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The Power of *Xenopus* Egg Extract for Reconstitution of Centromere and Kinetochore Function

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Abstract

Faithful transmission of genetic information during cell division requires attachment of chromosomes to the mitotic spindle via the kinetochore. In vitro reconstitution studies are beginning to uncover how the kinetochore is assembled upon the underlying centromere, how the kinetochore couples chromosome movement to microtubule dynamics, and how cells ensure the site of kinetochore assembly is maintained from one generation to the next. Here we give special emphasis to advances made in *Xenopus* egg extract, which provides a unique, biochemically tractable in vitro system that affords the complexity of cytoplasm and nucleoplasm to permit reconstitution of the dynamic, cell cycle-regulated functions of the centromere and kinetochore.

1 Introduction

All organisms must accurately segregate their genomes during cell division. In eukaryotes, duplicated sister chromosomes condense as cells enter mitosis, attach to the microtubules of the bipolar mitotic spindle, and then use microtubule-dependent forces to move to opposite spindle poles before separation into two new cells during cytokinesis. These events were first described nearly 150 years ago (Flemming 1880), yet we still lack a detailed molecular understanding of the processes that orchestrate chromosome segregation.

Central to the process of chromosome segregation is the multiprotein complex that links chromosomes to microtubules—the kinetochore. Kinetochores provide the primary interaction sites between microtubules and chromosomes for chromosome movement and monitor proper chromosome alignment through the activities of the spindle assembly checkpoint (Cheeseman 2014; Foley and Kapoor 2013). The process of kinetochore formation occurs in mitosis as numerous proteins and protein complexes localize to a specialized domain of the chromosome termed the centromere (McKinley and Cheeseman 2016; Westhorpe and Straight 2015). Centromeres are defined by a specialized chromatin domain in which the histone H3 variant CENP-A replaces histone H3 in nucleosomes. CENP-A nucleosomes recruit a complex of ~17 centromere-specific proteins, the constitutive centromere-associated network (CCAN), which associates with the chromosome throughout the cell cycle and provides the essential substrate for mitotic kinetochore

assembly (Fig. 1). The centromere and kinetochore thus play structural, mechanical, and signaling roles that are essential for proper chromosome segregation.

Over the last thirty years, efforts from numerous groups have identified many of the core components of centromeres and kinetochores. The first centromere proteins were discovered as autoantigens recognized by sera from scleroderma patients (Earnshaw and Rothfield 1985; Earnshaw et al. 1986, 1987). Subsequently, genetic studies of chromosome missegregation and biochemical purification (Foltz et al. 2006; Obuse et al. 2004; Izuta et al. 2006; Okada et al. 2006) have provided a relatively complete list of centromere and kinetochore proteins: by current estimates, more than 80 different proteins have been identified (Westhorpe and Straight 2015; McKinley and Cheeseman 2016).

As we now have many of the proteins of the centromere and kinetochore in hand, a major challenge going forward is to understand how those components come together to give rise to the activities that segregate chromosomes. In vitro reconstitution studies are beginning to uncover the biochemical functions of these components, how they are connected, and how their activities are regulated and coordinated to achieve proper chromosome segregation. As comprehensive coverage of the functions of the centromere and kinetochore is provided in other chapters of this book, the goal of this chapter is to highlight the specific contributions of reconstitution studies. Special emphasis is given to *Xenopus* egg extract, which provides a biochemically tractable in vitro system that affords the complexity of cytoplasm and nucleoplasm, uniquely permitting reconstitution of the dynamic, cell cycle-regulated functions of the centromere and kinetochore.

2 Reconstituting Centromere and Kinetochore Functions In Vitro

A key challenge in studying centromeres and kinetochores in vivo is that their activities are essential for cell viability. Thus deletion or mutation of protein components often results in cell death and give rise to phenotypes that are often difficult to interpret. Cell free or reconstituted systems bypass this problem and thus enable the study of centromere and kinetochore protein function without the limitations of cell viability.

Early studies of kinetochore activities in cell free systems showed that the kinetochores of purified mitotic chromosomes, when incubated with tubulin, bind microtubules, regulate microtubule dynamic instability, and couple chromosome movement to microtubule depolymerization (Mitchison and Kirschner 1985a, b; Koshland et al. 1988; Hyman and Mitchison 1990). In budding yeast, where a 125 bp DNA sequence is sufficient to define a centromere, incubation of centromere DNA containing plasmids in yeast extract can recapitulate the assembly of multiple centromere proteins on DNA thereby providing a biochemically tractable system that benefits from the wealth of mutants generated in genetic studies of chromosome segregation (Hyman et al. 1992; Kingsbury and Koshland 1991; Sorger et al. 1994; Sandall et al. 2006). These studies eventually led to the purification of intact kinetochore particles from yeast which made possible elegant structural, biophysical, and proteomic studies (Sorger et al. 1994; Akiyoshi et al. 2009, 2010; Gonen et al. 2012).

Although purification of kinetochores from cells has provided a powerful approach to studying the functions of kinetochore proteins, mutant analysis using this approach is still limited by host viability. Full reconstitution of kinetochores using purified components offers one potential solution to this problem. But despite major advances toward this goal (Pesenti et al. 2016), many centromere and kinetochore proteins have resisted expression and purification and it has been difficult to recapitulate post-translational regulation and cell cycle dependent assembly of centromeres and kinetochores in vitro.

Cell free approaches to centromere and kinetochore reconstitution that can capture the dynamic properties of centromeres and kinetochores and their cell cycle dependent regulation stand to provide significant new insight into their function. One of the most powerful approaches in this class is the use of extracts from *Xenopus* eggs, in which functional centromeres can be reconstituted de novo on defined chromatin templates, and which support spindle assembly, checkpoint control, and anaphase chromosome segregation. Below we discuss first the general advantages of egg extract for in vitro reconstitution studies, and then how its strengths have been specifically leveraged for the study of centromeres and kinetochores.

2.1 *Xenopus* Egg Extract as a Versatile System for In Vitro Reconstitution

Xenopus egg extracts were originally developed as a starting material for biochemical fractionation following the demonstration that cytoplasmic components were responsible for controlling cell cycle progress and for regulating the ensuing changes in chromosomes during DNA replication and mitosis (Gurdon and Woodland 1968; Gurdon 1968; Masui and Markert 1971; Benbow and Ford 1975; Benbow et al. 1975). *Xenopus* eggs are an ideal starting material for biochemical studies. First, because each egg contains ~1 μ L of cytoplasm and a single frog will lay 1000–2000 eggs, a few frogs will provide several milliliters of cytoplasm. Second, because new RNA synthesis does not start until the twelfth division after fertilization each egg is stockpiled with maternal proteins and RNAs to support chromatin assembly, nuclear assembly, and chromosome segregation (Newport and Kirschner 1982a, b). Therefore, extract from a batch of *Xenopus* eggs (Fig. 2a) which maintains RNA and protein concentrations at or near normal cytoplasmic levels provides abundant material for biochemical studies.

In addition to their biochemical advantages, egg extracts recapitulate complex cellular processes in vitro (Lohka and Masui 1983, 1984; Iwao and Katagiri 1984; Miake-Lye and Kirschner 1985). Lohka and Maller first demonstrated that addition of demembranated sperm chromatin to *Xenopus* egg extracts reconstituted pronuclear assembly, mitotic chromosome condensation, and spindle assembly (Lohka and Maller 1985). Over the following decades, reconstitution in *Xenopus* egg extracts has informed studies of sperm decondensation and chromatin assembly (Earnshaw et al. 1980; Almouzni and Mechali 1988a, b; Ohsumi and Katagiri 1991), nuclear envelope assembly (Sheehan et al. 1988), nucleocytoplasmic transport (Saitoh et al. 1996; Moore and Blobel 1992; Newmeyer and Forbes 1988), DNA replication (Blow and Laskey 1986), mitotic chromosome condensation (Shintomi et al. 2015), mitotic spindle assembly (Lohka and Maller 1985), spindle assembly checkpoint signaling (Minshull et al. 1994; Chen 2008), and anaphase chromosome

segregation (Murray et al. 1996). This is in addition to other activities recapitulated by extract including the DNA damage response (Kornbluth et al. 1992; Matsumoto and Bogenhagen 1989; Garner and Costanzo 2009; Lupardus et al. 2007), developmental signaling (Salic et al. 2000), apoptosis (Newmeyer et al. 1994; Kornbluth and Evans 2001), and organelle scaling (Levy and Heald 2015).

A significant advantage of working in egg extracts for dissecting such complex phenomena is that the cytoplasmic composition can be easily manipulated. Proteins can be removed by immunodepletion and complemented with in vitro translated or purified proteins at varying concentrations. Furthermore, complementation with mutants to assess phenotypes, fluorescently labeled proteins to image protein dynamics, inhibitors (small molecules, peptides, antibodies, etc.) to functionally manipulate the extract or radiolabeled metabolites to follow the flux of molecules through the extract are all straightforward.

A major advantage of the egg extract system for studying centromere and kinetochore function is the ability to manipulate the cell cycle state (Murray 1991; Desai et al. 1998) (Fig. 2b). *Xenopus* eggs are arrested at metaphase of meiosis II and initiate division once fertilized. Egg extracts prepared in the absence of calcium are naturally arrested in metaphase and can be cycled into interphase simply by addition of calcium to mimic fertilization. Sperm chromatin added to calcium-released extracts undergoes chromatid decondensation and subsequent DNA replication (Lohka and Maller 1985). Furthermore, interphase extracts can be driven back into metaphase by the addition of metaphase arrested extracts, thereby causing microtubule assembly around sperm chromatin to generate metaphase spindles with bioriented sister kinetochores that attach to opposite spindle poles and support spindle assembly checkpoint signaling (Sawin and Mitchison 1991; Minshull et al. 1994). Addition of calcium to these spindle-containing extracts drives anaphase chromosome segregation (Murray et al. 1996; Shamu and Murray 1992). Because of the short duration of embryonic cell cycles (30–90 min) (Newport and Kirschner 1982a), the events of multiple successive cell cycles can be tracked easily in egg extract (Chang and Ferrell 2013; Murray and Kirschner 1989).

The properties described above make *Xenopus* egg extract the ideal system for detailed dissection of complex cell cycle-regulated processes. In the next section, we discuss how the unique advantages *Xenopus* egg extract have and continue to contribute to the elucidation of various aspects of centromere and kinetochore function; including maintenance of centromere identity, recruitment of CCAN proteins onto chromatin, assembly of kinetochores during mitosis, and regulation of the spindle assembly checkpoint.

3 Application of Frog Egg Extracts to Study Centromere and Kinetochore Function

Xenopus egg extract is the only cell free system that assembles functional centromeres and kinetochores de novo (Desai et al. 1997). During spermatogenesis in frogs, CENP-A is retained on sperm chromatin while the rest of the centromere is disassembled (Milks et al. 2009; Palmer et al. 1990). Thus when demembranated sperm chromatin is added into egg extract it acts as a naïve template for centromere assembly. Because centromere proteins

must be assembled from the egg cytoplasm, depleting proteins from the extract makes it possible to assay depletion phenotypes within a single cell cycle without the confounding secondary effects often associated with RNAi or gene deletion experiments in living cells. These unique advantages of egg extract have enabled insights into several important aspects of centromere and kinetochore function.

