The Interaction Network between Centromeric Proteins during Kinetochore Formation

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Abstract

In eukaryotes, the kinetochore forms on the centromere to attach the microtubules from the mitotic spindle and is essential for chromosomal segregation. The centromere is specified by a sequence independent epigenetic mechanism in most eukaryotes and the centromere specific histone H3 variant CENP-A is the primary candidate as the epigenetic marker for kinetochore specification. Once the centromere is specified, various proteins are assembled on the centromere to establish the functional kinetochore. As these proteins associate with the centromere throughout the cell cycle, they are called the Constitutive Centromere Associated Network (CCAN) proteins. CCAN proteins also create a scaffold for the formation of the kinetochore and subsequently recruit outer kinetochore components that bind to the microtubules. Whereas considerable progress has been made in understanding the relation between CCAN components as well as between the inner and outer kinetochores, the exact temporal order, dependencies and the functional roles of each component are still unclear. While it has also been shown that CENP-C and CENP-T form two distinct associations with centromeric chromatin, questions remain as to whether there is any connection or interdependency between these pathways. Similarly, CENP-C and CENP-T form the two pathways for recruiting the outer kinetochore and subsequent microtubule attachment, yet it remains unknown whether they actively stabilize outer kinetochore – microtubule interaction or mainly function as platforms. Therefore, it is essential to clarify the organization of the CCAN proteins and the functional role of the CCAN in recruiting the outer kinetochore. The aim of this thesis is to study the various interaction networks during kinetochore formation to

1

elucidate; (i) How the CCAN components are organized during the recruitment of the kinetochore? (ii) Whether the CCAN proteins affect outer kinetochore – microtubule binding?

To answer these questions, I used chicken DT40 cells as a model system and first concentrated on the interactions between CENP-C and other CCAN components. I expressed various deletion mutants of CENP-C in CENP-C deficient cells and found that CENP-C appeared to localise to the centromere through multiple binding pathways. The middle region of CENP-C was essential for its localization to the centromere in a CCAN dependent manner whereas the CENP-C C-terminus localized to the centromere in a CCAN independent manner. I demonstrated that CENP-C localized to the centromere through direct interaction with CENP-A through its C-terminal region during M-phase, but also that it localized to the centromere via the CCAN during interphase. To analyse CENP-C interactions *in vitro* I used a biochemical approach and found a weak interaction of CENP-C with CENP-N/L. Subsequent 2-D NMR experiments confirmed the interaction between these proteins. I found that CENP-C lacking the middle region, which is suspected of being the interaction domain with CENP-N/L, could not rescue CENP-C deficient cells. In addition, cells expressing this deletion mutant showed a marked reduction in CENP-H localization to the centromere. These results suggest that CENP-C middle region is essential for centromere localization through interaction with other CCAN components including CENP-L/N and CENP-H in interphase, while CENP-C C-terminus largely contributes to centromere localization during mitosis via direct interaction with the CENP-A nucleosome. I propose that this dynamic change of CENP-C interaction may be essential for the formation of a functional kinetochore.

The second question is whether the CCAN proteins affect outer kinetochore-microtubule binding. For this question, I focused on the role of the Mis12 complex which is recruited by the CENP-C pathway and CENP-T on stabilizing the Ndc80 complex – microtubule interaction. Our laboratory has previously shown that CENP-C and CENP-T induce the formation of an artificial kinetochore when these proteins are localized to an ectopic site on chromosomes, whereas the Ndc80 complex does not induce an artificial kinetochore. In this study, I show that members of the chicken Mis12 complex did not induce an artificial kinetochore suggesting that a CCAN dependent pathway for the Ndc80 complex is required to form a functional kinetochore. Then, I prepared human CENP-T, and Mis12 complex *in-vitro* and tested whether these proteins affect the Ndc80 complex – microtubule binding based on single molecule total internal reflection microscopy (smTIRF), I demonstrate that both the Mis12 complex and CENP-T can enhance the microtubule binding ability of the Ndc80 complex. Thus, I show that a mechanism exists to regulate kinetochore – microtubule binding through CCAN components.

Acknowledgment

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Table of Contents

Chapter 1

Review of Literature

Adapted from:

Abstract

The kinetochore is an essential structure for the chromosome segregation machinery in eukaryotes; it serves as a bridge between the spindle microtubules and chromosomes. The kinetochore consists of multiple interconnecting components on the centromere; therefore, understanding its formation, molecular function, and regulation has remained an ongoing challenge. Recent studies have provided new insights into centromere identity, kinetochore assembly, and function. In this review, I discuss recent advances in our understanding of the function and regulation of key kinetochore components. I highlight the reciprocal localization dependencies of the different sub-complexes of the kinetochore and describe their regulation during the cell cycle.

1.1 Introduction

The proper segregation of genetic material during cellular division is essential for the maintenance of life. This process requires correct and timely attachment of spindle microtubules to the centromere, a specialized region on the chromosomes, during mitosis and meiosis. In this chapter I aim to provide details on the structure and regulation at the centromere as currently understood and provide context for the results shown in later chapters. A summary of this review can be found in section 1.2.

The centromere has been studied for over 100 years (Flemming 1882; Darlington 1936; Schrader 1939; Fukagawa and Earnshaw 2014) and it is essential for proper chromosome segregation. It is marked on the region of the chromosome, easily recognised as its primary point of constriction, which directs attachment of the chromosome to the spindle microtubules by the recruitment of a large protein complex called the kinetochore (Figure 1). From the onset of mitosis, the kinetochore becomes central to the alignment and segregation of the chromosomes, responsible for ensuring exclusive connection of sister chromatid to opposite spindle poles. The kinetochores also monitors inaccurate attachments, preventing anaphase entry until all chromosomes are correctly aligned through the spindle assembly checkpoint (SAC) (Musacchio and Salmon 2007; Cheeseman and Desai 2008; Varma and Salmon 2012; Foley and Kapoor 2012; London and Biggins 2014).

The actual mechanism behind kinetochore function started to emerge when electron microscopy of the kinetochore revealed a tri-layered structure that bound to microtubules (Jokelainen 1967). While a significant portion of the kinetochore is present constitutively on each chromosome, the full kinetochore is present on each duplicated chromosome only from late prophase to the end of mitosis during every cell cycle. Although the overall function of the

kinetochore is clear, the molecular mechanisms behind it are still not completely understood. The following sections provide an overview of our current understanding of centromere research and draw focus on the various unanswered question in the field.
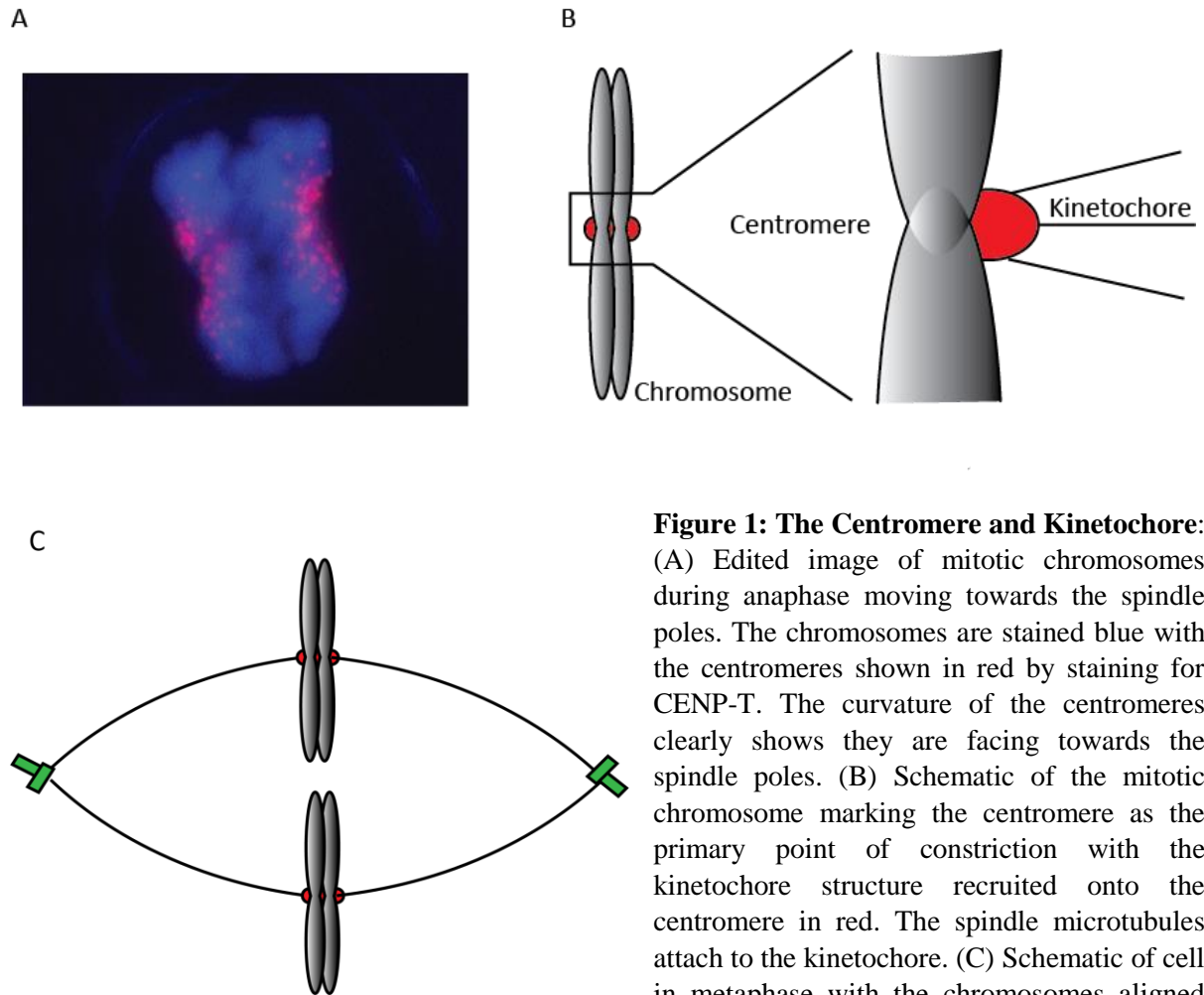
Figure 1: The Centromere and Kinetochore: (A) Edited image of mitotic chromosomes during anaphase moving towards the spindle poles. The chromosomes are stained blue with the centromeres shown in red by staining for CENP-T. The curvature of the centromeres clearly shows they are facing towards the spindle poles. (B) Schematic of the mitotic chromosome marking the centromere as the primary point of constriction with the kinetochore structure recruited onto the centromere in red. The spindle microtubules attach to the kinetochore. (C) Schematic of cell in metaphase with the chromosomes aligned along the spindle equator. Each sister chromatid is attached to the spindle microtubules. The spindle microtubules originate from the centrosomes shown in green.

1.2   Chapter Summary: The structure of the kinetochore

The molecular composition of the kinetochore remained a mystery until studies which attempted to determine its composition demonstrated that the sera of patients with scleroderma spectrum disease (CREST syndrome) recognized human centromeres (Moroi et al. 1980; Brenner et al. 1981). Characterization of these sera revealed three kinetochore components namely CENP-A, CENP-B, and CENP-C (Earnshaw and Rothfield 1985). Of these, CENP-A, a histone H3 variant (Palmer et al. 1987), emerged as the true marker for a functional centromere in the vast majority of eukaryotes. Its deposition is dependent on a complicated mechanism that require epigenetic factors, DNA sequences, and interaction with other kinetochore components. Since the discovery of CENP-A, B, and C, over 100 kinetochore components have been identified (Figure 2) (Sugata et al. 1999; Nishihashi et al. 2002; Obuse et al. 2004b; Foltz et al. 2006; Okada et al. 2006; Izuta et al. 2006; Hori et al. 2008a; Ohta et al. 2010; Perpelescu and Fukagawa 2011; Nishino et al. 2012; McKinley and Cheeseman 2016).

