

Kinetochores assembly: If you build it, they will come

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Abstract

Accurate chromosome segregation requires the interaction of chromosomes with the microtubules from the mitotic spindle. This interaction is mediated by the macro-molecular kinetochore complex, which assembles only at the centromeric region of each chromosome. However, how this site is specified and how assembly of the kinetochore structure is regulated in coordination with cell cycle progression remains unclear. Recent studies have begun to shed light on the mechanisms underlying assembly of this complex structure.

Introduction

During mitosis, cells must accurately partition their genome such that genetic information is transferred unperturbed to the progeny. In eukaryotes, accurate chromosome segregation requires each chromosome to interact appropriately with microtubules from the mitotic spindle that provides the structural framework upon which chromosome segregation occurs. This interaction is mediated by the macromolecular kinetochore complex, a structure of more than 90 proteins that assembles at the centromeric region of each chromosome during mitosis [1]. The kinetochore must facilitate the interaction between centromeric chromatin and dynamic microtubules to facilitate the biorientation of chromosomes on the metaphase plate and the segregation of sister chromatids at anaphase.

Conventional electron microscopy studies indicated that the kinetochore has a layered structure, with an electron dense inner plate that contacts centromeric chromatin, an outer plate that contacts microtubules, and a “fibrous corona” that extends away from the outer plate [2]. The majority of this structure is not present during interphase, assembling only upon entry into mitosis when the interaction with microtubules is required, and disassembling upon mitotic exit. In recent years, advances in proteomic approaches have greatly expanded the

catalogue of kinetochore components [1]. However, while the functions of many of these individual components have been defined, surprisingly little is known about how they are recruited to the centromere, and how they interact to assemble the complex kinetochore structure. Moreover, how this process is regulated to ensure timely assembly of the kinetochore upon mitotic entry, and disassembly of the structure upon completion of mitosis, is poorly understood. Here, we highlight recent advances in our understanding of kinetochore assembly and disassembly in human cells, as well as potential mechanisms that regulate this process.

Specifying the site of kinetochore assembly

For accurate chromosome segregation to occur, it is crucial the kinetochore assembles at only one site on each chromosome. The properties of the centromere that mark it as the site for kinetochore formation include a combination of epigenetic marks such as the presence of CENP-A (Centromere Associated Protein-A) containing nucleosomes, chromatin structure, and DNA sequence properties (reviewed in [3]). Deposition of the histone H3 variant CENP-A occurs predominantly at centromeres and is required for kinetochore assembly [4-6]. Recently, high resolution structural data for a CENP-A/H4 heterotetramer has been reported, showing significant structural differences between this, and canonical H3 containing nucleosomes. These differences are essential for centromeric incorporation of CENP-A nucleosomes [7], and may also reflect a contribution of CENP-A to a specialized chromatin structure at the base of kinetochores. Surprisingly, in mammalian cells, while CENP-A nucleosomes are required for kinetochore assembly, the presence of the alpha satellite DNA sequences that are typically associated with centromeres is not. In humans, this is demonstrated most strikingly by the rare occurrence of neo-

centromeres at ectopic chromosomal loci devoid of alpha satellite sequences [8]. At these sites CENP-A is deposited, along with a number of other kinetochore proteins, in the absence of alpha satellite sequences [9]

Unlike canonical histone molecules that are loaded into chromatin during DNA replication, CENP-A is equally segregated between sister chromatids during S phase, but new CENP-A is not incorporated into centromeric chromatin until telophase and G1 [10,11]. The significance of this unusual timing is unclear, but could represent a mechanism to guard against mis-incorporation and the consequent assembly of kinetochore structures at non-centromeric sites by temporally separating the incorporation of CENP-A from the replication-coupled incorporation of other histones. Ubiquitin-dependent proteolysis of CENP-A incorporated at non-centromeric sites may also act to guard against this misincorporation [12]. Other kinetochore components may also act to regulate this process. In *Drosophila*, tightly controlled levels of the CENP-A / CENP-C bridging factor Cal1 may prevent incorporation at non-centromeric sites [13]. However, an active mechanism for recruitment of CENP-A to centromeres is still essential. The Mis18 complex of proteins is required for the incorporation and maintenance of CENP-A [14-16]. The HJURP/Smc3 chaperone protein has also been implicated in the centromeric loading of CENP-A [17,18]. HJURP directly interacts with prenucleosomal CENP-A/H4 complexes via the centromere-targeting domain of CENP-A, and is required for the deposition of CENP-A in G1. HJURP is recruited to centromeres slightly later than Mis18, which is present from late anaphase to early G1. The Mis18 complex may act as a primer for the loading of CENP-A, which is in turn facilitated by a chaperone complex including HJURP (Figure 1). Thus, the mechanisms that initiate the processes of kinetochore assembly by specifying its a chromosomal position are beginning to be understood.