3.1 Epigenetic Maintenance of Centromere Identity

CENP-A nucleosomes appear to provide a true epigenetic mark that dictates the position of the centromere (McKinley and Cheeseman 2016). DNA sequence is neither necessary nor sufficient for centromere identity in vertebrates, whereas loss of CENP-A prevents centromere formation (McKinley and Cheeseman 2016). Moreover, targeting CENP-A to a noncentromeric locus promotes assembly of a neocentromere that is epigenetically maintained through subsequent generations (Mendiburo et al. 2011; McKinley and Cheeseman 2016). To understand the persistence of centromere identity, it is necessary to understand how CENP-A chromatin directs new CENP-A assembly. Perturbing the self-renewing mechanisms that maintain CENP-A chromatin is challenging in vivo because centromere inheritance is required for viability. *Xenopus* egg extract is the only in vitro system that recapitulates epigenetic CENP-A inheritance. Short cell cycles and facile control of cell cycle transitions have facilitated temporal dissection of the stages of CENP-A assembly. In addition, these studies benefit from the ability to manipulate the chromatin-associated and the soluble pools of CENP-A separately to understand the distinct mechanisms involved in recognizing template chromatin and in recognizing prenucleosomal CENP-A. Together, these features have permitted detailed studies of the mechanisms by which CENP-A chromatin is propagated, including what chaperones are involved, what proteins recognize the pre-existing centromere, and what features of CENP-A chromatin are recognized.

Extract-based CENP-A assembly assays recapitulate interphase- and centromere-specific CENP-A deposition on demembrated sperm chromatin that requires the CENP-A chaperone HJURP (Bernad et al. 2011; Moree et al. 2011; Foltz et al. 2009; Dunleavy et al. 2009) (Fig. 3a). Biochemical studies demonstrate that HJURP binds a region on CENP-A called the CATD (CENP-A Targeting Domain; Fig. 4b) first identified because transplanting this domain into histone H3 caused the chimeric molecule to assemble at centromeres (Black et al. 2004). Artificially tethering HJURP to an arbitrary chromosomal locus is sufficient to promote local CENP-A assembly (Barnhart et al. 2011). However, other H3 chaperones including RbAp46/48 have also been implicated in CENP-A assembly by studies in human cells and *Drosophila* (Dunleavy et al. 2009; Furuyama et al. 2006), though their roles have been difficult to study in vivo due to their importance in H3 chromatin assembly. In egg extract, inviability due to defective H3 chromatin assembly is not a concern. Depletion of RbAp48 from egg extract has no effect on CENP-A assembly, and depletion of CAF1p150 or HIRA—components of the H3.1 and H3.3 assembly complexes, respectively—have only mild effects (Bernad et al. 2011). Thus, HJURP appears to be the predominant CENP-A chaperone.

To understand how CENP-A serves as an epigenetic mark for centromere identity, it is necessary to understand how HJURP and the proteins that assemble new CENP-A recognize the pre-existing centromere. In egg extract, immunodepletion of CENP-C, which directly binds nucleosomal CENP-A, causes defective HJURP localization and decreased CENP-A assembly (Moree et al. 2011). The facile cell cycle control available in extract enabled identification of two roles for CENP-C in promoting new CENP-A assembly: recruitment of HJURP in inter-phase, and recruitment of another essential CENP-A assembly factor, the Mis18 complex, during mitosis (Moree et al. 2011) (Fig. 3b). The Mis18 complex, comprised of Mis18 α , Mis18 β , and M18BP1 (Maddox et al. 2007; Fujita et al. 2007) is also required for HJURP localization, as immunodepletion of M18BP1 causes a loss of both centromeric HJURP and CENP-A loading (Moree et al. 2011). Its mitotic function, however, remains mysterious. The Mis18 complex has been proposed to “prime” centromeric chromatin for assembly by modulating post-translational modification of chromatin (Fujita et al. 2007; Hayashi et al. 2004; Ohzeki et al. 2016; Kim et al. 2012) which could contribute to a proposed licensing mechanism for CENP-A assembly. Indeed, while M18BP1 is recruited to centromeres in metaphase egg extract by a direct interaction with CENP-C, M18BP1 switches to a CENP-C-independent mechanism for interphase localization (Moree et al. 2011; Westhorpe et al. 2015), possibly analogous to cell cycle-regulated licensing of DNA replication origins (Walter and Arias 2004). Facile cell cycle manipulation of egg extract and the ability to transfer chromatin between extracts of different composition or cell cycle states were essential for identifying the mechanisms of DNA replication licensing (Blow and Laskey 2016) and will likewise be valuable in elucidating the mechanisms that may license new CENP-A assembly.

While immunodepletion analysis has identified CENP-C as a key recognition factor for centromeric chromatin and ruled out other CCAN components such as CENP-T and CENP-W (Krizaic et al. 2015; Moree et al. 2011), the fundamental question underpinning the epigenetic inheritance of CENP-A is what features of CENP-A chromatin are recognized by the assembly machinery and how. Extract affords the only system in which the composition of the template chromatin can be freely manipulated to directly address this question. Chromatin reconstitution methods have been extensively developed over the past three decades, making it possible to assemble nucleosomes on arbitrary DNA sequences (Luger et al. 1999). This includes centromeric nucleosomes which have been reconstituted both as mononucleosomes and tandem nucleosome arrays (Guse et al. 2011; Yoda et al. 2000) (Fig. 4a). Using this approach, we altered both the composition and density of nucleosomes in the chromatin template underlying the centromere to probe the effects of centromeric chromatin composition on the CENP-A assembly process in egg extract.

The ability of CENP-A to promote the assembly of the centromere and kinetochore arises from key structural features of the CENP-A molecule that differ from H3 including the CATD—a loop and alpha helix within the histone fold domain (Black et al. 2004)—and a six amino acid C-terminal extension (the CENP-A C-terminus; CAC) (Carroll et al. 2010) (Fig. 4b). CENP-C and CENP-N are the two proteins known to selectively bind CENP-A nucleosomes. The CATD was shown to be the determinant in centromeric nucleosomes recognized by CENP-N (Carroll et al. 2009), and CENP-C recognizes the CAC (Carroll et al. 2010). Hydrophobic amino acids in the CAC contact hydrophobic residues in a conserved

domain of CENP-C termed the “CENP-C motif” while basic amino acids in the CENP-C motif interact with negatively charged amino acids on H2A/B termed the “acidic patch” (Kato et al. 2013).

By generating chromatin arrays that contained chimeric nucleosomes with the CATD domain and/or the CAC replacing the corresponding domains of histone H3, we determined the functions of those CENP-A domains in promoting CENP-A assembly. Consistent with the essential role of CENP-C in CENP-A assembly, reconstitution of CENP-A assembly on chimeric templates requires the CAC (Westhorpe et al. 2015). However, M18BP1 localization and CENP-A assembly are only supported by chimeras containing both the CATD and the CAC (Westhorpe et al. 2015). This suggests a CATD-binding protein like CENP-N—whose role has been implied in cell-based studies (Carroll et al. 2009)—may be required, or that CENP-A assembly machinery itself recognizes CATD-containing chromatin (Sandmann et al. 2017). As nascent CENP-A requires the CATD for HJURP-mediated incorporation into chromatin (Foltz et al. 2009; Fachinetti et al. 2013; Logsdon et al. 2015), extract reconstitution provides an advantage over similar cell-based experiments that cannot distinguish such separate roles for the CATD in HJURP recognition and localization of the CENP-A assembly machinery.

Reconstituted chromatin also affords the opportunity to ask how the density of CENP-A nucleosomes contributes to CENP-A assembly. Centromeric nucleosomes are partitioned equally between sister chromatids during DNA replication in the absence of new CENP-A assembly (Jansen et al. 2007), with H3.3 proposed to fill the “gaps” in centromeric chromatin left by this redistribution (Dunleavy et al. 2011) (Fig. 3c). Performing chromatin reconstitution with lower histone concentrations results in nucleosome arrays that are “subsaturated,” and egg extract assembles H3 nucleosomes in the gaps between CENP-A nucleosomes (Westhorpe et al. 2015) (Fig. 4c). While new CENP-A assembly occurs to some degree on chromatin templates that are saturated with CENP-A, it is twice as efficient on templates only 40% saturated with CENP-A (Westhorpe et al. 2015). This could reflect a role for H3 nucleosome disassembly in the CENP-A assembly process (Dunleavy et al. 2011), and may suggest the existence of mechanisms to distinguish CENP-A from H3 nucleosomes.

Reconstitution of nucleosome arrays with different histone compositions has provided several insights into centromere assembly, but relatively untapped is the role of DNA sequence in centromere specification. Nucleosome arrays can be reconstituted on arbitrary DNA sequences, providing an ideal tool for addressing this question. In cells, human artificial chromosome formation requires CENP-B box-containing alpha satellite sequences (Ohzeki et al. 2002; Masumoto et al. 1998). Recently, mass spectrometry analysis of bacterial artificial chromosomes (BACs) containing human α -satellite DNA recovered from egg extract revealed association of several centromere proteins including CENP-A, suggesting sequence-dependent, de novo centromere assembly may be recapitulated in egg extract (Aze et al. 2016). Understanding the role of DNA sequence in centromere maintenance and neocentromere formation represents an important goal for the field.

3.2 CCAN Assembly

An essential function of CENP-A chromatin is to assemble the CCAN which serves as the platform for kinetochore assembly in mitosis (Fig. 1). Experiments in frog egg extract in which purified plasmid DNA was coupled to beads and added to metaphase extract showed that DNA alone promotes the formation of bipolar microtubule spindles. However, these DNA beads could not support the formation of a centromere or kinetochore (Heald et al. 1996). However, if CENP-A nucleosomes are preassembled on DNA before addition to egg extract then beads coated with CENP-A chromatin acquire the capacity to form centromeres and kinetochores that attach to microtubules and elicit mitotic arrest in response to microtubule depolymerization (Guse et al. 2011). This reconstituted chromatin system demonstrates that CENP-A is sufficient to build a functional centromere in frog egg extract and provides a powerful tool to study how CENP-A specifies centromere and kinetochore assembly.

Two CCAN proteins, CENP-C and CENP-N, bind directly to CENP-A nucleosomes (see above) and, through interactions with other centromere proteins, recruit the other members of the CCAN to assemble the constitutive centromere (Fig. 1). A key advantage of egg extract is that their functions in CENP-A nucleosome assembly can be uncoupled from their functions in CENP-A nucleosome assembly. Whereas the epigenetic inheritance of CENP-A requires both the CATD and the CAC (see above), chromatin reconstituted with chimeric histones containing only the CAC is sufficient for centromere and kinetochore assembly in mitotic extracts (Guse et al. 2011). These kinetochores bind microtubules and exhibit spindle assembly checkpoint activity (Guse et al. 2011). Despite direct binding of CENP-N to the CATD (Carroll et al. 2009), CAC-containing chimeras recruit more CENP-N in extract than CATD-containing chimeras, suggesting that CENP-C provides the critical link between centromeric chromatin and the CCAN (Guse et al. 2011). Multivalent interactions between CENP-C and several CCAN components including CENP-N could stabilize their localization to centromeres. Work in several labs is beginning to elucidate this complex network of interactions with purified proteins and how their collective assembly gives rise to selective centromere formation at CENP-A chromatin (McKinley et al. 2015; Hinshaw and Harrison 2013; Nishino et al. 2012; Weir et al. 2016; Basilico et al. 2014; Pesenti et al. 2016; Klare et al. 2015).