Various proteins are recruited to establish the functional kinetochore, once the position of the centromere is specified. The kinetochore can be divided into three parts, based on localization and overall function: 1) the inner kinetochore, which includes proteins that recognize centromere chromatin and form a foundation for subsequent kinetochore assembly; 2) the outer kinetochore, which includes proteins that localize to the outer plate and are involved in microtubule binding; 3) regulatory proteins, which ensure proper spatial-temporal kinetochore-microtubule attachment (Figure 2).

Downstream of CENP-A is the Constitutive Centromere Associated Network (CCAN) which currently includes 16 components (CENP-C, CENP-H-I-K-M-L-N, CENP-O-P-Q-R-U,

and CENP-T-W-S-X) and forms the core of the inner kinetochore. The CCAN can be further divided into the following sub-groups based on their functions and biochemical characteristics (Okada et al. 2006; Hori et al. 2008a): CENP-C, the CENP-T group (CENP-T-W-S-X), the CENP-H group (CENP-H-I-K-L-M-N), and the CENP-O group (CENP-O-P-Q-R-U(50)). CENP-C and CENP-T-W-S-X bridge the centromere to the outer kinetochore (Hori et al. 2008a; Gascoigne et al. 2011; Przewloka et al. 2011; Screpanti et al. 2011; Nishino et al. 2013). The CENP-H group is located between the chromatin, CENP-C, and CENP-T-W-S-X. It plays roles in the maintenance and recruitment of CCAN (Cheeseman et al. 2008; Hori et al. 2013; Basilico et al. 2014). The CENP-O group localizes to the kinetochore, downstream of the CENP-H group, and plays roles in microtubule dynamics and kinetochore alignment (Hori et al. 2008b; Amaro et al. 2010; Hua et al. 2011; Eskat et al. 2012; Bancroft et al. 2015).

The CCAN and inner kinetochore are fully assembled onto the centromere by early G2 phase and acts as a platform for the assembly of several microtubule-binding proteins, which forms the outer kinetochore to establish full kinetochore function. The main microtubule binding interface is made up of the Knl1/Mis12 complex/Ndc80 complex (KMN) network, which assembles directly onto the CCAN (Cheeseman et al. 2004; Obuse et al. 2004a; Cheeseman et al. 2006). Among the KMN complex, the main component for microtubule binding is the four subunit Ndc80 complex (Spc24, Spc25, Nuf2 and Ndc80^{Hec1}). The Mis12 complex is a four-member complex made up of Nnf1, Mis12, Dsn1, and Nsl1 (Cheeseman et al. 2004; Obuse et al. 2004a; Kline et al. 2006). The Mis12 complex binds the Ndc80 complex (Petrovic et al. 2010) and links to CENP-C through direct interaction (Figure 2) (Przewloka et

al. 2011; Screpanti et al. 2011). The inner kinetochore protein CENP-T also directly binds the

Ndc80 complex (Gascoigne et al. 2011; Nishino et al. 2013).
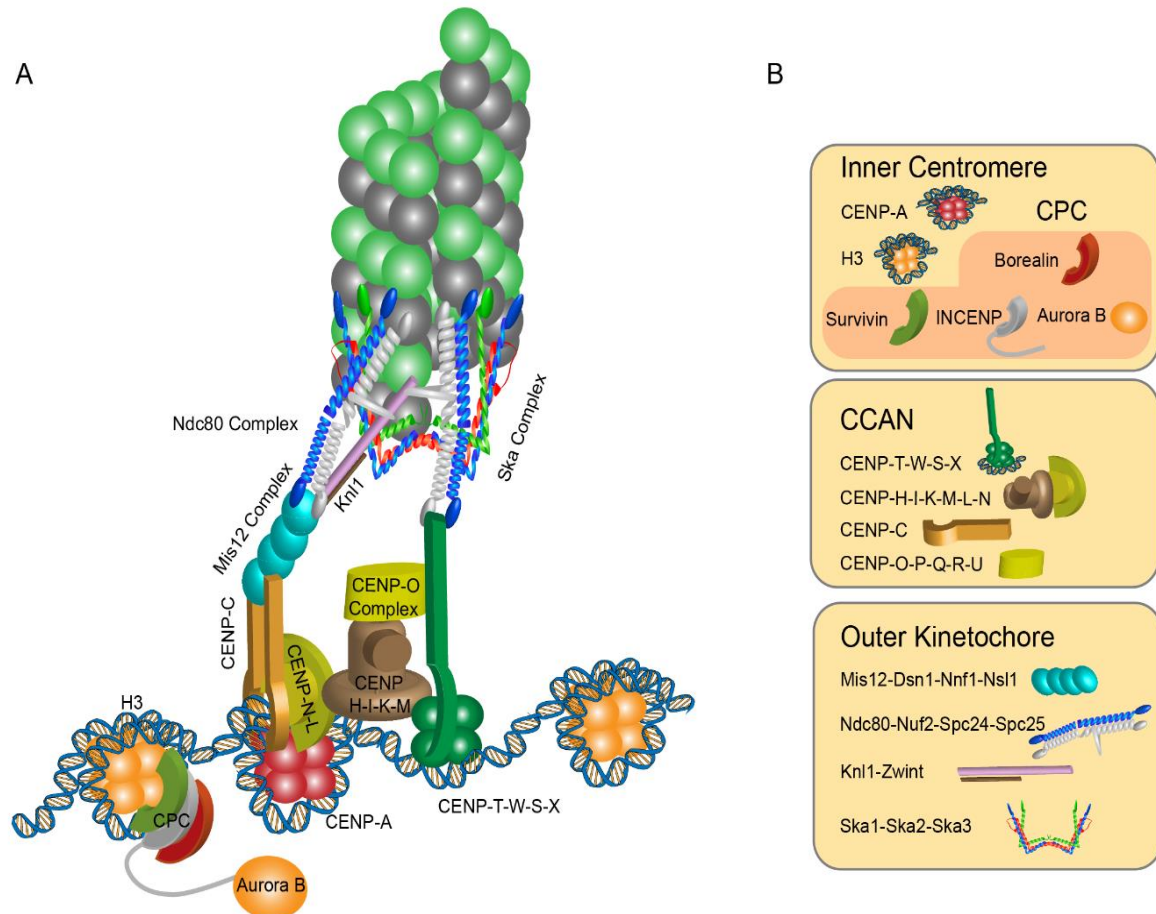
Figure 2: Model of the vertebrate mitotic kinetochore: (A) Schematic of the mitotic kinetochore showing simplified protein-protein interactions. The area between the sister chromatids is the inner centromere and includes the chromosome passenger complex (CPC) which included survivin, borealin, INCENP, and Aurora B. CENP-A mark the centromere for recruitment of the kinetochore. Downstream of CENP-A, the constitutive centromere associated network (CCAN) is assembled. It consists of CENP-C, CENP-T-W-S-X, CENP-H-I-K-M, CENP-N-L, and CENP-O-P-Q-R-U. Of these CENP-C and CENP-N are CENP-A binding proteins. CENP-T-W-S-X forms a nucleosome like structure and incorporates into the centromere region. CENP-N-L and CENP-H-I-K-M are proteins involved in regulatory and maintenance roles. CENP-I and CENP-C are involved in the recruitment of the CENP-A deposition machinery. CENP-H-I-K-M-L-N help recruit both CENP-C and CENP-T especially in interphase cells. Together CENP-C and CENP-T form two pathways for the recruitment of the Outer kinetochore whose role is to bind the spindle microtubules. CENP-C N-terminus binds to the Mis12 complex which subsequently recruits KNL1 and the microtubule binding protein Ndc80 complex. CENP-T N-terminus binds the Ndc80 complex directly. (B) List of major kinetochore components grouped into sub-complexes based on their functions and localization.

Apart from the ~30 core components of the kinetochore which bind to the centromeric chromatin and microtubules, there are several regulatory proteins such as the chromosome passenger complex (CPC), various histone modifiers, and multiple mitotic kinases including Aurora B, Plk1, Bub1, and CDK1 (Carmena et al. 2012; van der Horst and Lens 2014; Krenn and Musacchio 2015; Kitagawa and Lee 2015). In the following sections I shall discuss the different kinetochore components; their recruitment and regulation, in greater detail.

1.3   The Centromere

The centromere varies widely between different organisms and broadly fall into three types (Figure 3): point centromeres, regional centromeres and holocentromeres. Point centromeres such as those in *Saccharomyces cerevisiae* consist of a specific sequence of DNA (~125 bp) that is essential for centromere function (Clarke and Carbon 1980). Regional centromeres lack a definitive DNA region essential for centromere function, but consist of repetitive DNA sequences of up to 4 Mb, as found in humans (Willard and Waye 1987; Pluta et al. 1995). Repetitive centromeric DNA lacks conservation among species (Centola and Carbon 1994; Copenhaver et al. 1999; Sun et al. 2003). Holocentromeres extend along the entire length of the chromosome and are found in nematodes, plants, and some insects (Hughes-Schrader and Ris 1941). They can be considered to be dispersed but discretely localized regional centromeres (Steiner and Henikoff 2014). In this thesis, I will focus mainly on regional centromeres.

In the case of point centromeres, the CBF3 complex specifically binds to centromere DNA (Lechner and Carbon 1991) and is sufficient for centromere function. This varies significantly from both regional and holocentromeres where the underlying DNA is not strictly required for centromere activity (Earnshaw and Migeon 1985; Fukagawa and Earnshaw 2014; Fachinetti et al. 2015). In mammalian cells, regional centromeres have a conserved DNA-binding element, the CENP-B box, which associates with the centromeric protein CENP-B. In humans, the CENP-B box is a 17-bp motif found within the 171-bp α-satellite repeats (Masumoto et al. 1989). Constructs containing both α-satellite and CENP-B box DNA have been shown to be sufficient for the formation of artificial human chromosomes (Harrington et

14

al. 1997; Ikeno et al. 1998; Ohzeki et al. 2002). However, studies investigating human dicentric chromosomes containing α-satellite repeats (Earnshaw and Migeon 1985; Earnshaw et al. 1989) and functional neocentromeres lacking α-satellite DNA (Voullaire et al. 1993) revealed that α-satellite DNA is neither necessary nor sufficient for centromere activity. Moreover, the CENP-B box is not completely conserved among mammalian species (Haaf et al. 1995; Kipling et al. 1995). The presence of heritable neocentromeres, both induced and artificial, as well as the presence of dicentric chromosomes provides evidence that regional centromeres are specified epigenetically rather than according to the sequence (Earnshaw and Migeon 1985; Voullaire et al. 1993; Allshire and Karpen 2008).
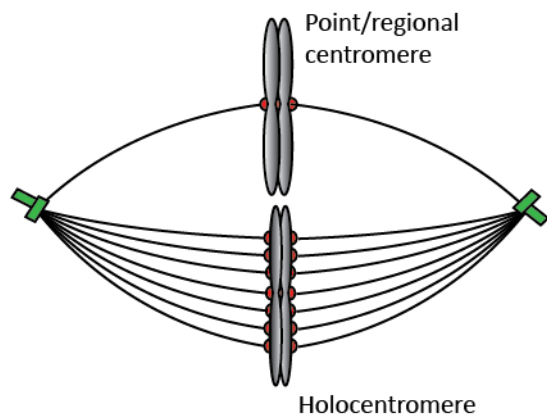
Figure 3: Different types of centromeres: The centromere can be categorized into three main types: point centromeres which are marked by a specific DNA sequence essential for centromere function onto which the kinetochore is loaded; Regional centromeres contain repetitive DNA sequences though these sequences are not essential for centromere function. They are instead marked epigenetically by the deposition of CENP-A; Holocentromeres consist of regional centromeres dispersed along the entire length of the chromosome.

