these components, no obvious ortholog for any of the kinetochore
462 proteins has been identified in prokaryotes, including Lokiarchaeota that is con-
463 sidered to be the closest sister group to eukaryotes (Spang et al. 2015). Therefore, it
464 is unclear whether the LECA utilized canonical kinetochore components, such as
465 CENP-A and Ndc80 that are found in nearly all extant species. It is formally
466 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 holo-centric 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

506 model organisms in relatively short space of time (Warren 2015; Kobayashi et al.
507 2015; Gladfelter 2015; Goldstein and King 2016). Insights into kinetochores from
508 diverse species outside of our current catalog of model organisms have a potential
509 to reveal fundamental design and working principles of the eukaryotic segregation
510 machines.

511 Note to Nomenclature

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

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