Cell-based studies have indicated that the mode of CENP-C recruitment changes from mitosis to interphase (Nagpal et al. 2015; Kwon et al. 2007), suggesting that CENP-C localization may be influenced either by cell cycle-regulated post-translational modification or by changes in the configuration of chromatin during chromosome condensation. In addition, the presence of H3 nucleosomes interspersed with CENP-A nucleosomes in centromeric chromatin may influence centromere assembly. Direct manipulation of chromatin configurations or of the amount of H3 at centromeres is not possible in vivo. But by reconstituting subsaturated chromatin templates (Fig. 4c), we have shown that the mechanism of CENP-C localization can change with the density of CENP-A nucleosomes at the centromere. Reconstituted chromatin saturated with CAC-containing chimeric nucleosomes recruits CENP-C similar to wild-type CENP-A chromatin (Westhorpe et al. 2015; Guse et al. 2011). In subsaturated arrays where chimeric CENP-A nucleosomes are

interspersed with H3 nucleosomes; however, the CAC is not sufficient for full CENP-C recruitment (Westhorpe et al. 2015). In this case, the CATD is also required to recruit the same amount of CENP-C as CENP-A chromatin. CENP-C contains two nucleosome binding motifs (Kato et al. 2013) in addition to a dimerization motif (Sugimoto et al. 1997) and therefore may be able to engage adjacent CENP-A nucleosomes and localize exclusively by the CAC where the CENP-A density is high. At lower CENP-A densities, it may require interactions with other CCAN components such as CENP-N for robust localization. Additional studies are required to understand this regulation, and more detailed studies of in vivo chromatin organization will shed light on the biological relevance of different saturation states. However, alteration of centromere protein recruitment in response to chromatin organization poses an exciting possibility.

3.3 Kinetochore Assembly

The primary function of the centromere and kinetochore in mitosis is to couple chromosomes to the mitotic spindle and translate the force generated by depolymerizing microtubules into chromosome segregation (Cheeseman 2014). For many years, it was unclear how chromosomes were coupled to depolymerizing micro-tubules (Gudimchuk et al. 2013; Lombillo et al. 1995). Eventually genetic studies in budding yeast demonstrated that the Ndc80 complex (composed of Ndc80, Nuf2, Spc24, and Spc25) was required for microtubule attachment during anaphase (He et al. 2001; Wigge and Kilmartin 2001) (Fig. 1). But while vertebrate homologues of Ndc80 and Nuf2 could be identified by homology, the vertebrate homologues of Spc24/25 remained elusive. Unlike Ndc80 and Nuf2 mutants, Spc24/25 mutants in yeast not only failed to bind microtubules but also failed to arrest at the spindle assembly checkpoint in response to lack of microtubule attachment (Janke et al. 2001), highlighting the importance of characterizing the entire vertebrate Ndc80 complex. And although many parts of the centromere and kinetochore are conserved from yeast to vertebrates, it was not clear the microtubule-binding mechanism would be the same between yeast point centromeres, in which a single CENP-A nucleosome form the microtubule-binding site, and the larger, regional centromeres of vertebrates.

Egg extract provides concentrated starting material for biochemical purification of intact protein complexes, facilitating the identification of functionally related proteins. Using antibodies raised against *Xenopus* Ndc80 and Nuf2, McClelland and colleagues purified the 190-kD Ndc80 complex from *Xenopus* egg extract and demonstrated it was composed of four species: Ndc80, Nuf2, and highly divergent homologues of Spc24/25 (McClelland et al. 2004). Purification and cloning of the *Xenopus* proteins facilitated the cloning of the human genes and dissection of their functions in chromosome microtubule attachment and metaphase alignment in vivo (Bharadwaj et al. 2004; Ciferri et al. 2008).

The Ndc80 complex is the major microtubule-binding component of the KMN network, comprised of three subcomplexes: KNL1, which also binds microtubules and serves as the platform for spindle assembly checkpoint signaling (see below); Mis12, which connects the KMN network to the underlying centromere; and Ndc80 (Fig. 1). Purification and reconstitution of the Ndc80 complex demonstrated that cooperative arrays of Ndc80 maintain attachment to individual microtubules (Alushin et al. 2010; Ciferri et al. 2008).

Reconstitution of the entire KMN network, however, synergistically enhances in vitro binding to purified microtubules (Cheeseman et al. 2006). An outstanding problem is understanding the basis for this synergy, as well as to understand how this cooperation plays out during chromosome segregation and gives rise to the mechanical properties of the kinetochore under load, a problem extract is well suited to address (see below). Overall, these studies support the model that microtubule binding by the kinetochore is largely conserved between yeast and vertebrates and the proposal that regional centromeres may effectively function as an array of point centromeres (Zinkowski et al. 1991).

Essential to proper chromosome segregation is that kinetochore assembly is restricted to the single centromeric locus (Westhorpe and Straight 2015). De novo centromere assembly in conjunction with immunodepletion studies have made extract an ideal system for dissecting how centromere proteins assemble the kinetochore. This has been particularly useful to address different models regarding the relative contributions of CENP-C and another essential CCAN component, CENP-T, to kinetochore recruitment (Basilico et al. 2014; Nishino et al. 2013; Rago et al. 2015) (Fig. 1). Centromeres on demembranated sperm rapidly assemble CENP-C upon incubation in metaphase extract (Milks et al. 2009). CENP-T does not localize to these centromeres until interphase, but then persists at centromeres after extract cycles into the next mitosis (Krizaic et al. 2015). This feature provides a convenient system for separating the respective contributions of CENP-C and CENP-T to kinetochore assembly.

In the absence of CENP-T, immunodepletion of CENP-C results in complete loss of Mis12 complex and spindle assembly checkpoint components in metaphase extract (Milks et al. 2009). By interacting with the CENP-C N-terminus, the Mis12 complex bridges the CCAN and the KMN network (Milks et al. 2009; Petrovic et al. 2010, 2014, 2016; Przewloka et al. 2011; Screpanti et al. 2011). In cycled mitotic extract in which both CENP-C and CENP-T localize to centromeres, CENP-C depletion only partially reduces Mis12 and Ndc80 localization (Krizaic et al. 2015). In turn, depletion of CENP-T also results in decreased Mis12 and Ndc80 localization (Krizaic et al. 2015), complementing in vitro studies where CENP-T binds both these components (Huis In 't Veld et al. 2016; Gascoigne et al. 2011; Nishino et al. 2013). While this supports a model in which CENP-C and CENP-T recruit Mis12 and Ndc80 by separate mechanisms, CENP-T localization is largely lost upon CENP-C depletion (Krizaic et al. 2015). This supports an emerging model that CENP-C plays a predominant role in organizing both the Mis12-Ndc80 and CENP-T-Ndc80 branches of kinetochore assembly (Klare et al. 2015), and suggests that additional factors contribute to kinetochore recruitment.

Our understanding of how the CCAN promotes kinetochore assembly and microtubule binding is still emerging. Efforts over the last decade to reconstitute kinetochore complexes with purified proteins have culminated in reconstitution of kinetochore particles comprising the KMN network bound to CENP-CHIKMLN bound to CENP-A mononucleosomes (Weir et al. 2016). This work demonstrated that the presence of the CCAN enhances in vitro microtubule binding by the KMN network, raising new questions about how these components integrate to form stable, load-bearing microtubule attachments. Moving forward, egg extract offers a powerful and unique system in which such detailed biochemical

analyses of microtubule binding and kinetochore structure can be directly coupled to phenotypic assays of spindle formation and chromosome segregation by supplementation of extract with purified proteins.

3.4 The Spindle Assembly Checkpoint

Another essential function of the kinetochore is its ability to monitor and regulate microtubule attachment to ensure biorientation, or attachment to opposite spindle poles (Cheeseman 2014). Inter-kinetochore tension generated at bioriented chromosomes by opposing microtubule-pulling forces has been proposed to regulate microtubule dynamics and attachment which, in turn, regulates the metaphase/anaphase transition via the spindle assembly checkpoint (SAC). The SAC delays exit from mitosis in response to unattached kinetochores by inhibiting the E3 ubiquitin ligase activity of the anaphase promoting complex (APC) to permit establishment of biorientation (Lara-Gonzalez et al. 2012). Disruption of the SAC in cells frequently results in chromosome missegregation and death.

Reconstituting spindle assembly checkpoint function in vitro generally poses a particular challenge because it requires reconstitution of both the kinetochore-microtubule interface and cell cycle regulation. These features, however, are easy to recapitulate in egg extract. Assembly of checkpoint protein complexes at kinetochores can be measured by immunofluorescence or biochemically. Furthermore, exit from mitosis in extract is easily monitored using radioactive kinase assays to measure the decline of Cdk activity where SAC activation presents as a delay in calcium-induced loss of Cdk phosphorylation.

Although the SAC is inactive in the early embryo and in extracts containing lower amounts of sperm chromatin, egg extract recapitulates the mitotic checkpoint when sperm is increased to 9000 sperm/ μ L (Minshull et al. 1994). SAC activation upon increased sperm concentration reflects a proportionality between the amount of checkpoint signal generated and the number of unattached kinetochores in the cytoplasm (Collin et al. 2013; Heinrich et al. 2013). While several genes required for spindle assembly checkpoint function had been identified in budding yeast (Hoyt et al. 1991; Li and Murray 1991), the molecular nature of the SAC signal and the mechanism by which the SAC monitored biorientation were unknown at that time (Minshull et al. 1994).

Xenopus Mad2 was among the first SAC components identified in vertebrates (Chen et al. 1996) (Fig. 5). Crucial to demonstrating the role of Mad2 in checkpoint arrest, immunodepletion of Mad2 from egg extract results in failure to delay mitotic exit upon nocodazole treatment, whereas supplementation with purified Mad2 stimulates mitotic arrest (Li et al. 1997; Fang et al. 1998). Unattached kinetochores recruit *Xenopus* Mad2 (Chen et al. 1996) and catalyze the formation of a soluble inhibitory complex: the mitotic checkpoint complex (MCC; Fig. 5), comprised of Mad2, Bub3, BubR1, and the APC co-activator subunit Cdc20 (Li et al. 1997; Fang et al. 1998; Rieder et al. 1995). Co-purification from extract and structural analyses have shown the MCC binds the APC as a pseudo-substrate, blocking cyclin degradation while simultaneously sequestering Cdc20 which is required to initiate anaphase (Fang et al. 1998; Izawa and Pines 2015). Association of the MCC with the APC ultimately leads to ubiquitination and degradation of Cdc20 followed by recycling of MCC components, resulting in robust but rapidly reversible inhibition of mitotic exit.