CENP-A, a histone H3 variant, is now considered to be the primary candidate as an epigenetic marker for centromere specification (Earnshaw and Rothfield 1985; Palmer et al. 1991; Sullivan et al. 1994). Its presence has been shown to be essential for kinetochore formation at active centromeres in most eukaryotes, including dicentric chromosomes and neocentromeres (Earnshaw et al. 1989; Warburton et al. 1997; Tyler-Smith et al. 1999; Marshall et al. 2008). This suggests that CENP-A is the foundation for further kinetochore assembly.

1.4   CENP-A: Marking the centromere

The formation and maintenance of an active kinetochore occurs in relatively few steps, namely, priming of the centromere chromatin, deposition of CENP-A, and maturation of CENP-A containing chromatin (Figure 4). These steps are necessary for recruitment of additional kinetochore components (Perpelescu and Fukagawa 2011; Fukagawa and Earnshaw 2014; McKinley and Cheeseman 2016).

Interestingly, the priming of native centromeric chromatin is achieved by interactions of pre-existing CENP-A with other kinetochore components, including CENP-C and the CENP-H group (Carroll et al. 2010; Hori et al. 2013; Falk et al. 2015; Shono et al. 2015; Guo et al. 2017). This interaction recruits other kinetochore components and licensing factors (Figure 4). This is seen most clearly in germ line cells in *Xenopus* (Milks et al. 2009; Krizaic et al. 2015) and *Drosophila* (Raychaudhuri et al. 2012), where CENP-A provides a template for fertilized zygote centromeres. In addition, disruption of the original centromere leads to neocentromere formation in the regions surrounding the original centromere. This is possibly due to the presence of a low level of mature CENP-A, which is referred to as the ectopic CENP-A cloud (Shang et al. 2013; Bodor et al. 2014). Modifications of the surrounding canonical histones may play some roles in priming the centromere, independent of CENP-A nucleosomes. Studies using human artificial chromosomes (HAC) have shown that heterochromatin in the pericentromere and modifications at H3 nucleosomes, such as H3K4 methylation or H3K9 acetylation in the centromeric chromatin, affect kinetochore assembly (Nakano et al. 2008; Cardinale et al. 2009; Ohzeki et al. 2012).
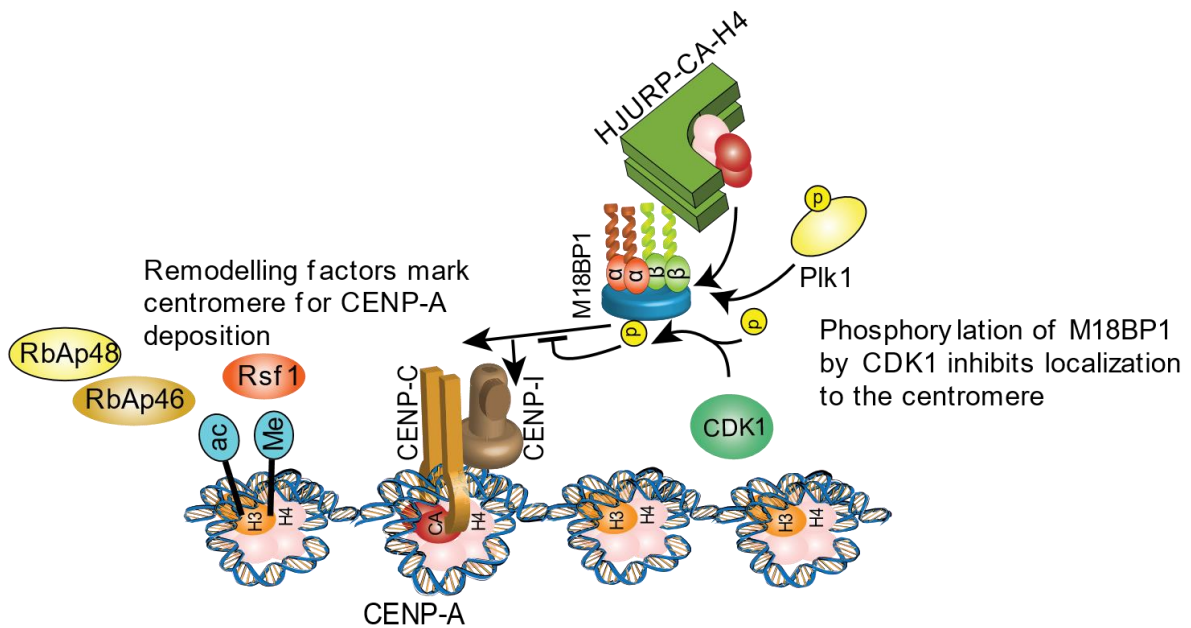
Figure 4: Maintenance of CENP-A chromatin: During DNA replication, CENP-A amount on each daughter chromosome is halved. This results in the cell undergoing mitosis with half its full CENP-A compliment with gaps filled with either H3.1/H3.3 or nucleosome free gaps. This requires the replenishment of CENP-A onto the centromere in early G1 phase in vertebrates. The propagation and maintenance of CENP-A at the centromere can be roughly divided into three stages: Priming, deposition and maturation. The priming step involves the action of various regulatory proteins such as RBAP46/48, RSF1, FACT, RNAPII etc. which act to create a conducive chromatin environment for new CENP-A recruitment through various post-translation modifications such as H3K4 methylation or H3K9 acetylation. The priming step also involves the recruitment of the Mis18 complex to the centromere. The localization of the Mis18 complex subunit Mis18BP1 is limited to mitosis by phosphorylation by CDK1 kinase which prevents its binding to CENP-C or CENP-I. After priming the next step is deposition of new CENP-A which is achieved through the CENP-A specific chaperone HJURP. HJURP binds soluble CENP-A-H4 and is recruited to the centromere by interaction with the Mis18 complex. During early G1 phase after loss of CDK1 activity, the Mis18 complex is phosphorylated by PLK1 which allows localization of the Mis18-HJURP complex for incorporation of new CENP-A. The deposition of new CENP-A also breaks down the Mis18 complex preventing its recruitment of new CENP-A till the next cell cycle.

Unlike that of canonical Histone H3, the actual deposition of new CENP-A in most eukaryotic cells is decoupled from DNA replication (Shelby et al. 1997; Shelby et al. 2000; Jansen et al. 2007). This results in cells undergoing mitosis with only half of its CENP-A compliment. The exact timing of CENP-A deposition varies amongst different species. CENP-A[CID] deposition in *Drosophila* occurs during anaphase or metaphase following the degradation of cyclin A (Schuh et al. 2007; Mellone et al. 2011). Vertebrate CENP-A is incorporated into the centromere during early G1 phase, following the loss of CDK activity (Figure 4) (Jansen et al. 2007; Silva et al. 2012).

The deposition of CENP-A is controlled by various chaperones and assembly factors. In most vertebrates, the Mis18 complex (formed by Mis18α, Mis18β, and M18BP1[Knl2]) (Hayashi et al. 2004; Fujita et al. 2007; Maddox et al. 2007) plays an important role in licensing centromeric chromatin for CENP-A deposition. In addition, HJURP, which is a CENP-A specific chaperone, mediates the deposition of CENP-A (Kato et al. 2007; Foltz et al. 2009; Dunleavy et al. 2009). The Mis18 complex is effectively deactivated by CDK phosphorylation of M18BP1[Knl2]. This event inhibits its localization to the centromere (Silva et al. 2012) (Figure 4). Localization of the Mis18 complex to the centromere during G1 phase and deposition of new CENP-A also require phosphorylation by PLK1 (Figure 4) (McKinley and Cheeseman 2014). The Mis18 complex is recruited to centromeres by interacting with CENP-C (Moree et al. 2011; Dambacher et al. 2012). This provides a degree of temporal and spatial regulation for active centromeres; however, the Mis18 complex has not been reported as CENP-A binding proteins (Obuse et al. 2004b; Foltz et al. 2006; Izuta et al. 2006; Fujita et al. 2007). The actual deposition of CENP-A occurs through recognition of the Mis18 complex by the HJURP-CENP-A-H4 complex (Figure 4) (Nardi et al. 2016). In humans, the N-terminal region of HJURP specifically binds CENP-A (Shuaib et al. 2010; Hu et al. 2011; Bassett et al. 2012).

The interaction is sufficient for CENP-A deposition, when artificially tethered to ectopic non-centromeric regions (Barnhart et al. 2011; Hori et al. 2013). The C-terminal region of HJURP interacts with the Mis18α–β heterotetramer for its centromere recognition (Wang et al. 2014; Perpelescu et al. 2015; Nardi et al. 2016). The Mis18α–β heterotetramer is disrupted immediately after binding to HJURP in centromeric chromatin, which limits continuous CENP-A deposition (Nardi et al. 2016). Studies in Mis18 knockout mice revealed that the Mis18 complex alters the centromere chromatin by recruitment of DNA methyltransferase and histone acetyltransferase (Kim et al. 2012). These data strongly suggest that the Mis18 complex binds to the HJURP-CENP-A complex to mark CENP-A for deposition, although further examination is required

Once deposited, CENP-A activity must be rigidly controlled to prevent formation of multiple centromeres, segregation errors, and DNA damage. In fact, most of the chromosomal CENP-A has been found at ectopic sites outside the active centromere region (Shang et al. 2013; Bodor et al. 2014). Artificial targeting of CENP-A to ectopic regions is insufficient to trigger centromere activity (Brinkley and Stubblefield 1966; Gascoigne et al. 2011). This is attributed to negative feedback control of CENP-A expression, whereby high CENP-A levels restrict new CENP-A synthesis (Jansen et al. 2007). Linkage of this feedback mechanism to regulation, via Mis18/HJURP interaction, limits CENP-A deposition to active centromeres. A possible candidate factor for controlling active CENP-A is H4 Lys20 monomethylation (H4K20me1) of the CENP-A chromatin. H4K20me1 has been shown to occur specifically at active centromeric CENP-A, and its reduction results in mislocalization of CENP-H and CENP-T (Hori et al. 2014). The factors that control H4K20me1, as well as additional potential modifications of the centromeric nucleosomes responsible for maturation, remain an important and active area of study.