Architecture of the Human Kinetochore

Although CENP-A is sufficient to drive kinetochore formation in *Drosophila* [19], in human cells the presence of CENP-A is not sufficient for complete kinetochore assembly during mitosis [20]. Additional proteins are also found constitutively at the human centromere throughout the cell cycle, and are required for correct assembly of the mitotic structure. In particular, functional and proteomic approaches have identified a group of 15 proteins known as the Constitutive Centromere Associated Network (CCAN) [21-23-25] (Figure 1). Functional analyses have suggested that these proteins primarily play a structural role in forming a stable base for dynamic kinetochore assembly, as well as providing an appropriate environment for the incorporation of new CENP-A [21,23,25-28]. However, a recent study also suggested that the CCAN may also function to directly control microtubule dynamics [24]. Dissecting the role of these proteins in structural support and assembly versus a direct role in microtubule interactions will be an important for future work.

The CCAN proteins are found at the kinetochore-chromatin interface, and several of these proteins have been shown to have DNA binding activity [23,29,30] or directly interact with CENP-A [31,32]. As these proteins remain associated with centromeric chromatin throughout the cell cycle, in conjunction with CENP-A they may form a stable, permissive environment for the assembly of the mitotic kinetochore structure. The outer kinetochore plate and fibrous corona assemble upon entry into mitosis, and contain proteins required for interactions with microtubules. This includes proteins with direct microtubule binding activity, such as the KMN (KNL1, Mis12, Ndc80) network (reviewed in [1], the Ska1 complex [33], and CENP-E [34], as well as more transient factors that modulate this interaction or monitor attachment status (Figure 1).

Recent work using super-resolution light microscopy techniques [35] has mapped the internal architecture of the kinetochore in the presence and absence of tension across kinetochore pairs, and identified surprising changes in the organization of the structure. For example, in the absence of tension, the distance between inner kinetochore proteins, such as CENP-C, and the microtubule interacting Ndc80 complex was reduced. However, the localization of inner kinetochore proteins with respect to each other remained unchanged. Reduction in the tension across kinetochores also caused a striking rearrangement of components of the KMN network, suggesting some kinetochore protein complexes are more compliant than others in response to forces exerted by microtubule interactions.

FRET (fluorescence resonance energy transfer) based approaches have also been used to position proteins with respect to each other within the kinetochore ultrastructure [36]. FRET measurements between selected pairs of CCAN proteins indicated that during interphase, specific components of the CCAN complex are located close together at the centromere. For example, high FRET measurements were observed between CENP-U and CENP-I, and CENP-U and CENP-B. In the future, the use of this approach to systematically map the localization of known kinetochore components, in parallel with proteomic and functional analysis, will allow the formation of a detailed picture of kinetochore structure and assembly pathways.

Turnover of Kinetochore Proteins

While at first glance the bulk of the kinetochore structure appears stable at the centromere throughout mitosis, recent work suggests that kinetochore proteins are actually highly dynamic. Outer kinetochore components recruited in mitosis can be divided into two groups; 1) those that form the stable core of the

kinetochore responsible for achieving and maintaining microtubule attachment, and 2) those whose association with the kinetochore structure is more transient and is influenced by the attachment state of the kinetochore. Outer kinetochore proteins involved in sensing attachments and spindle checkpoint signaling are very dynamic. FRAP (Fluorescence Recovery After Photo-bleaching) experiments indicated that the checkpoint protein Mad2 has a half-life at the kinetochore of just seconds [37,38] (figure 1). In contrast, these studies indicate that stable components, such as CENP-C or Ndc80 (Figure 1), have much longer residencies, remaining immobile at the kinetochore throughout mitosis [37,39]. Recent work has indicated that even some constitutive kinetochore proteins are dynamic. For example, during interphase, pools of Mis12, CENP-C and CENP-B have relatively short half-lives at kinetochores [40].

Driving kinetochore assembly

The overall kinetochore composition is highly dynamic through the cell cycle. Inner kinetochore proteins such as the CCAN are present at the centromere throughout the cell cycle, while some outer kinetochore proteins such as Mis12 complex and KNL1 are recruited in G2 [25] (Figure 1). This assembly prior to mitosis presumably acts to 'prime' the centromere for assembly of the remaining components, which occurs at prophase and prometaphase to generate a structure capable of binding to microtubules.