The ability to directly supplement egg extract with purified proteins afforded the first opportunity to investigate the role of Mad2 conformational changes in checkpoint control. Assembly of Mad2 into the MCC requires its conversion at unattached kinetochores from an “open” conformation to a “closed” conformation (Lara-Gonzalez et al. 2012; Luo et al. 2004) (Fig. 5). Purification of recombinant Mad2 from bacteria yields two forms: a monomeric, inactive form and an active, oligomeric form (Fang et al. 1998). Addition of monomeric Mad2 (“open” Mad2) to egg extract fails to stimulate checkpoint arrest; however, the conformation of oligomeric Mad2 recapitulates that of “closed” Mad2 and inhibits APC activity (Fang et al. 1998). The ease with which kinetochores can be manipulated in extract and chromatin isolated has facilitated dissection of the catalytic cycle occurring on kinetochores by which Mad2 coverts between “open” to “closed” forms, a cycle which largely governs the responsiveness of the SAC to microtubule attachment.

Arguably the most important question regarding the spindle assembly checkpoint is how the kinetochore senses the status of its attachment to microtubules. This appears to be mediated in part by a kinase, Mps1, which binds competitively to the microtubule-binding surface of Ndc80 to identify unattached kinetochores (Hiruma et al. 2015; Ji et al. 2015) (Fig. 5). Mps1 phosphorylation of KNL1 promotes assembly of SAC proteins including Mad2 (Abrieu et al. 2001; Chen 2002; Zhao and Chen 2006; Hewitt et al. 2010). In addition, the absence of tension between kinetochores that are not bioriented promotes disassembly of improper microtubule attachments, contributing indirectly to SAC function. Because tension and microtubule attachment are intimately linked in vivo, however, it has been challenging to dissect their separate contributions to regulation of microtubule attachment and to SAC function. Indeed, elongation of Ndc80 experiencing tension along the kinetochore axis may directly affect SAC function by separating Mps1 from KNL1 and preventing its phosphorylation (Wan et al. 2009; Aravamudan et al. 2015). While the role of tension in stabilizing kinetochore microtubules and regulating anaphase onset was first described fifty years ago in micromanipulation studies of grasshopper spermatocyte chromosomes (Nicklas and Koch 1969), in the absence of straightforward methods to simultaneously manipulate tension applied to kinetochores and perturb centromere/kinetochore composition, tension-dependent changes in kinetochore function have been challenging to dissect.

Purification and micromanipulation of yeast kinetochores have begun to provide insight into novel mechanisms by which tension stabilizes kinetochore micro-tubules (Akiyoshi et al. 2009, 2010; Miller et al. 2016). However, tolerance of the host to mutation of kinetochore components is still a limitation of this method. This limitation does not apply in egg extract, where tension can be applied directly to sperm or reconstituted kinetochores (Almagro and Dimitrov 2005; Yan et al. 2007). Extract also affords the unique opportunity to understand how cytoplasmic factors or those that do not copurify with kinetochores may contribute to their response to tension. In egg extract, Aurora substrate phosphorylation or SAC activation can be monitored directly, permitting careful measurement of force-response curves that would provide a quantitative, molecular description of how microtubule dynamics/attachment and cell cycle arrest are regulated by tension, measurements that have been challenging to make in vivo. Biophysical studies of kinetochores in egg extract will also provide a unique method for comparing the mechanical properties of regional vertebrate centromeres to the point centromeres of budding yeast.

4 Conclusion

Reconstitution studies have given us tremendous insight into the many functions of the centromere and kinetochore. Egg extract in particular has provided a versatile system for probing these questions biochemically because of the ease with which the composition of centromere, the composition of the cytoplasm, and the cell cycle state can be controlled. Extract is unique in its ability to capture the dynamically regulated aspects of this structure in vitro.

Historically, extract has been valued for the opportunity to validate observations by comparing processes reconstituted in vitro with the corresponding processes in *Xenopus* tissue culture cells or in intact embryos. Because of the difficulties associated with genetic manipulations in *Xenopus*, however, most in vivo work has shifted to human tissue culture cells or other model systems. But with the recent publication of the *Xenopus laevis* genome (Session et al. 2016) and rapid innovations in gene editing technologies, a new dawn for in vivo studies of chromosome segregation in frogs could lie ahead. The ability to genetically encode tagged centromere proteins would also circumvent the often laborious process of antibody generation that precedes most work in extract.

Our ability to reconstitute functional kinetochores from purified components has progressed from a pipe dream of the 1960s and 70s to reality. Although many questions remain regarding centromere recognition, centromere propagation, and kinetochore assembly and function, integrating reconstitution of specific structures from purified proteins with reconstitution of dynamic and cell cycle-regulated aspects of chromosome segregation in extract offers a wide array of possibilities for probing their mechanisms.

References

- Abrieu A, Magnaghi-Jaulin L, Kahana JA, Peter M, Castro A, Vigneron S, Lorca T, Cleveland DW, Labbe J. Mps1 is a kinetochore-associated kinase essential for the vertebrate mitotic checkpoint. *Cell*. 2001; 106:83–93. [PubMed: 11461704]
- Akiyoshi B, Nelson CR, Ranish JA, Biggins S. Quantitative proteomic analysis of purified yeast kinetochores identifies a PP1 regulatory subunit. *Genes Dev*. 2009; 23(24):2887–2899. DOI: 10.1101/gad.1865909 [PubMed: 19948764]
- Akiyoshi B, Sarangapani KK, Powers AF, Nelson CR, Reichow SL, Arellano-Santoyo H, Gonen T, Ranish JA, Asbury CL, Biggins S. Tension directly stabilizes reconstituted kinetochore-microtubule attachments. *Nature*. 2010; 468(7323):576–579. DOI: 10.1038/nature09594 [PubMed: 21107429]
- Almagro S, Dimitrov S. Assembly and micromanipulation of *Xenopus* in vitro-assembled mitotic chromosomes. *Curr Protoc Cell Biol*. 2005; Chapter 22(Unit 22):29.doi: 10.1002/0471143030.cb2209s26
- Almouzni G, Mechali M. Assembly of spaced chromatin involvement of ATP and DNA topoisomerase activity. *EMBO J*. 1988a; 7(13):4355–4365. [PubMed: 2854062]
- Almouzni G, Mechali M. Assembly of spaced chromatin promoted by DNA synthesis in extracts from *Xenopus* eggs. *EMBO J*. 1988b; 7(3):665–672. [PubMed: 3396538]
- Alushin GM, Ramey VH, Pasqualato S, Ball DA, Grigorieff N, Musacchio A, Nogales E. The Ndc80 kinetochore complex forms oligomeric arrays along microtubules. *Nature*. 2010; 467(7317):805–810. DOI: 10.1038/nature09423 [PubMed: 20944740]
- Aravamudhan P, Goldfarb AA, Joglekar AP. The kinetochore encodes a mechanical switch to disrupt spindle assembly checkpoint signalling. *Nat Cell Biol*. 2015; 17(7):868–879. DOI: 10.1038/ncb3179 [PubMed: 26053220]

- Aze A, Sannino V, Soffientini P, Bachi A, Costanzo V. Centromeric DNA replication reconstitution reveals DNA loops and ATR checkpoint suppression. *Nat Cell Biol.* 2016; 18(6):684–691. DOI: 10.1038/ncb3344 [PubMed: 27111843]
- Barnhart MC, Kuich PH, Stellfox ME, Ward JA, Bassett EA, Black BE, Foltz DR. HJURP is a CENP-A chromatin assembly factor sufficient to form a functional de novo kinetochore. *J Cell Biol.* 2011; 194(2):229–243. DOI: 10.1083/jcb.201012017 [PubMed: 21768289]
- Basilico F, Maffini S, Weir JR, Prumbaum D, Rojas AM, Zimniak T, De Antoni A, Jeganathan S, Voss B, van Gerwen S, Krenn V, Massimiliano L, Valencia A, Vetter IR, Herzog F, Raunser S, Pasqualato S, Musacchio A. The pseudo GTPase CENP-M drives human kinetochore assembly. *Elife.* 2014; 3:e02978.doi: 10.7554/eLife.02978 [PubMed: 25006165]
- Benbow RM, Ford CC. Cytoplasmic control of nuclear DNA synthesis during early development of *Xenopus laevis*: a cell-free assay. *Proc Natl Acad Sci USA.* 1975; 72(6):2437–2441. [PubMed: 1056038]
- Benbow RM, Pestell RQW, Ford CC. Appearance of DNA-polymerase activities during early development of *Xenopus-Laevis*. *Dev Biol.* 1975; 43(1):159–174. DOI: 10.1016/0012-1606(75)90138-4
- Bernad R, Sanchez P, Rivera T, Rodriguez-Corsino M, Boyarchuk E, Vassias I, Ray-Gallet D, Arnaoutov A, Dasso M, Almouzni G, Losada A. *Xenopus* HJURP and condensin II are required for CENP-A assembly. *J Cell Biol.* 2011; 192(4):569–582. DOI: 10.1083/jcb.201005136 [PubMed: 21321101]
- Bharadwaj R, Qi W, Yu H. Identification of two novel components of the human NDC80 kinetochore complex. *J Biol Chem.* 2004; 279(13):13076–13085. DOI: 10.1074/jbc.M310224200 [PubMed: 14699129]
- Black BE, Foltz DR, Chakravarthy S, Luger K, Woods VL Jr, Cleveland DW. Structural determinants for generating centromeric chromatin. *Nature.* 2004; 430(6999):578–582. DOI: 10.1038/nature02766 [PubMed: 15282608]
- Blow JJ, Laskey RA. Initiation of DNA replication in nuclei and purified DNA by a cell-free extract of *Xenopus* eggs. *Cell.* 1986; 47(4):577–587. [PubMed: 3779837]
- Blow JJ, Laskey RA. *Xenopus* cell-free extracts and their contribution to the study of DNA replication and other complex biological processes. *Int J Dev Biol.* 2016; 60(7-8-9):201–207. DOI: 10.1387/ijdb.160142jb [PubMed: 27759151]
- Carroll CW, Silva MC, Godek KM, Jansen LE, Straight AF. Centromere assembly requires the direct recognition of CENP-A nucleosomes by CENP-N. *Nat Cell Biol.* 2009; 11(7):896–902. DOI: 10.1038/ncb1899 [PubMed: 19543270]
- Carroll CW, Milks KJ, Straight AF. Dual recognition of CENP-A nucleosomes is required for centromere assembly. *J Cell Biol.* 2010; 189(7):1143–1155. DOI: 10.1083/jcb.201001013 [PubMed: 20566683]
- Chang JB, Ferrell JE Jr. Mitotic trigger waves and the spatial coordination of the *Xenopus* cell cycle. *Nature.* 2013; 500(7464):603–607. DOI: 10.1038/nature12321 [PubMed: 23863935]
- Cheeseman IM. The kinetochore. *Cold Spring Harb Perspect Biol.* 2014; 6(7):a015826.doi: 10.1101/cshperspect.a015826 [PubMed: 24984773]
- Cheeseman IM, Chappie JS, Wilson-Kubalek EM, Desai A. The conserved KMN network constitutes the core microtubule-binding site of the kinetochore. *Cell.* 2006; 127(5):983–997. DOI: 10.1016/j.cell.2006.09.039 [PubMed: 17129783]
- Chen RH. BubR1 is essential for kinetochore localization of other spindle checkpoint proteins and its phosphorylation requires Mad1. *J Cell Biol.* 2002; 158(3):487–496. DOI: 10.1083/jcb.200204048 [PubMed: 12163471]
- Chen RH. The spindle checkpoint in *Xenopus laevis*. *Front Biosci.* 2008; 13:2231–2237. [PubMed: 17981705]
- Chen RH, Waters JC, Salmon ED, Murray AW. Association of spindle assembly checkpoint component XMAP215 with unattached kinetochores. *Science.* 1996; 274(5285):242–246. [PubMed: 8824188]
- Ciferri C, Pasqualato S, Screpanti E, Varetto G, Santaguida S, Dos Reis G, Maiolica A, Polka J, De Luca JG, De Wulf P, Salek M, Rappsilber J, Moores CA, Salmon ED, Musacchio A. Implications

for kinetochore-microtubule attachment from the structure of an engineered Ndc80 complex. *Cell*. 2008; 133(3):427–439. DOI: 10.1016/j.cell.2008.03.020 [PubMed: 18455984]