19

1.5 Assembling the Kinetochore: The CCAN

The first of the inner kinetochore proteins was identified along with CENP-A, using the sera from patients with CREST (calcinosis, Raynaud phenomenon, esophageal dysmotility, sclerodactyly, and telangiectasia) syndrome (Earnshaw and Rothfield 1985). Additional proteins, which might form the inner kinetochore, were isolated from HeLa cells by immunoprecipitation with a CENP-A-specific antibody; they were termed the interphase centromere complex proteins (ICEN) (Obuse et al. 2004b; Izuta et al. 2006). A majority of these might be associated with centromere maintenance, but some of them are not actual components of the kinetochore. Additional immunoprecipitation assays, using antibodies specific for CENP-A, CENP-H, CENP-I. CENP-T or CENP-S (a member of ICEN), have revealed that 16 proteins including some ICEN proteins, localize to the centromere throughout the cell cycle. They are now defined as the CCAN (Foltz et al. 2006; Okada et al. 2006; Izuta et al. 2006; Hori et al. 2008b; Amano et al. 2009; Nishino et al. 2012). The CCAN makes up the core of the kinetochore, which is responsible for recruitment of the microtubule-associated outer kinetochore proteins during mitosis (Cheeseman et al. 2008; Hori et al. 2008a). Depletion of any member of CENP-C, the CENP-H group, or CENP-T-W-S-X results in cell death following strong mitotic defects (Fukagawa 1997; Kalitsis et al. 1998; Fukagawa et al. 1999; Fukagawa et al. 2001; Nishihashi et al. 2002; Kwon et al. 2007; Hori et al. 2008a; Gascoigne et al. 2011; Basilico et al. 2014; Klare et al. 2015).

As the CCAN is constitutively bound to the centromere, it is difficult to clarify how and when its different components are recruited to CENP-A-containing chromatin. Recent studies suggest that there is no clear hierarchy for CCAN recruitment and maintenance (Hori

et al. 2008a; Fang et al. 2015; McKinley et al. 2015), although there are active debates on this issue (Carroll et al. 2010; Basilico et al. 2014; Klare et al. 2015). Various dynamic interactions between the different CCAN sub-complexes result in an extensive meshwork of interactions (Figure 5) (Kwon et al. 2007; Klare et al. 2015; McKinley et al. 2015). This situation creates separate hierarchies during *de novo* CCAN recruitment and for maintenance of CCAN components at various cell cycle stages. CENP-C and CENP-N bind directly to CENP-A nucleosomes *in vitro* (Carroll et al. 2009; Carroll et al. 2010; Kato et al. 2013; Falk et al. 2015; Fang et al. 2015; Falk et al. 2016; Guo et al. 2017) and localize near the CENP-A chromatin *in vivo* (Figure 5) (Wan et al. 2009). CENP-C appears to be upstream of all other CCAN complexes, including the CENP-H group in human cells (Klare et al. 2015; Tachiwana et al. 2015). Studies using *Xenopus* egg extract showed that CENP-C is the first CCAN component recruited to the centromere of sperm chromatin alongside new CENP-A (Milks et al. 2009; Krizaic et al. 2015). This suggests that CENP-C functions upstream of other CCAN components during *de novo* CCAN recruitment. However, localization of CENP-T-W-S-X and the CENP-H group appears to be distinct from that of CENP-C in chicken DT40 cells (Hori et al. 2008a); therefore, additional studies may be necessary.

CENP-C depletion in human cells causes loss of other CCAN components, especially in mitotic cells (Klare et al. 2015; McKinley et al. 2015; Tachiwana et al. 2015). However, the CENP-H and CENP-T groups are only mildly reduced in CENP-C-deficient chicken DT40 cells (Kwon et al. 2007; Hori et al. 2008a). By contrast, depletion of the CENP-H group causes consistent loss of CENP-T-W-S-X, although reduction levels are variable among species (Hori et al. 2008a; Klare et al. 2015; McKinley et al. 2015).
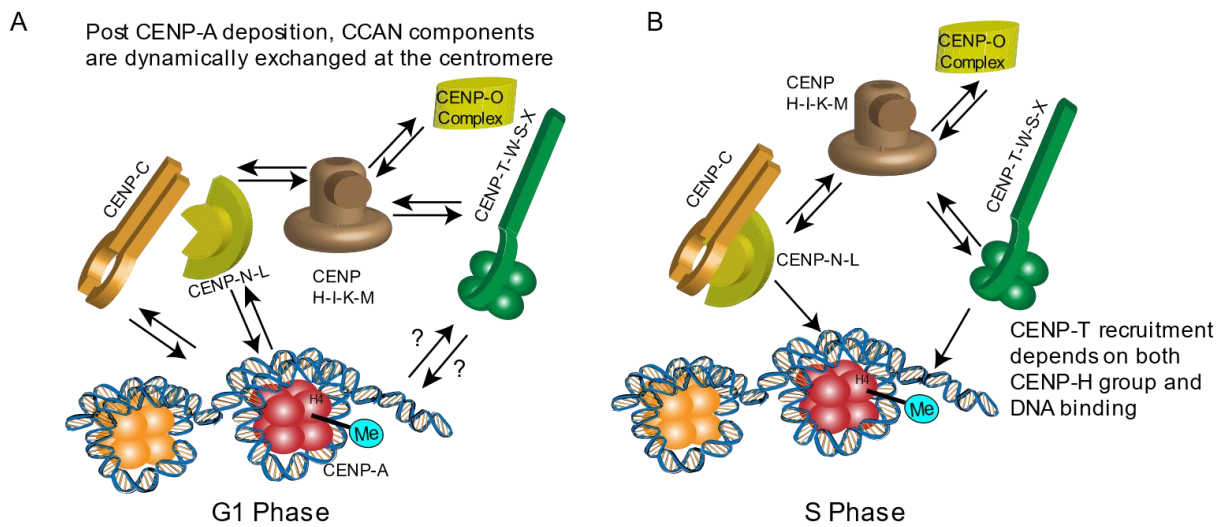
Figure 5: Model for assembly of the inner kinetochore: The 16-member CCAN can be further divided into 4 sub-groups: CENP-C, CENP-T-W-S-X, CENP-H-I-K-N-L and CENP-O-P-Q-R-U. These proteins are constitutively found at the centromere and have multiple pathways for recruitment to the kinetochore. (A) In G1 phase after new CENP-A incorporation, most CCAN components are dynamically exchanged at the centromere. CENP-C recruitment appears dependent on CENP-N-L. CENP-H group is also dynamically exchanged coupled with CENP-C. The CENP-O group is recruited downstream of the CENP-H group. Recruitment of CENP-T in G1 is currently not well understood. CENP-T depends on the CENP-H group for localization but may not be able to stably bind at the kinetochore. (B) In S-phase possibly due to changes in chromatin structure or changes in phosphorylation states of the CCAN, CCAN proteins begin to bind more strongly to the centromere. CENP-N and CENP-T are especially enriched at this stage in the cell cycle. CENP-C still appears downstream of CENP-N-L though this is debated. In Late G2/ Early M Phase (Figure 2), BubR1 dependant phosphorylation of H2-T120 helps recruit the CPC. I will show changes in the CCAN hierarchy with CENP-C becoming upstream of CENP-N and the CENP-H group and also strong binding of the CCAN to the centromere in metaphase unlike the dynamic exchange seen during interphase. The mechanism behind this change is currently unknown and remains an important question.

FRAP (Fluorescence Recovery After Photobleaching) studies have shown that CENP-C and CENP-H are stably bound to centromeres only during S-phase and mitosis, and undergo dynamic exchange during G1 (Hemmerich et al. 2008). This profile corresponds to the loading of CENP-N to the kinetochore (Hellwig et al. 2011). CENP-C interacts with either CENP-H (Klare et al. 2015) and/or CENP-L-N during interphase (Hinshaw and Harrison 2013; McKinley et al. 2015), which may be required for CENP-C stabilization (Figure 5). In the following sections I will also show that CENP-C in mitotic cells directly recognizes CENP-A

distinct from the CENP-H-group. Inversely, CENP-N localizes to centromeres independent of CENP-C in interphase, but is dependent on CENP-C during mitosis (McKinley et al. 2015). Thus, dynamic CENP-C during G1 plays a role in recruiting diffuse downstream CCAN components to the kinetochore. Subsequently, interactions between CENP-C and the CENP-H group facilitate maintenance of the CCAN structure in interphase.

Kinetochore localization of CENP-T-W-S-X depends on the CENP-H group (Figure 5) (Hori et al. 2008a; Basilico et al. 2014; McKinley et al. 2015). However, CENP-T localization is not completely lost in CENP-H-deficient DT40 cells (Hori et al. 2008a). CENP-T expression in *Xenopus* was not completely lost after depletion of CENP-C (Krizaic et al. 2015). CENP-T-W-S-X contains histone fold domains, which confer an innate DNA-binding ability (Hori et al. 2008a; Nishino et al. 2012; Takeuchi et al. 2014). Mutation of the CENP-T-W-S-X in its DNA-binding domain greatly reduces centromere localization, without affecting CENP-H group association (Nishino et al. 2012). These observations suggest that direct DNA interaction and the CENP-H group are both required for centromere localization of CENP-T-W-S-X. Experiments in *Xenopus* showed that *de novo* CENP-T recruitment occurs during S-phase, but it is not coupled with DNA replication (Krizaic et al. 2015). FRAP experiments using human cells suggest that CENP-T recruitment occurs in S-phase (Prendergast et al. 2011; Dornblut et al. 2014).

The molecular mechanisms by which CCAN dynamics are controlled are interesting, and gaining a better understanding of these mechanisms is an important and active topic of research in this field.

1.6   Recruiting the Outer kinetochore

The key function of the kinetochore is binding to the microtubules for proper chromosome segregation. The inner acts as a platform for the assembly of several microtubule-binding proteins, which forms the outer kinetochore to establish full kinetochore function. The main microtubule binding interface is made up of the Knl1/Mis12 complex/Ndc80 complex (KMN) network, which assembles directly onto the CCAN (Cheeseman et al. 2004; Obuse et al. 2004a; Cheeseman et al. 2006). A four-subunit Ndc80 complex (Spc24, Spc25, Nuf2, and Ndc80[Hec1]) directly binds microtubules (Cheeseman et al. 2006; DeLuca et al. 2006). The Ndc80 complex forms a rod-shaped structure (Ciferri et al. 2008) in which the Nuf2-Ndc80[Hec1] end directly binds microtubules (Wei et al. 2007). The Spc24-Spc25 complex faces the inner kinetochore (Petrovic et al. 2010) and binds both the Mis12 and CENP-T-W-S-X group.

The Mis12 complex is a four-member rod-shaped complex made up of Nnf1, Mis12, Dsn1, and Nsl1 (Cheeseman et al. 2004; Obuse et al. 2004a; Kline et al. 2006). The Mis12 complex binds the Ndc80 complex through its Nsl1 subunit (Petrovic et al. 2010) and links to CENP-C through direct interaction (Figure 4) (Przewloka et al. 2011; Screpanti et al. 2011; Petrovic et al. 2016a). The inner kinetochore protein CENP-T also directly binds the Ndc80 complex (Gascoigne et al. 2011; Nishino et al. 2013). In yeast, the Ndc80 complex and Mis12 complex share an extensive interaction network (Kudalkar et al. 2015) and the CENP-T homologue Cnn1 and the yeast Mis12 complex competitively bind the yeast Ndc80 complex (Malvezzi et al. 2013). It has been suggested that CENP-T and the Mis12 complex may also competitively bind the Ndc80 complex in vertebrates (Gascoigne et al. 2011; Nishino et al. 2013).