Work on kinetochore assembly has primarily used reciprocal dependency relationships to define the requirements for localization to kinetochores and to place proteins within the 'kinetochore assembly hierarchy' [25,28,41,42]. For example, depletion of CENP-A from *C. elegans* prevents the localization of CENP-C to chromosomes, while CENP-C depletion has no effect on CENP-A localization suggesting that CENP-A functions upstream of CENP-C [6,43]. Using

such pairwise assays, it has been possible to construct extensive maps of the dependency relationships for the yeast, *C. elegans*, *Drosophila*, and human kinetochores. While there are some minor differences in these relationships in different organisms (for example, the localization of CENP-A and CENP-C localization is interdependent in *Drosophila* [44]), the fundamental principles of this organization are largely conserved. Importantly, directed biochemical experiments defining the physical interactions between components of the kinetochore has largely supported these dependency relationships [21,26,27,31,45].

Based on the work that has been conducted to date, we suggest that there are four primary types of proteins that contribute to kinetochore assembly; linkers, scaffolds, chaperones, and structural stabilizers. As might be expected based on the more than 90 proteins that are stably associated with the mitotic kinetochore, a subset of proteins must function to build the molecular connectivity between centromeric DNA and microtubule polymers. These proteins, which we term “linkers”, would bind to both a DNA proximal protein and a more peripheral protein to hold these together. For example, the Mis12 complex associates with inner kinetochore proteins, and also binds to the outer kinetochore proteins KNL1 and the Ndc80 complex [46] (Figure 1). However, it is important to note that such linker proteins do not need to simply bridge molecular interactions. In the case of the Mis12 complex, our recent work has suggested that it functions to position the KNL1 and Ndc80 complexes in a way that promotes their individual microtubule binding activities [47].

While proteins such as the Mis12 complex would function to link together components of the kinetochore, “scaffold” proteins would provide a broader platform for the assembly of distinct components of the kinetochore. In this context, KNL1 (also named Spc105 or Blinkin), appears to provide such a

scaffold within the kinetochore. To date, KNL1 has been demonstrated to bind directly to the Mis12 complex, the Ndc80 complex, the spindle assembly checkpoint proteins Bub1 and BubR1, the outer kinetochore protein Zwint, the protein phosphatase PP1, and microtubules [46,48-50]. In this way, KNL1 can coordinate multiple different activities at the outer kinetochore (Figure 1).

A subset of proteins appear to function in kinetochore assembly despite not stably localizing to the kinetochore themselves. These kinetochore “chaperones” function to bring proteins to the kinetochore, or stabilize their proper incorporation. For example, hJURP appears to function as a CENP-A-specific nucleosome assembly factor, facilitating its recruitment kinetochores [17,18]. Recent work has also implicated Skp1 and Hsp90 in kinetochore assembly by acting to recruit and stabilize the Mis12 complex [51] (Figure 1).

Although the process of building a kinetochore by recruiting proteins to the centromere is critical for chromosome segregation during mitosis, it is also important that the final assembled structure be capable of withstanding the large forces that are exerted upon it. Indeed, the formation of bi-oriented attachments is capable of stretching an individual kinetochore structure by more than 60 nm. Recent work has suggested that a subset of kinetochore proteins, including a complex of CENP-S and CENP-X, may function to maintain structural rigidity, but not directly contribute to the localization of other components [52]. Thus, CENP-S/X may function as structural “stabilizers” such that their depletion results in a kinetochore structure that is much less rigid and compact despite an apparently normal complement of kinetochore proteins (Figure 1).

The control of kinetochore assembly and disassembly by post-translational modifications

A striking feature of the vertebrate kinetochore is the massive reorganization that takes place during mitosis. During a time span of less than one hour, the kinetochore must recruit more than 40 mitosis-specific components to facilitate attachment to microtubules, and then subsequently disassemble these proteins to return to an interphase state. There are several possibilities for how the association of these proteins with the kinetochore could be controlled. First, the presence of the nuclear envelope may restrict a subset of proteins from the nucleus such that they are unable to associate with the kinetochore until Nuclear Envelope Break Down (NEBD). For example, nuclear pore complex components such as the Nup107-160 complex relocate from the nuclear envelope to the kinetochore upon NEBD, and then leave the kinetochore during early anaphase as the nuclear envelope reforms [53,54]. Disassembly of the nuclear envelope likely liberates this complex, making it available for kinetochore binding. Similarly, the reformation of the nuclear envelope on mitotic exit could then sequester the complex away from the centromere (Figure 1). The nuclear envelope may similarly act to block the assembly of other mitosis-specific proteins. However, recent work has demonstrated that at least a subset of proteins are present within the nucleus at times when they do not localize to kinetochores [25], suggesting that this is not true in every case.