Collin P, Nashchekina O, Walker R, Pines J. The spindle assembly checkpoint works like a rheostat rather than a toggle switch. *Nat Cell Biol*. 2013; 15(11):1378–1385. DOI: 10.1038/ncb2855 [PubMed: 24096242]

Desai A, Deacon HW, Walczak CE, Mitchison TJ. A method that allows the assembly of kinetochore components onto chromosomes condensed in clarified *Xenopus* egg extracts. *Proc Natl Acad Sci USA*. 1997; 94:12378–12383. [PubMed: 9356457]

Desai A, Murray A, Mitchison TJ, Walczak CE. Chapter 20 The use of *xenopus* egg extracts to study mitotic spindle assembly and function in vitro. *Methods Cell Biol*. 1998; 61:385–412. DOI: 10.1016/s0091-679x(08)61991-3

Dunleavy EM, Roche D, Tagami H, Lacoste N, Ray-Gallet D, Nakamura Y, Daigo Y, Nakatani Y, Almouzni-Pettinotti G. HJURP is a cell-cycle-dependent maintenance and deposition factor of CENP-A at centromeres. *Cell*. 2009; 137(3):485–497. DOI: 10.1016/j.cell.2009.02.040 [PubMed: 19410545]

Dunleavy EM, Almouzni G, Karpen GH. H3.3 is deposited at centromeres in S phase as a placeholder for newly assembled CENP-A in G(1) phase. *Nucleus*. 2011; 2(2):146–157. DOI: 10.4161/nucl.2.2.15211 [PubMed: 21738837]

Earnshaw WC, Rothfield N. Identification of a family of human centromere proteins using autoimmune sera from patients with scleroderma. *Chromosoma*. 1985; 91(3–4):313–321. DOI: 10.1007/Bf00328227 [PubMed: 2579778]

Earnshaw WC, Honda BM, Laskey RA, Thomas JO. Assembly of nucleosomes: the reaction involving *X. Laevis* nucleoplasmin. *Cell*. 1980; 21(2):373–383. [PubMed: 7407918]

Earnshaw W, Bordwell B, Marino C, Rothfield N. Three human chromosomal autoantigens are recognized by sera from patients with anti-centromere antibodies. *J Clin Invest*. 1986; 77(2):426–430. DOI: 10.1172/JCI112320 [PubMed: 3511098]

Earnshaw WC, Sullivan KF, Machlin PS, Cooke CA, Kaiser DA, Pollard TD, Rothfield NF, Cleveland DW. Molecular cloning of cDNA for CENP-B, the major human centromere autoantigen. *J Cell Biol*. 1987; 104(4):817–829. [PubMed: 2435739]

Fachinetti D, Diego Folco H, Nechemia-Arbely Y, Valente LP, Nguyen K, Wong AJ, Zhu Q, Holland AJ, Desai A, Jansen LE, Cleveland DW. A two-step mechanism for epigenetic specification of centromere identity and function. *Nat Cell Biol*. 2013; 15(9):1056–1066. DOI: 10.1038/ncb2805 [PubMed: 23873148]

Fang G, Yu H, Kirschner MW. The checkpoint protein MAD2 and the mitotic regulator CDC20 form a ternary complex with the anaphase-promoting complex to control anaphase initiation. *Genes Dev*. 1998; 12(12):1871–1883. [PubMed: 9637688]

Flemming W. Beiträge zur Kenntniss der Zelle und ihrer Lebenserscheinungen, Theil II. *Archiv für Mikroskopische Anatomie*. 1880; 18:151–259.

Foley EA, Kapoor TM. Microtubule attachment and spindle assembly checkpoint signalling at the kinetochore. *Nat Rev Mol Cell Biol*. 2013; 14(1):25–37. DOI: 10.1038/nrm3494 [PubMed: 23258294]

Foltz DR, Jansen LE, Black BE, Bailey AO, Yates JR 3rd, Cleveland DW. The human CENP-A centromeric nucleosome-associated complex. *Nat Cell Biol*. 2006; 8(5):458–469. DOI: 10.1038/ncb1397 [PubMed: 16622419]

Foltz DR, Jansen LE, Bailey AO, Yates JR 3rd, Bassett EA, Wood S, Black BE, Cleveland DW. Centromere-specific assembly of CENP-A nucleosomes is mediated by HJURP. *Cell*. 2009; 137(3):472–484. DOI: 10.1016/j.cell.2009.02.039 [PubMed: 19410544]

Fujita Y, Hayashi T, Kiyomitsu T, Toyoda Y, Kokubu A, Obuse C, Yanagida M. Priming of centromere for CENP-A recruitment by human hMis18alpha, hMis18beta, and M18BP1. *Dev Cell*. 2007; 12(1):17–30. DOI: 10.1016/j.devcel.2006.11.002 [PubMed: 17199038]

Furuyama T, Dalal Y, Henikoff S. Chaperone-mediated assembly of centromeric chromatin in vitro. *Proc Natl Acad Sci USA*. 2006; 103(16):6172–6177. DOI: 10.1073/pnas.0601686103 [PubMed: 16601098]

- Garner E, Costanzo V. Studying the DNA damage response using in vitro model systems. *DNA Repair* (Amst). 2009; 8(9):1025–1037. DOI: 10.1016/j.dnarep.2009.04.015 [PubMed: 19482562]
- Gascoigne KE, Takeuchi K, Suzuki A, Hori T, Fukagawa T, Cheeseman IM. Induced ectopic kinetochore assembly bypasses the requirement for CENP-A nucleosomes. *Cell*. 2011; 145(3):410–422. [PubMed: 21529714]
- Gonen S, Akiyoshi B, Iadanza MG, Shi D, Duggan N, Biggins S, Gonen T. The structure of purified kinetochores reveals multiple microtubule-attachment sites. *Nat Struct Mol Biol*. 2012; 19(9):925–929. DOI: 10.1038/nsmb.2358 [PubMed: 22885327]
- Gudimchuk N, Vitre B, Kim Y, Kiyatkin A, Cleveland DW, Ataullakhanov FI, Grishchuk EL. Kinetochore kinesin CENP-E is a processive bi-directional tracker of dynamic microtubule tips. *Nat Cell Biol*. 2013; 15(9):1079–1088. DOI: 10.1038/ncb2831 [PubMed: 23955301]
- Gurdon JB. Changes in somatic cell nuclei inserted into growing and maturing amphibian oocytes. *J Embryol Exp Morphol*. 1968; 20(3):401–414. [PubMed: 5750105]
- Gurdon JB, Woodland HR. The cytoplasmic control of nuclear activity in animal development. *Biol Rev Camb Philos Soc*. 1968; 43(2):233–267. [PubMed: 4869950]
- Guse A, Carroll CW, Moree B, Fuller CJ, Straight AF. In vitro centromere and kinetochore assembly on defined chromatin templates. *Nature*. 2011; 477(7364):354–358. DOI: 10.1038/nature10379 [PubMed: 21874020]
- Hayashi T, Fujita Y, Iwasaki O, Adachi Y, Takahashi K, Yanagida M. Mis16 and Mis18 are required for CENP-A loading and histone deacetylation at centromeres. *Cell*. 2004; 118(6):715–729. DOI: 10.1016/j.cell.2004.09.002 [PubMed: 15369671]
- He X, Rines DR, Espelin CW, Sorger PK. Molecular analysis of kinetochore-microtubule attachment in budding yeast. *Cell*. 2001; 106(2):195–206. [PubMed: 11511347]
- Heald R, Tournebise R, Blank T, Sandaltzopoulos R, Becker P, Hyman A, Karsenti E. Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature*. 1996; 382(6590):420–425. DOI: 10.1038/382420a0 [PubMed: 8684481]
- Heinrich S, Geissen EM, Kamenz J, Trautmann S, Widmer C, Drewe P, Knop M, Radde N, Hasenauer J, Hauf S. Determinants of robustness in spindle assembly checkpoint signalling. *Nat Cell Biol*. 2013; 15(11):1328–1339. DOI: 10.1038/ncb2864 [PubMed: 24161933]
- Hewitt L, Tighe A, Santaguida S, White AM, Jones CD, Musacchio A, Green S, Taylor SS. Sustained Mps1 activity is required in mitosis to recruit O-Mad2 to the Mad1-C-Mad2 core complex. *J Cell Biol*. 2010; 190(1):25–34. DOI: 10.1083/jcb.201002133 [PubMed: 20624899]
- Hinshaw SM, Harrison SC. An Iml3-Chl4 heterodimer links the core centromere to factors required for accurate chromosome segregation. *Cell Rep*. 2013; doi: 10.1016/j.celrep.2013.08.036
- Hiruma Y, Sacristan C, Pachis ST, Adamopoulos A, Kuijt T, Ubbink M, von Castelmur E, Perrakis A, Kops GJPL. Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling. *Science*. 2015; 348(6240):1264–1267. DOI: 10.1126/science.aaa4055 [PubMed: 26068855]
- Hoyt MA, Totis L, Roberts BT. *S. cerevisiae* genes required for cell cycle arrest in response to loss of microtubule function. *Cell*. 1991; 66(3):507–517. [PubMed: 1651171]
- Huis In 't Veld PJ, Jeganathan S, Petrovic A, Singh P, John J, Krenn V, Weissmann F, Bange T, Musacchio A. Molecular basis of outer kinetochore assembly on CENP-T. *Elife*. 2016; 5:e21007.doi: 10.7554/eLife.21007 [PubMed: 28012276]
- Hyman AA, Mitchison TJ. Modulation of microtubule stability by kinetochores in vitro. *J Cell Biol*. 1990; 110(5):1607–1616. [PubMed: 2186046]
- Hyman AA, Middleton K, Centola M, Mitchison TJ, Carbon J. Microtubule-motor activity of a yeast centromere-binding protein complex. *Nature*. 1992; 359(6395):533–536. DOI: 10.1038/359533a0 [PubMed: 1406970]
- Iwao Y, Katagiri C. In vitro induction of sperm nucleus decondensation by cytosol from mature toad eggs. *J Exp Zool*. 1984; 230(1):115–124. DOI: 10.1002/jez.1402300115 [PubMed: 6427388]
- Izawa D, Pines J. The mitotic checkpoint complex binds a second CDC20 to inhibit active APC/C. *Nature*. 2015; 517(7536):631–634. DOI: 10.1038/nature13911 [PubMed: 25383541]
- Izuta H, Ikeno M, Suzuki N, Tomonaga T, Nozaki N, Obuse C, Kisu Y, Goshima N, Nomura F, Nomura N, Yoda K. Comprehensive analysis of the ICEN (Interphase Centromere Complex)