These data clearly indicate that there are two mechanisms for recruitment of the Ndc80 complex to the kinetochore. One is through the direct recruitment of Ndc80 by CENP-T. The second mechanism entails indirect recruitment of Ndc80 by the Mis12 complex, which associates with CENP-C (Przewloka et al. 2011; Screpanti et al. 2011; Petrovic et al. 2016a). Knockout of either CENP-C or CENP-T shows a significant reduction in Ndc80 levels (Suzuki et al. 2015; Rago et al. 2015), which suggests that the two mechanisms may not be redundant. Studies using CENP-T and CENP-C mutants have shown that CENP-T is able to recruit the entire KMN network, possibly due to indirect recruitment of the Mis12 complex through the Ndc80 complex (Kim and Yu 2015; Rago et al. 2015). However, the Ndc80 complex shows mutually exclusive binding to the Mis12 complex and CENP-T *in vitro* (Malvezzi et al. 2013; Nishino et al. 2013). Replacement of the CENP-C N-terminus with the CENP-T N-terminus (Ndc80 binding domain) rescues CENP-C depletion (Suzuki et al. 2015). Artificially tethered CENP-C or CENP-T is able to independently recruit the KMN complex to form functional kinetochores (Gascoigne et al. 2011; Hori et al. 2013). Taken together, the two mechanisms for recruitment of the Ndc80 complex seem to be independent in artificial conditions. However, the two mechanisms may function cooperatively to recruit sufficient Ndc80 complexes to native kinetochores.

The Knl1 complex is recruited to the kinetochore through interactions with the Mis12 complex (Cheeseman et al. 2008; Petrovic et al. 2010); it consists of two components, Knl1[Blinkin] and Zwint (Petrovic et al. 2010). Knl1 can bind microtubules directly through its N-terminus (Cheeseman et al. 2006), although this microtubule-binding activity is dispensable in *C. elegans* (Espeut et al. 2012). However, the main role of Knl1 is regulation of the kinetochore-microtubule interaction, by recruiting other outer kinetochore components associated with microtubule dynamics and the SAC (Cheeseman and Desai 2008; Varma and

Salmon 2012; Ghongane et al. 2014). Knl1[Blinkin] is the key protein for recruitment of the mitotic checkpoint proteins Bub1 and BubR1 (Kiyomitsu et al. 2007; Kiyomitsu et al. 2011; Faesen and Musacchio 2015; Silió et al. 2015; Vleugel et al. 2015; Caldas et al. 2015). Zwint is thought to be responsible for recruiting the Rod/Zwilch/ZW10 (RZZ) complex (Wang et al. 2004; Kops et al. 2005; Varma et al. 2013), but recent studies suggest that RZZ recruitment occurs through Bub1 (Zhang et al. 2015). Bub1, BubR1, and the RZZ complex recruit Spindly and the Mad1-Mad2 complex (Kops et al. 2005), which are essential components of the SAC.

It remains unclear how microtubule plus-end dynamics are regulated by the outer kinetochore. The Dam/Dash complex confers the ability to track the plus end of dynamic microtubules in budding yeast and in other fungi (Lampert et al. 2010). A vertebrate homologue for the Dam/Dash complex has not been found, but the Ska complex has been proposed as the functional homologue (Welburn et al. 2009; Schmidt et al. 2012). The Ska complex is necessary for stable kinetochore microtubule attachments (Hanisch et al. 2006; Gaitanos et al. 2009; Welburn et al. 2009; Theis et al. 2009; Daum et al. 2009). It may play a role in silencing the SAC (Hanisch et al. 2006; Daum et al. 2009), possibly by enhancing binding of the APC/C to the chromosomes (Sivakumar et al. 2014). The Ska complex is able to track plus-end microtubules, like the Dam/Dash complex (Welburn et al. 2009; Schmidt et al. 2012). Recent study on the Ska complex structure shows that it forms a W-shaped heterodimer with monomeric Ska1, Ska2, and Ska3 subunits(Figures 6) (Jeyaprakash et al. 2012). The Ska complex binds microtubules in multiple locations and orientations (Abad et al. 2014); thus, it may have a function in proper kinetochore-microtubule attachments. The relationship of the Ska complex with the KMN network remains unclear. However, it has been shown to bind the Mis12 complex and Ndc80[Hec1] in an Aurora B-dependent manner (Chan et al. 2012b). Other study suggests that the Ska complex binds to the inner loop of Ndc80[Hec1] (Zhang et al. 2012)

or through its N-terminal Tail (Cheerambathur et al. 2017). Further characterization of this complex is essential to consider regulation of proper kinetochore-microtubule interactions
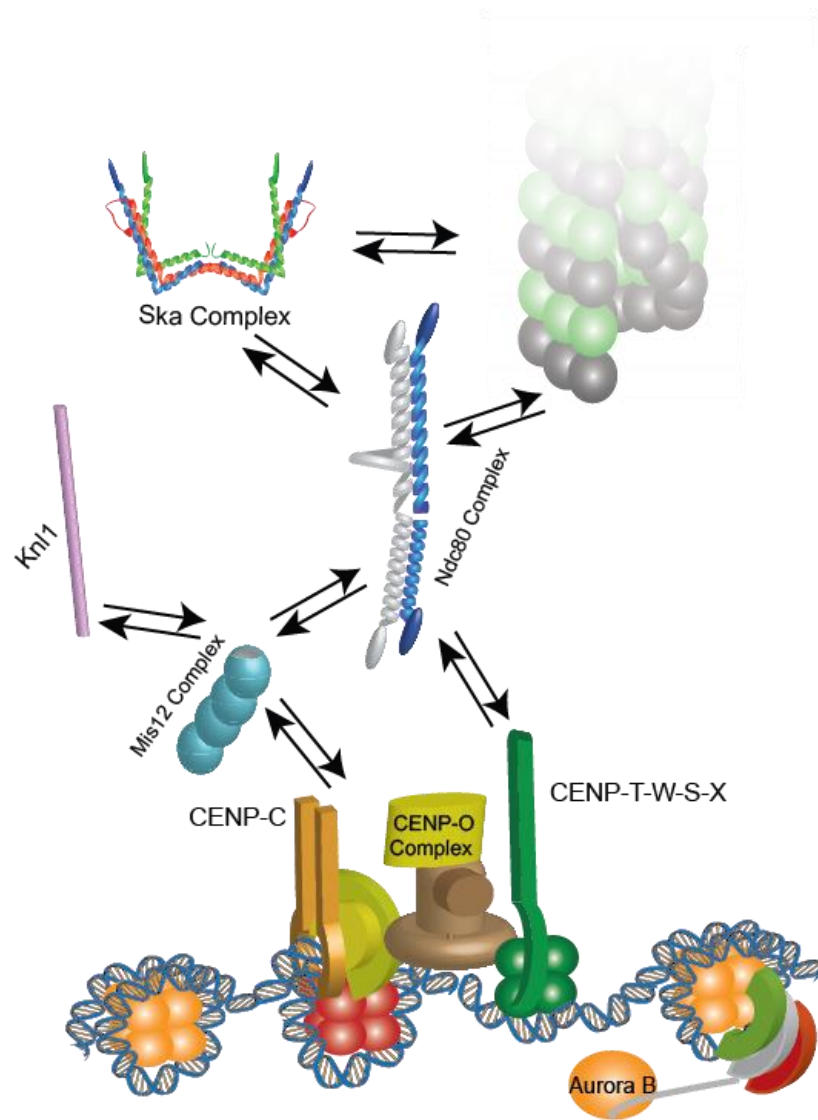
Figure 6: Model for assembly of the outer kinetochore: The inner kinetochore acts as a platform for the assembly of several microtubule-binding proteins, which forms the outer kinetochore to establish full kinetochore function. The main microtubule binding interface is made up of the Knl1/Mis12 complex/Ndc80 complex (KMN) network, which assembles directly onto the CCAN. A four-subunit Ndc80 complex forms a rod-shaped structure and directly binds microtubules, the Mis12, and the CENP/T/W/S/X complex. The Mis12 complex is a four-member rod-shaped complex that binds the Ndc80 complex and links it to CENP-C through direct interaction. The Knl1 complex is recruited to the kinetochore through interactions with the Mis12 complex. The main role of Knl1 is regulation of the kinetochore-microtubule interaction, by recruiting other outer kinetochore components associated with microtubule dynamics and the SAC. The relationship of the Ska complex with the KMN network remains unclear. However, it has been shown to bind the Mis12 complex and Ndc80[Hec1] in an Aurora B-dependent manner. The Ska complex is necessary for stable kinetochore microtubule attachments. It may play a role in silencing the SAC and is also able to track plus-end microtubules.

1.7   Regulation of the kinetochore

Proper recruitment of the kinetochore components is necessary for correct chromosome segregation. As explained in previous sections there exist many complicated and overlapping feedback mechanisms for recruitment of the kinetochore, many of which are poorly understood and under active study.

Phosphorylation by regulatory kinases are a prime example of such regulation. Many kinetochore components are phosphorylated. For example, the CENP-T N-terminus is phosphorylated by CDK1, and this phosphorylation event promotes recruitment of the Ndc80 complex to the kinetochore during early M phase (Figure 7A) (Nishino et al. 2013; Rago et al. 2015). The overall control of outer kinetochore recruitment is dependent on CDK1 kinase activity. CDK1 is also involved in recruitment of Aurora B kinase, a member of the chromosome passenger complex (CPC), to the kinetochore (Tsukahara et al. 2010). The mechanism by which the CPC is recruited to the centromere consists of various feedback loops among the CPC components, phosphorylation of histones H3 and H2A, and multiple mitotic kinases, including Haspin, Aurora B, Plk1, Bub1, and CDK1. The recruitment of inner kinetochore components such as CENP-T, CENP-C, and CENP-N may also be dependent on phosphorylation to control recruitment to the centromere at various cell cycle stages though this requires further study.
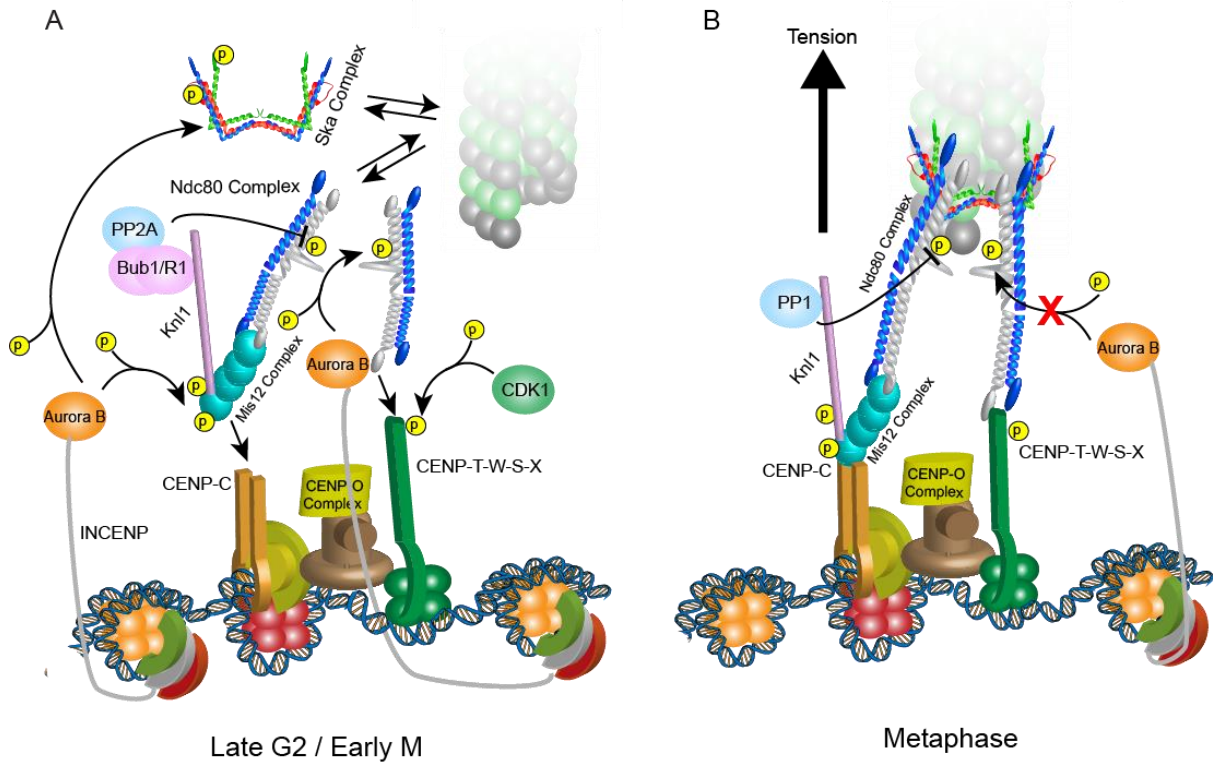
Figure 7: Attachment and regulation of the outer kinetochore: (A) The outer kinetochore builds onto CENP-C and CENP-T during late G2/Early M Phase. Phosphorylation of the Mis12 complex by the Aurora B kinase is necessary for its recruitment onto CENP-C as well as for recruitment of KNL1. The Mis12 complex subsequently recruits Ndc80. Phosphorylation of CENP-T by CDK1 is necessary for recruitment of Ndc80 to CENP-T. Together these form the two pathways for recruiting Ndc80 to the centromere. (B) The regulation of microtubule binding until proper bi-orientation is achieved by a feedback mechanism consisting of phosphorylation of the Ndc80 complex by Aurora B and dephosphorylation by PP2A-B56 which respectively decrease and increase Ndc80-microtubule binding. After proper Bi-orientation is achieved and the kinetochore is under tension, Aurora B activity is decreased due to increased intra-kinetochore distance and recruitment of PP1 phosphatase dramatically increase Ndc80-microtubule binding.