While the presence of the nuclear envelope may function to control the assembly of some kinetochore proteins, it is also possible that the expression of mitotic kinetochore proteins may be coupled with the cell cycle such that these proteins are not present during interphase. However, in the cases tested so far, constitutive basal expression of GFP kinetochore protein fusions has revealed identical localization to that of the endogenous counterparts as detected by immunofluorescence [21,25,42,46], suggesting that this does not play a major role in controlling kinetochore assembly.

A third possibility is that the stability of individual proteins may be altered during mitosis, such as by targeting specific proteins for degradation. Recent work has demonstrated that the ubiquitin-like protein SUMO modifies the kinetochore protein CENP-I [55]. When CENP-I is extensively SUMOylated, it is targeted for degradation. Thus, the correct incorporation of CENP-I into kinetochores requires the action of the SUMO protease SENP6 to remove this SUMOylation (Figure 1). In addition, several kinetochore components including Aurora B, Polo Like Kinase-1 (PLK-1), CENP-E, and CENP-F are known to be degraded at the end of mitosis via the Anaphase Promoting Complex (APC) ubiquitin ligase and the proteasome [56,57].

Finally, it is possible that kinetochore proteins are also carefully regulated by post-translational modifications, such as phosphorylation, in coordination with the cell cycle to control their assembly and disassembly occurs at specific times. Indeed, there have been several recent reports that have implicated post-translational modifications in the control of kinetochore assembly. The most obvious candidate for a kinase that would affect the mitotic assembly state of the kinetochore is Cyclin Dependent Kinase (CDK). However, due to the numerous roles of CDK in controlling cell cycle progression, thus far it has not been possible to define a specific role for CDK phosphorylation in kinetochore assembly. In contrast, although Aurora B kinase has been primarily implicated in controlling kinetochore-microtubule attachments by modulating the microtubule binding activity of key kinetochore proteins [45,58,59], studies in budding yeast and *Xenopus* extracts have suggested that Aurora B activity is also required for proper kinetochore assembly [60]. However, the effects observed in *Xenopus* extracts do not appear to be as dramatic following Aurora B inhibition in human cells [47]. Thus, the relative contribution of Aurora B to kinetochore assembly remains to be determined. Finally, recent work has demonstrated the Bub1

kinase phosphorylates a conserved site on Histone H2A [**61]. Phosphorylation of this site appears to create a binding site for the regulatory protein Sgo1 to allow it to target to kinetochores. In total, it is likely that a coordinated combination of mechanisms acts to regulate the highly ordered process of kinetochore assembly and disassembly.

Conclusions and Future directions

Recent work to define the composition of the kinetochore and the nearly complete parts list of its components has yielded important information regarding the organization and function of this vital mitotic structure. As highlighted in this review, while some regulatory events that control kinetochore assembly have been identified, it remains a key goal to define the mechanisms by which this dynamic multi-protein assembly is built and disassembled during mitosis.

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Figure 1

Schematic representation of kinetochore assembly in human cells. A) The CCAN proteins are present at the centromere throughout the cell cycle. In late telophase and early G1, new CENP-A containing nucleosomes are deposited via the Mis18 complex and the HJURP “chaperone”. B) During G2, a subset of kinetochore components such as the Mis12 complex and KNL1 assemble at the centromere, while others maybe excluded by the nuclear envelope. The Sgt/HSP90 complex acts as a “chaperone” to promote recruitment of the Mis12 complex, while the SENP6 SUMO protease regulates assembly of the CCAN protein CENP-I. C) On entry into mitosis the bulk of the kinetochore is recruited to the centromere. This

process is likely regulated by phosphorylation by mitotic kinases. Some components, such as the Mis12 complex, act as “linkers”, while others such as KNL1 act as “scaffolds” for the recruitment of multiple other components. Finally, other proteins such as the CENP-S/X complex act to “stabilize” the structure. Kinetochore components display different dynamics during mitosis, with some proteins such as Mad2 turning over rapidly, but others such as Ndc80, turning over more slowly.

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