- components enriched in the CENP-A chromatin of human cells. *Genes Cells*. 2006; 11(6):673–684. DOI: 10.1111/j.1365-2443.2006.00969.x [PubMed: 16716197]
- Janke C, Ortiz J, Lechner J, Shevchenko A, Shevchenko A, Magiera MM, Schramm C, Schiebel E. The budding yeast proteins Spc24p and Spc25p interact with Ndc80p and Nuf2p at the kinetochore and are important for kinetochore clustering and checkpoint control. *EMBO J*. 2001; 20(4):777–791. DOI: 10.1093/emboj/20.4.777 [PubMed: 11179222]
- Jansen LE, Black BE, Foltz DR, Cleveland DW. Propagation of centromeric chromatin requires exit from mitosis. *J Cell Biol*. 2007; 176(6):795–805. [PubMed: 17339380]
- Ji Z, Gao H, Yu H. Kinetochore attachment sensed by competitive Mps1 and microtubule binding to Ndc80C. *Science*. 2015; 348(6240):1260–1264. DOI: 10.1126/science.aaa4029 [PubMed: 26068854]
- Kato H, Jiang J, Zhou BR, Rozendaal M, Feng H, Ghirlando R, Xiao TS, Straight AF, Bai Y. A conserved mechanism for centromeric nucleosome recognition by centromere protein CENP-C. *Science*. 2013; 340(6136):1110–1113. DOI: 10.1126/science.1235532 [PubMed: 23723239]
- Kim IS, Lee M, Park KC, Jeon Y, Park JH, Hwang EJ, Jeon TI, Ko S, Lee H, Baek SH, Kim KI. Roles of Mis18alpha in epigenetic regulation of centromeric chromatin and CENP-A loading. *Mol Cell*. 2012; 46(3):260–273. DOI: 10.1016/j.molcel.2012.03.021 [PubMed: 22516971]
- Kingsbury J, Koshland D. Centromere-dependent binding of yeast minichromosomes to microtubules in vitro. *Cell*. 1991; 66(3):483–495. [PubMed: 1868546]
- Klare K, Weir JR, Basilico F, Zimniak T, Massimiliano L, Ludwigs N, Herzog F, Musacchio A. CENP-C is a blueprint for constitutive centromere-associated network assembly within human kinetochores. *J Cell Biol*. 2015; 210(1):11–22. DOI: 10.1083/jcb.201412028 [PubMed: 26124289]
- Kornbluth S, Evans EK. Analysis of apoptosis using *Xenopus* egg extracts. *Curr Protoc Cell Biol*. 2001; Chapter 11(Unit 11):12.doi: 10.1002/0471143030.cb1112s09
- Kornbluth S, Smythe C, Newport J. In vitro cell cycle arrest induced by using artificial DNA templates. *Mol Cell Biol*. 1992; 12(7):3216–3223. [PubMed: 1320197]
- Koshland DE, Mitchison TJ, Kirschner MW. Polewards chromosome movement driven by microtubule depolymerization in vitro. *Nature*. 1988; 331(6156):499–504. DOI: 10.1038/331499a0 [PubMed: 3340202]
- Krizaic I, Williams SJ, Sanchez P, Rodriguez-Corsino M, Stukenberg PT, Losada A. The distinct functions of CENP-C and CENP-T/W in centromere propagation and function in *Xenopus* egg extracts. *Nucleus*. 2015; 6(2):133–143. DOI: 10.1080/19491034.2014.1003509 [PubMed: 25569378]
- Kwon MS, Hori T, Okada M, Fukagawa T. CENP-C is involved in chromosome segregation, mitotic checkpoint function, and kinetochore assembly. *Mol Biol Cell*. 2007; 18(6):2155–2168. DOI: 10.1091/mbc.E07-01-0045 [PubMed: 17392512]
- Lara-Gonzalez P, Westhorpe FG, Taylor SS. The spindle assembly checkpoint. *Curr Biol*. 2012; 22(22):R966–R980. DOI: 10.1016/j.cub.2012.10.006 [PubMed: 23174302]
- Levy DL, Heald R. Biological scaling problems and solutions in amphibians. *Cold Spring Harb Perspect Biol*. 2015; 8(1):a019166.doi: 10.1101/cshperspect.a019166 [PubMed: 26261280]
- Li R, Murray AW. Feedback control of mitosis in budding yeast. *Cell*. 1991; 66(3):519–531. [PubMed: 1651172]
- Li Y, Gorbea C, Mahaffey D, Rechsteiner M, Benezra R. MAD2 associates with the cyclosome/anaphase-promoting complex and inhibits its activity. *Proc Natl Acad Sci USA*. 1997; 94(23):12431–12436. [PubMed: 9356466]
- Logsdon GA, Barrey EJ, Bassett EA, DeNizio JE, Guo LY, Panchenko T, Dawicki-McKenna JM, Heun P, Black BE. Both tails and the centromere targeting domain of CENP-A are required for centromere establishment. *J Cell Biol*. 2015; 208(5):521–531. DOI: 10.1083/jcb.201412011 [PubMed: 25713413]
- Lohka MJ, Masui Y. Formation in vitro of sperm pronuclei and mitotic chromosomes induced by amphibian ooplasmic components. *Science*. 1983; 220(4598):719–721. [PubMed: 6601299]
- Lohka MJ, Masui Y. Effects of Ca^{2+} ions on the formation of metaphase chromosomes and sperm pronuclei in cell-free preparations from unactivated *Rana pipiens* eggs. *Dev Biol*. 1984; 103(2):434–442. [PubMed: 6427039]

- Lohka MJ, Maller JL. Induction of nuclear envelope breakdown, chromosome condensation, and spindle formation in cell-free extracts. *J Cell Biol.* 1985; 101(2):518–523. [PubMed: 3926780]
- Lombillo VA, Stewart RJ, McIntosh JR. Minus-end-directed motion of kinesin-coated microspheres driven by microtubule depolymerization. *Nature.* 1995; 373(6510):161–164. DOI: 10.1038/373161a0 [PubMed: 7816099]
- Luger K, Rechsteiner TJ, Richmond TJ. Expression and purification of recombinant histones and nucleosome reconstitution. *Methods Mol Biol.* 1999; 119:1–16. DOI: 10.1385/1-59259-681-9:1 [PubMed: 10804500]
- Luo X, Tang Z, Xia G, Wassmann K, Matsumoto T, Rizo J, Yu H. The Mad2 spindle checkpoint protein has two distinct natively folded states. *Nat Struct Mol Biol.* 2004; 11(4):338–345. DOI: 10.1038/nsmb748 [PubMed: 15024386]
- Lupardus PJ, Van C, Cimprich KA. Analyzing the ATR-mediated checkpoint using *Xenopus* egg extracts. *Methods.* 2007; 41(2):222–231. DOI: 10.1016/j.ymeth.2006.07.024 [PubMed: 17189864]
- Maddox PS, Hyndman F, Monen J, Oegema K, Desai A. Functional genomics identifies a Myb domain-containing protein family required for assembly of CENP-A chromatin. *J Cell Biol.* 2007; 176(6):757–763. DOI: 10.1083/jcb.200701065 [PubMed: 17339379]
- Masui Y, Markert CL. Cytoplasmic control of nuclear behavior during meiotic maturation of frog oocytes. *J Exp Zool.* 1971; 177(2):129–145. DOI: 10.1002/jez.1401770202 [PubMed: 5106340]
- Matsumoto Y, Bogenhagen DF. Repair of a synthetic abasic site in DNA in a *Xenopus laevis* oocyte extract. *Mol Cell Biol.* 1989; 9(9):3750–3757. [PubMed: 2779565]
- Masumoto H, Ikeno M, Nakano M, Okazaki T, Grimes B, Cooke H, Suzuki N. Assay of centromere function using a human artificial chromosome. *Chromosoma.* 1998; 107(6–7):406–416. DOI: 10.1007/s004120050324 [PubMed: 9914372]
- McClelland ML, Kallio MJ, Barrett-Wilt GA, Kestner CA, Shabanowitz J, Hunt DF, Gorbsky GJ, Stukenberg PT. The Vertebrate Ndc80 complex contains Spc24 and Spc25 homologs, which are required to establish and maintain kinetochore-microtubule attachment. *Curr Biol.* 2004; 14(2):131–137. DOI: 10.1016/j.cub.2003.12.058 [PubMed: 14738735]
- McKinley KL, Cheeseman IM. The molecular basis for centromere identity and function. *Nat Rev Mol Cell Biol.* 2016; 17(1):16–29. DOI: 10.1038/nrm.2015.5 [PubMed: 26601620]
- McKinley KL, Sekulic N, Guo LY, Tsinman T, Black BE, Cheeseman IM. The CENP-L-N complex forms a critical node in an integrated meshwork of interactions at the centromere-kinetochore interface. *Mol Cell.* 2015; 60(6):886–898. DOI: 10.1016/j.molcel.2015.10.027 [PubMed: 26698661]
- Mendiburo MJ, Padeken J, Fulop S, Schepers A, Heun P. *Drosophila* CENH3 is sufficient for centromere formation. *Science.* 2011; 334(6056):686–690. DOI: 10.1126/science.1206880 [PubMed: 22053052]
- Miake-Lye R, Kirschner MW. Induction of early mitotic events in a cell-free system. *Cell.* 1985; 41(1):165–175. [PubMed: 3888406]
- Milks KJ, Moree B, Straight AF. Dissection of CENP-C-directed centromere and kinetochore assembly. *Mol Biol Cell.* 2009; 20(19):4246–4255. DOI: 10.1091/mbc.E09-05-0378 [PubMed: 19641019]
- Miller MP, Asbury CL, Biggins S. A TOG protein confers tension sensitivity to kinetochore-microtubule attachments. *Cell.* 2016; 165(6):1428–1439. DOI: 10.1016/j.cell.2016.04.030 [PubMed: 27156448]
- Minshull J, Sun H, Tonks NK, Murray AW. A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell.* 1994; 79(3):475–486. [PubMed: 7954813]
- Mitchison TJ, Kirschner MW. Properties of the kinetochore in vitro. I. Microtubule nucleation and tubulin binding. *J Cell Biol.* 1985a; 101(3):755–765. [PubMed: 4030893]
- Mitchison TJ, Kirschner MW. Properties of the kinetochore in vitro. II. Microtubule capture and ATP-dependent translocation. *J Cell Biol.* 1985b; 101(3):766–777. [PubMed: 4030894]
- Moore MS, Blobel G. The two steps of nuclear import, targeting to the nuclear envelope and translocation through the nuclear pore, require different cytosolic factors. *Cell.* 1992; 69(6):939–950. [PubMed: 1606616]