Other regulatory mechanisms involve various histone methylation and acetylation which affect CENP-A deposition and maturation. For example, H4K20me1 has been shown to occur specifically at active centromeric CENP-A, and its reduction results in mislocalization of CENP-H and CENP-T (Hori et al. 2014). These factors also affect transcription and heterochromatin formation at the pericentromeric region. Studies using human artificial chromosomes (HAC) have shown that heterochromatin in the pericentromere and

modifications at H3 nucleosomes, such as H3K4 methylation or H3K9 acetylation in the centromeric chromatin, affect kinetochore assembly (Nakano et al. 2008; Cardinale et al. 2009; Ohzeki et al. 2012). Furthermore, transcription levels have been shown to affect the centromere, in which excessive transcription disrupts centromere activity (Bergmann et al. 2012). Other studies suggest that some amount of transcription activity may be required for centromere function (Rošić and Erhardt 2016); centromere chromatin contains many transcriptionally active markers such as H3K4me2 and H3K36me2 (Bergmann et al. 2011; Rošić and Erhardt 2016), and resists silencing mediated by H3K27me3/K9me3 (Martins et al. 2015). Furthermore, RNAPII localizes to active centromeres, and its disruption results in decreased centromere transcripts and subsequent kinetochore defects (Chan et al. 2012a; Liu et al. 2015). The exact nature of the effect of transcription and heterochromatin on centromere formation remains an interesting question.

The regulatory control of the outer kinetochore is comparatively better understood. Aurora B is a key player in controlling kinetochore microtubule attachments (Biggins et al. 1999; Cheeseman et al. 2002; Tanaka et al. 2002; Cheeseman et al. 2006; DeLuca et al. 2006; Liu et al. 2009; Kim et al. 2010; Welburn et al. 2010). Aurora B, localized to the kinetochore, directly phosphorylates the KMN network and the Ska complex (Cimini et al. 2006; Welburn et al. 2010) (Figure 7A). Aurora B phosphorylation of Ndc80 reduces its microtubule-binding affinity, which prevents erroneous attachment of kinetochores to microtubules (Cheeseman et al. 2006; DeLuca et al. 2006; Liu et al. 2009; Alushin et al. 2010; Lampson and Cheeseman 2011; Zaytsev et al. 2015). Ndc80 phosphorylation is finely controlled until proper bi-orientation is established under tension during prometaphase (Magidson et al. 2011; Sikirzhytski et al. 2014). This stable attachments between kinetochores and microtubules are mediated by a feedback mechanism, which balances phosphorylation of Ndc80 by Aurora B

and its dephosphorylation by B56-PP2A or PP1 phosphatases (Foley et al. 2011; Suijkerbuijk et al. 2012). Once the kinetochores are under tension, the increased inter-kinetochore distance reduces the ability of Aurora B to phosphorylate Ndc80, which increases binding activity of Ndc80 to microtubules. PP1 phosphatase is also recruited to Knl1 (Liu et al. 2010; Meadows et al. 2011; Rosenberg et al. 2011) and to Ska1(Sivakumar et al. 2016) (Figure 7B) after proper bi-orientation is achieved and acts as a second feedback mechanism, further enhancing the Ndc80-microtubule interaction. The intra-kinetochore stretch (Maresca and Salmon 2009; Uchida et al. 2009; Suzuki et al. 2011) and its resulting changes to kinase availability may also facilitate silencing of the SAC.

Aurora B also phosphorylates the Mis12 complex, which may facilitate kinetochore assembly through a tight CENP-C-Mis12 complex interaction (Yang et al. 2008; Welburn et al. 2010; Kim and Yu 2015; Rago et al. 2015). Furthermore, phosphorylation of Knl1 helps to stabilize the Knl1-Mis12 complex interaction and promotes recruitment of BUB1 and PP1 (Liu et al. 2010; Welburn et al. 2010; Vleugel et al. 2015). This contributes to dynamic control of microtubule attachment until proper chromosome bi-orientation is established. There is a significant number of substrates for various mitotic kinases in the kinetochore complex; thus, it is crucial to address the regulation mechanisms of kinetochore assembly by mitotic kinases.

Chapter 2

Dynamic changes in the CCAN through CENP-C

Adapted from:

Abstract

The kinetochore is a crucial structure for faithful chromosome segregation during mitosis. It is formed in the centromeric region of each chromosome. The 16-subunit protein complex known as the constitutive centromere associated network (CCAN) forms the foundation for kinetochore assembly on the centromeric chromatin. Although the CCAN can be divided into several sub-groups, it remains unclear how CCAN proteins are organized to form the functional kinetochore. In particular, the organization may vary during the cell cycle. To address this question, I analyzed the relationship of CENP-C with the CENP-H group during progression of the cell cycle. I demonstrated that the middle portion of chicken CENP-C (CENP-C$^{166-324}$) is sufficient for centromere localization during interphase, potentially through association with the CENP-L-N complex. The C-terminus of CENP-C (CENP-C$^{601-864}$) is essential for centromere localization during mitosis, through binding to CENP-A nucleosomes, independent of the CENP-H group. Based on these results, I propose that CCAN organization changes dynamically during progression of the cell cycle.

2.1   Introduction

As described in chapter 1, the kinetochore is a multi-protein complex responsible for the equal distribution of genetic material to the daughter cells by coupling the chromosomes to the spindle microtubules. The kinetochore assembles on a section of the chromosome called the centromere which is marked by the histone H3 variant CENP-A (chapter 1.4). The inner layer of the kinetochore is recruited downstream of CENP-A and consists of a 16-subunit protein complex known as the constitutive centromere associated network (CCAN) (chapter 1.5). The CCAN can be further divided into several subcomplexes based on their functions and biochemical characteristics. As the CCAN is constitutively bound to the centromere, it is difficult to clarify how and when its different components are recruited to CENP-A-containing chromatin. Recent studies suggest that there is no clear hierarchy for CCAN recruitment and maintenance (Hori et al. 2008a; Fang et al. 2015; McKinley et al. 2015), although this assertion is heavily debated (Carroll et al. 2010; Basilico et al. 2014; Klare et al. 2015). Whereas considerable progress has been made in understanding the relation between CCAN components as well between the inner and outer kinetochores, the exact temporal order and dependencies are still unclear. Of particular interest are the CENP-C and CENP-T pathways which form two distinct associations with centromeric chromatin, questions remain as to the role played by the CENP-H-I-K-L-M-N group on these pathways. In this Chapter I show that CENP-C is recruited to the kinetochore in both a CCAN dependent and independent manner and that the CENP-C recruitment pathway varies based on the cell cycle. I also show that a connection exists between the CENP-C and CENP-T pathways through the CENP-N-L complex and that this connection creates a co-dependence necessary for the proper maintenance and functioning of the kinetochore.

2.2 The middle portion and C-terminus of CENP-C can localize to the centromere

Previous studies have shown that depletion of CENP-H or CENP-K in DT40 cells results in loss of CENP-C localization to interphase nuclei (Fukagawa et al. 2001; Kwon et al. 2007) but not in mitotic nuclei where CENP-C appears to localize independently of the CCAN. This suggests that CENP-C has more than one mechanism to localize to the kinetochore and that interphase localization must be related to the CENP-H group. To test this hypothesis, I prepared various chicken GFP-fused CENP-C-truncated mutants (Figure 8A) and introduced them into CENP-C-deficient cells and examined the localization of each mutant CENP-C in both interphase and mitosis (Figure 8B). Please note that the number of centromere foci in interphase nuclei are less than those in mitotic cells, because centromere foci are clustered in interphase nuclei.

CENP-C has various functional domains. These domains were based on previous studies of CENP-C as well as by identifying various conserved regions between different species. The N-terminal end is required for Mis12 binding (Petrovic et al. 2010; Przewloka et al. 2011; Petrovic et al. 2016b); the C-terminal end contains the CENP-C (or Mif2) motif alongside a dimerization domain which is important for CENP-A binding (Kato et al. 2013; Falk et al. 2015). The Mis12 binding domain, CENP-C motif and dimerization domain are relatively conserved. The middle of CENP-C is suspected of being another CENP-A–binding region in humans (Falk et al. 2016) though this region is not well conserved (Figure 8A). A fifth region spanning the 166–324 aa region of chicken CENP-C is also relatively conserved, however its function remained unclear.

I found that CENP-C$^{601-864}$ preferentially localized to centromeres in mitotic cells, but that it became diffuse in interphase nuclei (Figure 8B). In contrast, CENP-C$^{1-643}$, which lacks the CENP-C motif and dimerization domain, preferentially localized to centromeres in interphase cells, but its centromeric localization in mitotic cells was lower than full length CENP-C localization in wild-type cells (Figure 8B). These data suggest that the entire N-terminal portion of CENP-C (aa 1–643) is primarily responsible for centromere localization in interphase nuclei, and the C-terminal dimerization domain of CENP-C is critical for centromeric localization during mitosis. I further analyzed which region of N-terminal CENP-C is responsible for centromere localization in interphase nuclei and found that dimeric CENP-C$^{166-324}$-LacI is sufficient for centromeric localization in interphase nuclei (Figure 8B). As the cells lack LacO domains I conclude the localization is due to CENP-C$^{166-324}$. As CENP-C$^{166-324}$-LacI could not rescue CENP-C depletion, I visualized its localization in the presence of endogenous CENP-C, and found that the clear punctate signals of GFP-CENP-C$^{166-324}$-LacI in interphase were lost in mitosis and were visible again in next G1 cells by live-cell imaging (Figure 8C). Based on these results, I conclude that the middle portion of CENP-C (aa 166–324 shown as conserved domain in Figure 8A) is potentially responsible for interphase centromeric localization and that the C-terminus of CENP-C (aa 601–864) is critical for mitotic centromeric localization.
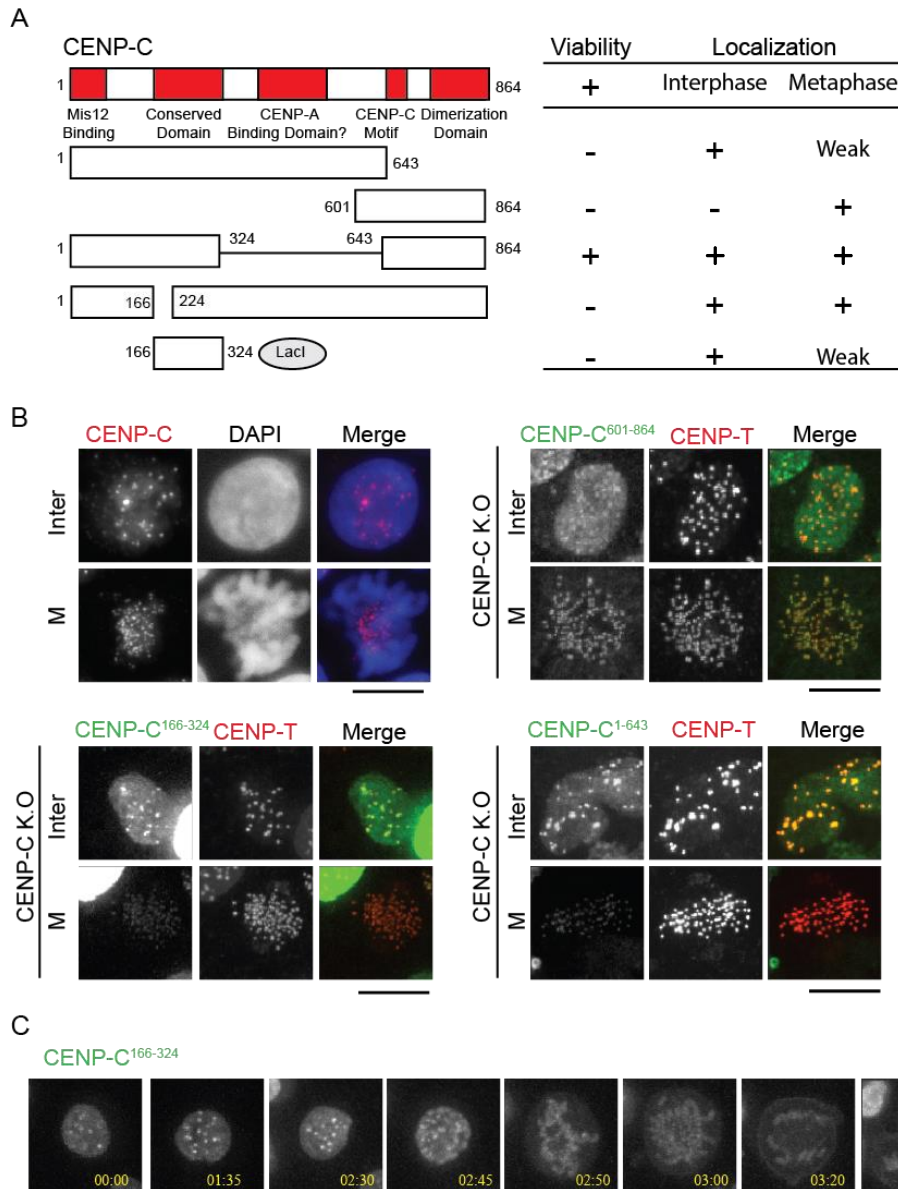
Figure 8: CENP-C$^{166-324}$ and CENP-C$^{601-864}$ localize to interphase and mitotic centromeres, respectively: (A) Schematic showing CENP-C domains (red) and the constructs used, along with their ability to rescue CENP-C depletion and localization throughout the cell cycle (+ for viable or localized, - for not viable or not localized). (B) Representative images showing wild-type CENP-C (using an anti-ggCENP-C antibody), GFP-CENP-C$^{601-864}$, GFP-CENP-C$^{166-324}$ and GFP-CENP-C$^{1-643}$ localization during interphase and metaphase. Kinetochores in CENP-C conditional knockout cells expressing GFP constructs were visualized by CENP-T immunostaining at 36 hrs after tetracycline addition. Bars, 10 μm. (C) Live-cell imaging of cells expressing GFP-CENP-C$^{166-324}$-LacI.

2.3   The central putative CENP-A binding region is dispensable for chicken CENP-C function

Human CENP-C contains two CENP-A binding regions (Kato et al. 2013). The first is the C-terminal dimerization domain and CENP-C motif, which are highly conserved among species. The second is the middle of human CENP-C (hsCENP-C$^{444\text{-}537}$; aa 444–537 in human CENP-C); no clearly homologous region is present between the middle domain of chicken CENP-C and hsCENP-C$^{444\text{-}537}$. To determine the function of the putative corresponding region of chicken CENP-C (aa 325-643), I introduced a CENP-C$^{\Delta325–643}$ mutant into CENP-C–deficient cells and investigated cell viability. As shown in Figure 9A, CENP-C$^{\Delta325–643}$ rescued the CENP-C deficiency, suggesting that the middle region of chicken CENP-C (aa 325–643) is dispensable for its function, suggesting that this region may not be required for centromeric localization of CENP-C.
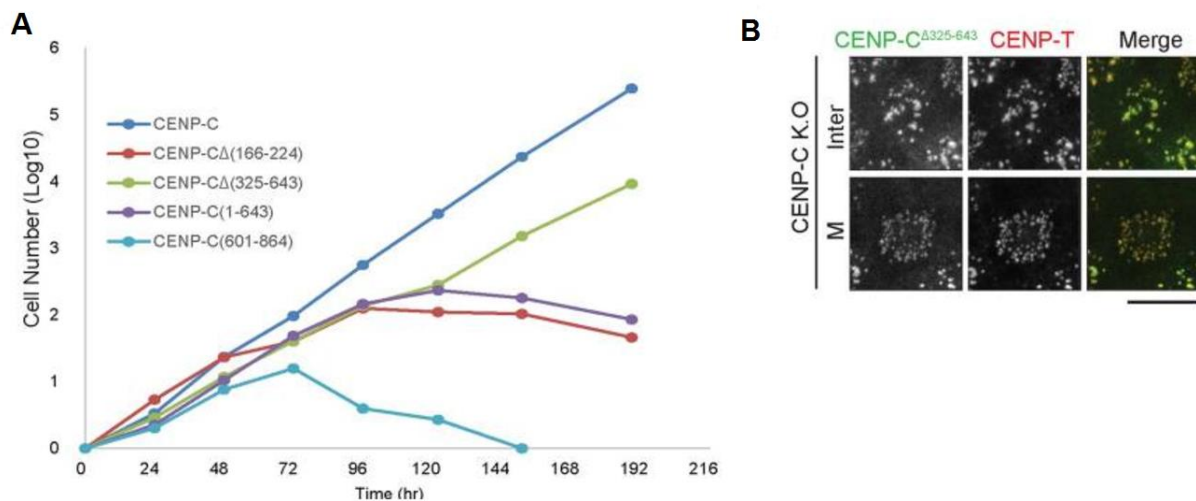
Figure 9: Putative CENP-A binding domain CENP-C$^{325\text{-}643}$ is dispensable for chicken CENP-C function: (A) Growth curves for CENP-C–deficient cells expressing the indicated CENP-C constructs. Tetracycline was added at $t = 0$, and the number of live cells was counted. (B) Representative image of localization of CENP-C$^{\Delta325–643}$ and CENP-T in CENP-C–deficient cells expressing CENP-C$^{\Delta325–643}$. Bars, 10 μm.

I also confirmed that CENP-C$^{\Delta 325–643}$ and CENP-T showed clear centromere localization in CENP-C-deficient cells expressing CENP-C$^{\Delta 325–643}$ (Figure 9B). In contrast, CENP-C$^{1–643}$ did not rescue the CENP-C deficiency (Figure 9A), because the C-terminus of CENP-C is responsible for CENP-A binding. These results suggest that the putative CENP-A binding region of chicken CENP-C (aa 325–643) is dispensable for cell viability and may not be involved in CENP-A binding in chicken cells.