- Moree B, Meyer CB, Fuller CJ, Straight AF. CENP-C recruits M18BP1 to centromeres to promote CENP-A chromatin assembly. *J Cell Biol.* 2011; 194(6):855–871. DOI: 10.1083/jcb.201106079 [PubMed: 21911481]
- Murray AW. Chapter 30 cell cycle extracts. 1991; 36:581–605. DOI: 10.1016/s0091-679x(08)60298-8
- Murray AW, Kirschner MW. Cyclin synthesis drives the early embryonic-cell cycle. *Nature.* 1989; 339(6222):275–280. DOI: 10.1038/339275a0 [PubMed: 2566917]
- Murray AW, Desai AB, Salmon ED. Real time observation of anaphase in vitro. *Proc Natl Acad Sci USA.* 1996; 93(22):12327–12332. [PubMed: 8901580]
- Nagpal H, Hori T, Furukawa A, Sugase K, Osakabe A, Kurumizaka H, Fukagawa T. Dynamic changes in the CCAN organization through CENP-C during cell-cycle progression. *Mol Biol Cell.* 2015; 26(21):3768–3776. [PubMed: 26354420]
- Newmeyer DD, Forbes DJ. Nuclear import can be separated into distinct steps in vitro, nuclear pore binding and translocation. *Cell.* 1988; 52(5):641–653. [PubMed: 3345567]
- Newmeyer DD, Farschon DM, Reed JC. Cell-free apoptosis in *Xenopus* egg extracts: inhibition by Bcl-2 and requirement for an organelle fraction enriched in mitochondria. *Cell.* 1994; 79(2):353–364. [PubMed: 7954801]
- Newport J, Kirschner M. A major developmental transition in early *xenopus* embryos: I. characterization and timing of cellular changes at the midblastula stage. *Cell.* 1982a; 30(3):675–686. [PubMed: 6183003]
- Newport J, Kirschner M. A major developmental transition in early *Xenopus* embryos: II. Control of the onset of transcription. *Cell.* 1982b; 30(3):687–696. [PubMed: 7139712]
- Nicklas RB, Koch CA. Chromosome micromanipulation. 3. Spindle fiber tension and the reorientation of mal-oriented chromosomes. *J Cell Biol.* 1969; 43(1):40–50. [PubMed: 5824068]
- Nishino T, Takeuchi K, Gascoigne KE, Suzuki A, Hori T, Oyama T, Morikawa K, Cheeseman IM, Fukagawa T. CENP-T-W-S-X forms a unique centromeric chromatin structure with a histone-like fold. *Cell.* 2012; 148(3):487–501. DOI: 10.1016/j.cell.2011.11.061 [PubMed: 22304917]
- Nishino T, Rago F, Hori T, Tomii K, Cheeseman IM, Fukagawa T. CENP-T provides a structural platform for outer kinetochore assembly. *EMBO J.* 2013; 32(3):424–436. DOI: 10.1038/emboj.2012.348 [PubMed: 23334297]
- Obuse C, Yang H, Nozaki N, Goto S, Okazaki T, Yoda K. Proteomics analysis of the centromere complex from HeLa interphase cells: UV-damaged DNA binding protein 1 (DDB-1) is a component of the CEN-complex, while BMI-1 is transiently co-localized with the centromeric region in interphase. *Genes Cells.* 2004; 9(2):105–120. [PubMed: 15009096]
- Ohsumi K, Katagiri C. Characterization of the ooplasmic factor inducing decondensation of and protamine removal from toad sperm nuclei—involvement of nucleoplasm. *Dev Biol.* 1991; 148(1):295–305. DOI: 10.1016/0012-1606(91)90338-4 [PubMed: 1936566]
- Ohzeki J, Nakano M, Okada T, Masumoto H. CENP-B box is required for de novo centromere chromatin assembly on human alphoid DNA. *J Cell Biol.* 2002; 159(5):765–775. DOI: 10.1083/jcb.200207112 [PubMed: 12460987]
- Ohzeki J, Shono N, Otake K, Martins NM, Kugou K, Kimura H, Nagase T, Larionov V, Earnshaw WC, Masumoto H. KAT7/HBO1/MYST2 regulates CENP-A chromatin assembly by antagonizing Suv39h1-mediated centromere inactivation. *Dev Cell.* 2016; 37(5):413–427. DOI: 10.1016/j.devcel.2016.05.006 [PubMed: 27270040]
- Okada M, Cheeseman IM, Hori T, Okawa K, McLeod IX, Yates JR 3rd, Desai A, Fukagawa T. The CENP-H-I complex is required for the efficient incorporation of newly synthesized CENP-A into centromeres. *Nat Cell Biol.* 2006; 8(5):446–457. DOI: 10.1038/ncb1396 [PubMed: 16622420]
- Palmer DK, O'Day K, Margolis RL. The centromere specific histone CENP-A is selectively retained in discrete foci in mammalian sperm nuclei. *Chromosoma.* 1990; 100(1):32–36. DOI: 10.1007/Bf00337600 [PubMed: 2101350]
- Pesenti ME, Weir JR, Musacchio A. Progress in the structural and functional characterization of kinetochores. *Curr Opin Struct Biol.* 2016; 37:152–163. DOI: 10.1016/j.sbi.2016.03.003 [PubMed: 27039078]
- Petrovic A, Pasqualato S, Dube P, Krenn V, Santaguida S, Cittaro D, Monzani S, Massimiliano L, Keller J, Tarricone A, Maiolica A, Stark H, Musacchio A. The MIS12 complex is a protein

interaction hub for outer kinetochore assembly. *J Cell Biol.* 2010; 190(5):835–852. DOI: 10.1083/jcb.201002070 [PubMed: 20819937]

Petrovic A, Mosalaganti S, Keller J, Mattiuzzo M, Overlack K, Krenn V, De Antoni A, Wohlgemuth S, Cecatiello V, Pasqualato S, Raunser S, Musacchio A. Modular assembly of RWD domains on the Mis12 complex underlies outer kinetochore organization. *Mol Cell.* 2014; 53(4):591–605. DOI: 10.1016/j.molcel.2014.01.019 [PubMed: 24530301]

Petrovic A, Keller J, Liu Y, Overlack K, John J, Dimitrova YN, Jenni S, van Gerwen S, Stege P, Wohlgemuth S, Rombaut P, Herzog F, Harrison SC, Vetter IR, Musacchio A. Structure of the MIS12 complex and molecular basis of its interaction with CENP-C at human kinetochores. *Cell.* 2016; 167(4):1028–1040. e1015. DOI: 10.1016/j.cell.2016.10.005 [PubMed: 27881301]

Przewloka MR, Venkei Z, Bolanos-Garcia VM, Debski J, Dadlez M, Glover DM. CENP-C is a structural platform for kinetochore assembly. *Curr Biol.* 2011; 21(5):399–405. DOI: 10.1016/j.cub.2011.02.005 [PubMed: 21353555]

Rago F, Gascoigne KE, Cheeseman IM. Distinct organization and regulation of the outer kinetochore KMN network downstream of CENP-C and CENP-T. *Curr Biol.* 2015; 25(5):671–677. DOI: 10.1016/j.cub.2015.01.059 [PubMed: 25660545]

Rieder CL, Cole RW, Khodjakov A, Sluder G. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J Cell Biol.* 1995; 130(4):941–948. DOI: 10.1083/jcb.130.4.941 [PubMed: 7642709]

Saitoh H, Cooke CA, Burgess WH, Earnshaw WC, Dasso M. Direct and indirect association of the small GTPase ran with nuclear pore proteins and soluble transport factors: studies in *Xenopus laevis* egg extracts. *Mol Biol Cell.* 1996; 7(9):1319–1334. [PubMed: 8885229]

Salic A, Lee E, Mayer L, Kirschner MW. Control of beta-catenin stability: reconstitution of the cytoplasmic steps of the wnt pathway in *Xenopus* egg extracts. *Mol Cell.* 2000; 5(3):523–532. [PubMed: 10882137]

Sandall S, Severin F, McLeod IX, Yates JR 3rd, Oegema K, Hyman A, Desai A. A Bir1-Sli15 complex connects centromeres to microtubules and is required to sense kinetochore tension. *Cell.* 2006; 127(6):1179–1191. DOI: 10.1016/j.cell.2006.09.049 [PubMed: 17174893]

Sandmann M, Talbert PD, Demidov Kuhlmann M, Rutten T, Conrad U, Lermontova I. Targeting of A. thaliana KNL2 to centromeres depends on the conserved CENPC-k motif in its C-terminus. *Plant Cell.* 2017; doi: 10.1105/tpc.16.00720

Sawin KE, Mitchison TJ. Mitotic spindle assembly by two different pathways in vitro. *J Cell Biol.* 1991; 112(5):925–940. [PubMed: 1999463]

Screpanti E, De Antoni A, Alushin GM, Petrovic A, Melis T, Nogales E, Musacchio A. Direct binding of Cenp-C to the Mis12 complex joins the inner and outer kinetochore. *Curr Biol.* 2011; 21(5): 391–398. DOI: 10.1016/j.cub.2010.12.039 [PubMed: 21353556]

Session AM, Uno Y, Kwon T, Chapman JA, Toyoda A, Takahashi S, Fukui A, Hikosaka A, Suzuki A, Kondo M, van Heeringen SJ, Quigley I, Heinz S, Ogino H, Ochi H, Hellsten U, Lyons JB, Simakov O, Putnam N, Stites J, Kuroki Y, Tanaka T, Michiue T, Watanabe M, Bogdanovic O, Lister R, Georgiou G, Paranjpe SS, van Kruijsbergen I, Shu S, Carlson J, Kinoshita T, Ohta Y, Mawaribuchi S, Jenkins J, Grimwood J, Schmutz J, Mitros T, Mozaffari SV, Suzuki Y, Haramoto Y, Yamamoto TS, Takagi C, Heald R, Miller K, Haudenschield C, Kitzman J, Nakayama T, Izutsu Y, Robert J, Fortriede J, Burns K, Lotay V, Karimi K, Yasuoka Y, Dichmann DS, Flajnik MF, Houston DW, Shendure J, DuPasquier L, Vize PD, Zorn AM, Ito M, Marcotte EM, Wallingford JB, Ito Y, Asashima M, Ueno N, Matsuda Y, Veenstra GJ, Fujiyama A, Harland RM, Taira M, Rokhsar DS. Genome evolution in the allotetraploid frog *Xenopus laevis*. *Nature.* 2016; 538(7625):336–343. DOI: 10.1038/nature19840 [PubMed: 27762356]

Shamu CE, Murray AW. Sister chromatid separation in frog egg extracts requires DNA topoisomerase II activity during anaphase. *J Cell Biol.* 1992; 117(5):921–934. [PubMed: 1315785]

Sheehan MA, Mills AD, Sleeman AM, Laskey RA, Blow JJ. Steps in the assembly of replication-competent nuclei in a cell-free system from *Xenopus* eggs. *J Cell Biol.* 1988; 106(1):1–12. [PubMed: 3339085]

- Shintomi K, Takahashi TS, Hirano T. Reconstitution of mitotic chromatids with a minimum set of purified factors. *Nat Cell Biol.* 2015; 17(8):1014–1023. DOI: 10.1038/ncb3187 [PubMed: 26075356]
- Sorger PK, Severin FF, Hyman AA. Factors required for the binding of reassembled yeast kinetochores to microtubules in vitro. *J Cell Biol.* 1994; 127(4):995–1008. [PubMed: 7962081]
- Sugimoto K, Kuriyama K, Shibata A, Himeno M. Characterization of internal DNA-binding and C-terminal dimerization domains of human centromere/kinetochore autoantigen CENP-C in vitro: role of DNA-binding and self-associating activities in kinetochore organization. *Chromosome Res.* 1997; 5(2):132–141. [PubMed: 9146917]
- Walter JC, Arias EE. Initiation of DNA replication in *Xenopus* egg extracts. *Front Biosci.* 2004; 9:3029–3045. [PubMed: 15353335]
- Wan X, O'Quinn RP, Pierce HL, Joglekar AP, Gall WE, DeLuca JG, Carroll CW, Liu ST, Yen TJ, McEwen BF, Stukenberg PT, Desai A, Salmon ED. Protein architecture of the human kinetochore microtubule attachment site. *Cell.* 2009; 137(4):672–684. DOI: 10.1016/j.cell.2009.03.035 [PubMed: 19450515]
- Weir JR, Faesen AC, Klare K, Petrovic A, Basilico F, Fischbock J, Pentakota S, Keller J, Pesenti ME, Pan D, Vogt D, Wohlgemuth S, Herzog F, Musacchio A. Insights from biochemical reconstitution into the architecture of human kinetochores. *Nature.* 2016; 537(7619):249–253. DOI: 10.1038/nature19333 [PubMed: 27580032]
- Westhorpe FG, Straight AF. The centromere: epigenetic control of chromosome segregation during mitosis. *Cold Spring Harb Perspect Biol.* 2015; 7(1):a015818.doi: 10.1101/cshperspect.a015818
- Westhorpe FG, Fuller CJ, Straight AF. A cell-free CENP-A assembly system defines the chromatin requirements for centromere maintenance. *J Cell Biol.* 2015; 209(6):789–801. DOI: 10.1083/jcb.201503132 [PubMed: 26076692]
- Wigge PA, Kilmartin JV. The Ndc80p complex from *Saccharomyces cerevisiae* contains conserved centromere components and has a function in chromosome segregation. *J Cell Biol.* 2001; 152(2):349–360. DOI: 10.1083/jcb.152.2.349 [PubMed: 11266451]
- Yan J, Maresca TJ, Skoko D, Adams CD, Xiao B, Christensen MO, Heald R, Marko JF. Micromanipulation studies of chromatin fibers in *Xenopus* egg extracts reveal ATP-dependent chromatin assembly dynamics. *Mol Biol Cell.* 2007; 18(2):464–474. DOI: 10.1091/mbc.E06-09-0800 [PubMed: 17108322]
- Yoda K, Ando S, Morishita S, Houmura K, Hashimoto K, Takeyasu K, Okazaki T. Human centromere protein A (CENP-A) can replace histone H3 in nucleosome reconstitution in vitro. *P Natl Acad Sci USA.* 2000; 97(13):7266–7271. DOI: 10.1073/pnas.130189697
- Zhao Y, Chen RH. Mps1 phosphorylation by MAP kinase is required for kinetochore localization of spindle-checkpoint proteins. *Curr Biol.* 2006; 16(17):1764–1769. DOI: 10.1016/j.cub.2006.07.058 [PubMed: 16950116]
- Zinkowski RP, Meyne J, Brinkley BR. The centromere-kinetochore complex: a repeat subunit model. *J Cell Biol.* 1991; 113(5):1091–1110. [PubMed: 1828250]

Fig. 1.

General schematic of a vertebrate kinetochore. The point of attachment between a chromosome and the microtubules of the mitotic spindle is a region called the centromere. During mitosis, the centromere recruits a multiprotein complex called the kinetochore that physically links chromosomes to microtubules, monitors its own attachment to microtubules, and delays anaphase onset until chromosomes are properly attached to opposite poles of the spindle (*SAC* the spindle assembly checkpoint). At the heart of the kinetochore is the KMN network: KNL1, which can bind microtubules and serves as the hub for SAC signaling; the Mis12 complex (Mis12, Nnf1, Nsl1, Dsn1) which anchors the kinetochore to the centromere; and the Ndc80 complex (Ndc80, Nuf2, Spc24, Spc25) which provides the core microtubule-binding activity of the kinetochore. The location of the centromere is specified by chromatin containing nucleosomes in which histone H3 has been replaced with the centromere-specific histone H3 variant CENP-A. CENP-A nucleosomes are bound directly by the constitutive centromere-associated network (CCAN), a complex of ~17 “CENP” proteins that associate with the centromere throughout the cell cycle and provide the platform for kinetochore assembly in mitosis. Of these, only CENP-C and CENP-N are known to directly interact with the CENP-A nucleosome. The CCAN is further divided into several subcomplexes: CENP-C, CENP-LN, CENP-HIKM, CENP-OPQRU, CENP-TWSX

Fig. 2.

Extract and cell cycle manipulation. Preparation and manipulation of extracts from *Xenopus laevis* eggs. **a** Preparation of egg extract. *Xenopus laevis* females are injected with human chorionic gonadotropin (hCG) to stimulate ovulation. Eggs laid overnight are collected, treated with cysteine to remove their sticky jelly coat, and subjected to a low-speed packing spin to remove interstitial buffer. Centrifugation at higher speed gently ruptures the cell membrane and causes the maternal chromosomes to pellet with the pigment granules, resulting in chromatin-free cytoplasmic extract that contains soluble cytoplasmic components including ribosomes as well as the membranes required to support nuclear envelope assembly. Egg extract is isolated following centrifugation by side puncture using a syringe. **b** Cell cycle manipulation of extract. Crushing unfertilized eggs in the presence of the calcium-chelator EGTA results in extract that preserves the metaphase II meiotic arrest of the intact eggs. This metaphase extract assembles bipolar spindles around unreplicated chromosomes following addition of demembranated sperm chromatin. Addition of calcium mimics fertilization and causes release of the metaphase II arrest and entry into interphase. Chromosomes decondense, form interphase nuclei, and undergo DNA replication. Addition of fresh metaphase extract to interphase extract drives it forward into the next mitosis. Replicated chromosomes condense and form bioriented spindles in this cycled mitotic extract. Addition of calcium again drives exit from mitosis, with replicated chromosomes undergoing microtubule-direct anaphase segregation.

Fig. 3.

CENP-A assembly. Epigenetic maintenance of CENP-A. **c** Changes in centromeric CENP-A content during the cell cycle. During S-phase, parental nucleosomes are partitioned equally between the daughter chromatids as DNA replicates. While new H3 nucleosome assembly is coupled to the replication machinery, new CENP-A nucleosome assembly does not proceed until the following G1. This results in halving of centromeric CENP-A from G1 to G2, indicated by transition of the *pink circles* to *pink half-circles* at the centromere. **b** Schematic of CENP-A loading. In *Xenopus*, initial recruitment of the CENP-A assembly machinery takes place in mitosis. The Mis18 complex (Mis18 α , Mis18 β , and M18BP1) localizes to centromeres via a cell cycle-regulated interaction between CENP-C and M18BP1. In interphase, CENP-C and the Mis18 complex interact with the CENP-A-specific chaperone HJURP to recruit a prenucleosomal complex of CENP-A/H4 to the centromere. The details of CENP-A nucleosome assembly following HJURP recruitment remain unclear, but may entail disassembly of H3 nucleosomes at sites where new CENP-A nucleosomes will form. **a** Schematic of CENP-A assembly assays in egg extract. Because CENP-A is retained on sperm chromatin through spermatogenesis, tagged CENP-A is added to extract to differential new and old CENP-A. Demembranated sperm chromatin or reconstituted CENP-A chromatin can serve as the template for CENP-A assembly. The assembly reaction is initiated by the addition of calcium and the CENP-A chaperone HJURP, recapitulating the in vivo requirements for assembly. Incorporation of tagged CENP-A at centromeres is assessed by immunofluorescence

Reconstitution of chromatin arrays. Reconstitution of kinetochore function with reconstituted chromatin arrays. **a** Schematic of chromatin reconstitution and kinetochore assembly assay. Purified CENP-A/H4 tetramer and H2A/B dimer are mixed in high salt buffer with biotinylated DNA containing high affinity nucleosome positioning sequences. Gradual dialysis of the assembly reaction to low salt causes spontaneous assembly of octameric nucleosomes on the DNA, which can then be coupled to streptavidin-coated magnetic beads. Following their recovery from egg extract, centromere and kinetochore protein recruitment or spindle assembly on chromatin-coated beads is assessed by immunofluorescence. **b** Schematic of CENP-A and H3 domains. Reconstitution of chromatin from chimeric histones in which domains of CENP-A have been exchanged with the corresponding domains of H3 provides a method for understanding the roles of specific CENP-A domains in centromere function. **c** The DNA substrate for chromatin reconstitution contains nineteen high affinity “601” nucleosome positioning sequences. Performing the reconstitution with higher concentrations of histone (2 μ M) results in “saturated” chromatin arrays in which every positioning site is occupied by a CENP-A nucleosome. At lower concentrations (0.5 μ M), only a subset of the sites are occupied, yielding “subsaturated” chromatin arrays. In egg extract, H3 nucleosomes assemble at these unoccupied sites resulting in mixed CENP-A/H3 arrays that may more accurately reflect the admixture of CENP-A and H3 nucleosomes observed at centromeres in vivo

Fig. 5.

Spindle assembly checkpoint regulation at kinetochores. Spindle assembly checkpoint (SAC) signaling prevents anaphase onset in response to unattached kinetochores. KNL1 serves as the platform for assembling SAC proteins. In the absence of microtubules, the checkpoint kinase Mps1 binds to Ndc80 and phosphorylates KNL1. Phosphorylated KNL1 recruits Bub3:BubR1, Mad2, and the APC coactivator Cdc20 to unattached kinetochores. Mad2 exists in both an "open" inactive conformation, and a "closed" active conformation. Open Mad2 must be converted to closed Mad2 at unattached kinetochores in order to bind Cdc20 and promote SAC arrest. Bub3, BubR1, Mad2, and Cdc20 assemble into the mitotic checkpoint complex (MCC) which inhibits the anaphase promoting complex by sequestering Cdc20, promoting Cdc20 degradation, and preventing cyclin degradation