2.4 CENP-C$^{166\text{-}324}$ depends on the CENP-H group for its centromere localization

CENP-C$^{166–324}$ preferentially localizes to interphase centromeres (Figure 8B, C). As our lab have previously shown, interphase CENP-C localization depends on the CENP-H group (Fukagawa et al. 2001; Kwon et al. 2007), and thus interphase centromeric localization of CENP-C$^{166–324}$ may depend on the CENP-H group. To test this possibility, I expressed CENP-C$^{166–324}$-GFP in CENP-N conditional knockout cells. CENP-N conditional knockout cells cause complete loss of CENP-H, -I, -K, -L, and -M signal and a reduction of CENP-T signals (Figure 10A, B) at the kinetochore. This phenotype is similarly seen in both CENP-H or CENP-K knockout cells (Fukagawa et al. 2001; Okada et al. 2006; Kwon et al. 2007; Hori et al. 2008a). Although GFP-CENP-C$^{166–324}$-LacI localized to interphase centromeres in the presence of CENP-N, this localization was abolished in the absence of CENP-N (Figure 10C). I also confirmed that interphase centromere localization of CENP-C$^{1–643}$ depends on CENP-N (Figure 10C). Based on these results, I conclude that interphase centromeric localization of CENP-C$^{166–324}$ depends on the CENP-H group.
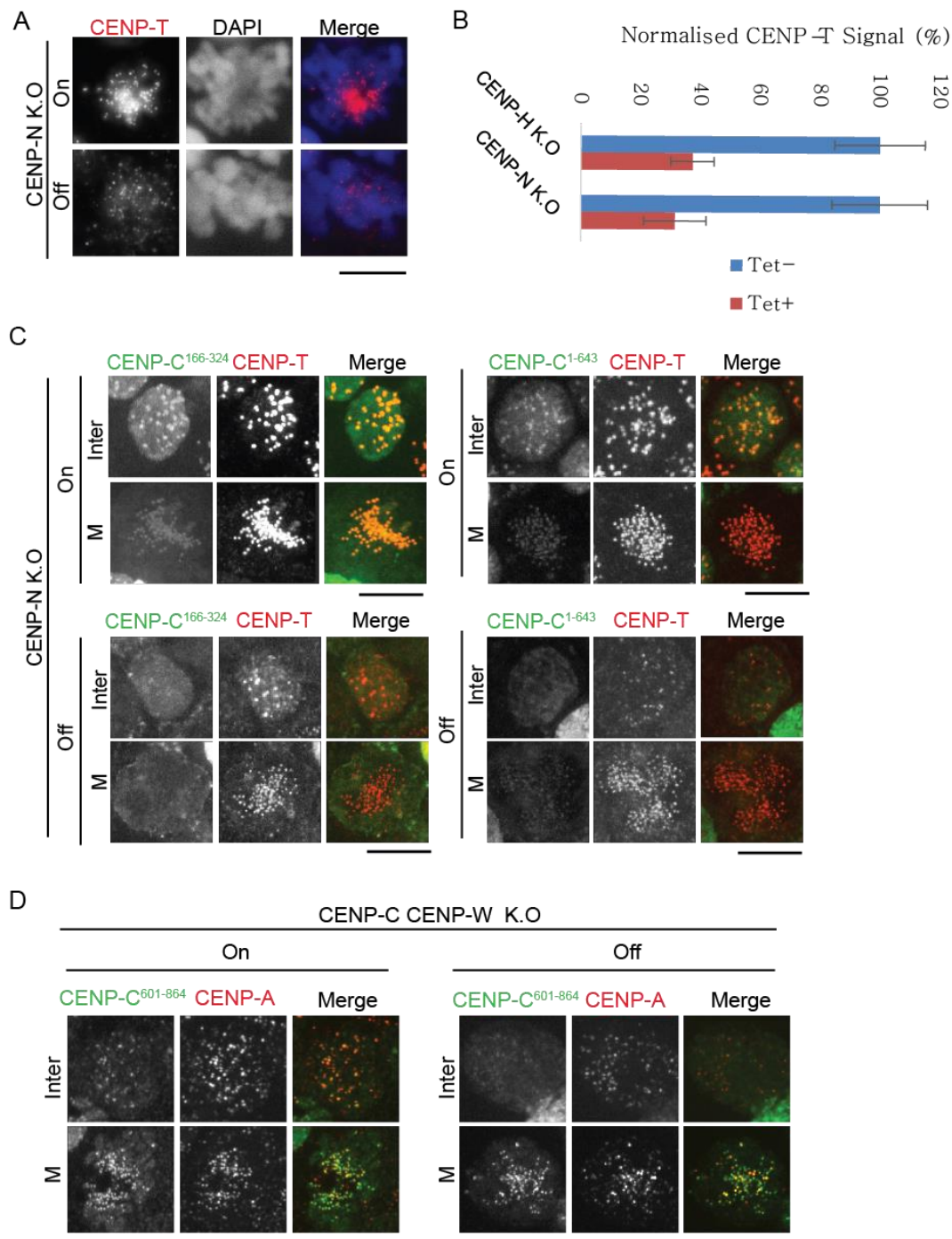
Figure 10: Centromere localization of CENP-C$^{166-324}$ depends on CENP-N during interphase and that of CENP-C$^{601-864}$ depends on CENP-A during mitosis: (A) Representative images showing loss of CENP-T on CENP-N depletion (before (On) and 36 hrs. (Off) after addition of Tetracycline). (B) Quantification of loss of CENP-T on CENP-H or CENP-N depletion. Results were plotted as an average of the signal intensities of 20 kinetochores per cell (n > 7 cells). Error bars indicate standard deviation (S.D.). (C) Representative images showing loss of centromere localization during the cell cycle for CENP-C N-terminal constructs (green) on CENP-N depletion (before (On) and 36 hrs. (Off) after addition of tetracycline). Kinetochores were visualized by CENP-T immunostaining (red). Bars, 10 μm. (D) CENP-C$^{601-864}$ (green) localizes to the centromeres in mitotic cells after CENP-C and CENP-W double depletion. Bars, 10μm.

Figure 11: Centromeric CENP-H levels also depend on CENP-C: (A) Immunostaining for CENP-H (red) in CENP-C–deficient cells expressing various GFP–CENP-C. Images before (On) and 36 hrs. (Off) after addition of tetracycline. (B) Quantification of CENP-H in DT40 cells expressing the indicated GFP–CENP-C constructs in control and CENP-C–deficient cells. Results were plotted as an average of the signal intensities of 20 kinetochores per cell (n > 7 cells). Error bars indicate standard deviation (S.D.). (C, E) Immunostaining for CENP-T (red) in CENP-N and CENP-C-deficient cells expressing GFP-CENP-C$^{\Delta166-224}$. (D) Quantification of CENP-T in deficient cells expressing GFP-CENP-C$^{\Delta166-224}$. (F) Cell cycle distribution of CENP-C deficient cells with and without expression of CENP-C$^{1-643}$ at 0, 48, and 72 hrs after tetracycline addition.

40

Given that CENP-C$^{601-864}$ localizes to mitotic centromeres and mitotic CENP-C localization does not depend on the CENP-H group (Kwon et al. 2007), it is likely that CENP-C$^{601-864}$ localizes to the mitotic centromere even after depletion of proteins in the CENP-H-group. To test this hypothesis, I expressed mutant CENP-C$^{601-864}$ in CENP-C and CENP-W double-knockout. Our lab have previously demonstrated that CENP-H group proteins do not localize to centromeres in CENP-C and CENP-W double-knockout cells (Hori et al. 2008a). By using this cell line, I can also avoid the formation of CENP-C$^{601-864}$ and wild-type CENP-C heterodimers. In control cells (in the presence of endogenous CENP-C and CENP-W), I observed centromere localization of CENP-C$^{601-864}$ in both interphase and mitotic cells (Figure 10D left). When expression of both genes was turned off, interphase localization CENP-C$^{601-864}$ was abolished, but mitotic localization was not altered (Figure 10D, right). This indicates that mitotic localization of CENP-C$^{601-864}$ does not depend on the CENP-H group. Because amongst the CCAN components only CENP-A is present in the centromeric region in CENP-C and CENP-W double-knockout cells (Hori et al. 2008a), it is possible that CENP-C$^{601-864}$ directly recognizes CENP-A.

Finally, I tested how CENP-H localization depends on each mutant CENP-C. I used CENP-C conditional knockout cells at 36 hrs after induction of CENP-C knockout by addition of tetracycline, which does not cause CENP-H signal reduction at kinetochores (Figure 11A, B). Unlike in CENP-C knockout cells, When I expressed CENP-C$^{\Delta 1-324}$ or CENP-C$^{\Delta 166-224}$ alongside performing CENP-C knockout, CENP-H was reduced to 30–40% of its levels before induction of CENP-C knockout (Figure 11A, B), suggesting a dominant-negative effect on CENP-H localization, I also observed a 40-50% reduction in CENP-T signal at the kinetochores consistent with the loss of CENP-H (Figure 10B, 11C). CENP-C$^{\Delta 166-224}$ did not rescue CENP-C deficiency (Figure 8A), and strong mitotic defects were observed similar to

41

the phenotype caused with the loss of CENP-H group proteins. I noted that while CENP-C$^{166-324}$ is sufficient for centromere localization in interphase cells, CENP-C$^{\Delta166-224}$ still localize to interphase centromeres (Figure 11E), suggesting that additional N-terminal region of CENP-C may be responsible for centromere localization in interphase cells. On the other hand, compared to CENP-C$^{\Delta166-224}$ or CENP-C$^{\Delta1-324}$, CENP-C-deficient cells expressing CENP-C$^{1-643}$ showed no change in CENP-H and CENP-T levels at the kinetochore as compared to those in control cells (Figure 8B, 11A, B, D). However, consistent with the observation that CENP-C$^{1-643}$ does not localize to the centromere during mitosis, CENP-C-deficient cells expressing CENP-C$^{1-643}$ are not viable with a similar mitotic index to CENP-C-deficient cells (Figure 11F), suggesting that proper kinetochore structure is not formed even if CENP-H localizes to centromeres. Based on these data, I can conclude that the CENP-H group is involved in centromeric localization of the middle portion of CENP-C (aa 166-324), and that the C-terminus of CENP-C (aa 601-864) is independent of the CCAN and directly downstream of CENP-A.

2.5 The middle-conserved domain of CENP-C binds directly to CENP-N-L and C-terminus binds the CENP-A nucleosome

As I have established in the previous sections that the middle portion of CENP-C (aa 166-324) is related to the CENP-H group. The yeast homologue of CENP-C, Mif2, directly interacts with Iml3-Chl4 dimers, the counterpart of the CENP-N-L complex (Hinshaw and Harrison 2013), a member of the CENP-H group. Therefore, to test this connection, I focused on the CENP-N-L complex. The 166–224 aa region of chicken CENP-C is relatively conserved as shown in Figure 12A.

I first expressed and purified a complex of full-length CENP-L and the C-terminal domain of CENP-N fused with the maltose binding protein MBP (MBP- CENP-N^{ct}-L) (Figure 12B). I also prepared recombinant CENP-$C^{166–224}$ or CENP-$C^{166–324}$ fused with MBP and His (MBP-CENP-$C^{166–224}$-His or MBP-CENP-$C^{166–324}$-His) (Figure 12B).

To test for direct interaction of CENP-C with the CENP-N-L complex, I immobilized MBP-CENP-$C^{166–324}$-His on Ni-sepharose beads and used it as bait to pull down MBP-CENP-N^{ct}-L. I observed reproducible pull-down of MBP-CENP-N^{ct}-L (Figure 12C). I also observed that MBP-CENP-N^{ct}-L-His interacted with MBP-CENP-$C^{166–324}$. I also tested for interactions between the CENP-N-L complex and other domains of CENP-C, and found that other domains, including the C-terminus of CENP-C, did not bind the CENP-N-L complex (Figure 12C).

To verify the interaction and identify the residues responsible for it, my collaborators performed NMR experiments with ^{13}C and ^{15}N labelled CENP-$C^{166–324}$ and found that it was disordered alone. On addition of unlabeled CENP-N^{ct}-L, NMR peaks for various residues of CENP-$C^{166-324}$ were changes (moved, increased in intensity, or disappeared). As CENP-$C^{166–324}$ was slightly aggregated in the NMR sample tube, my collaborators repeated the experiment using CENP-$C^{166–224}$, which showed a much clearer NMR signal as well as clear variations in the peaks on addition of unlabeled CENP-N^{ct}-L, indicating a clear interaction between CENP-$C^{166–224}$ and CENP-N^{ct}-L (Figure 13A). I have highlighted some of the CENP-$C^{166-224}$ residues that showed shifted or abolished peaks when complexed with CENP-N^{ct}-L in Figure 13A and specified which residues where moved, disappeared, or whose signals where increased in Figure 13B.
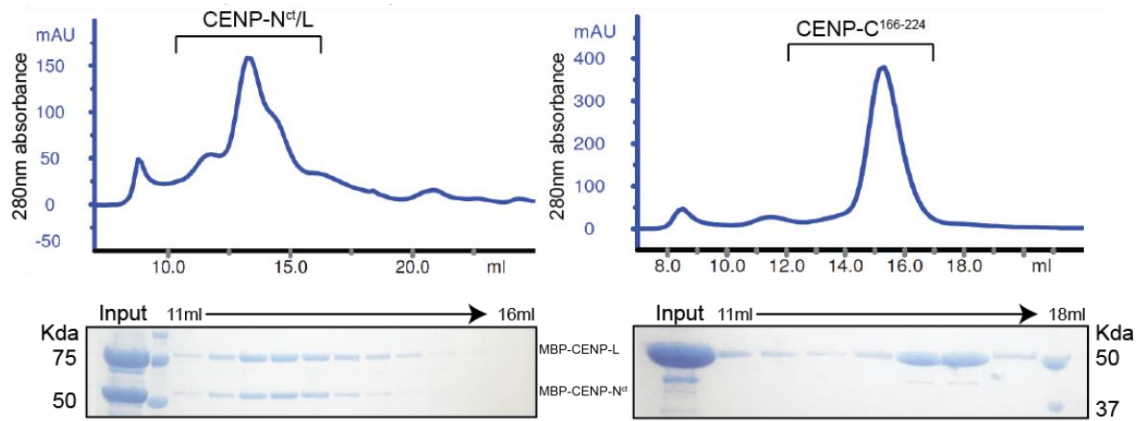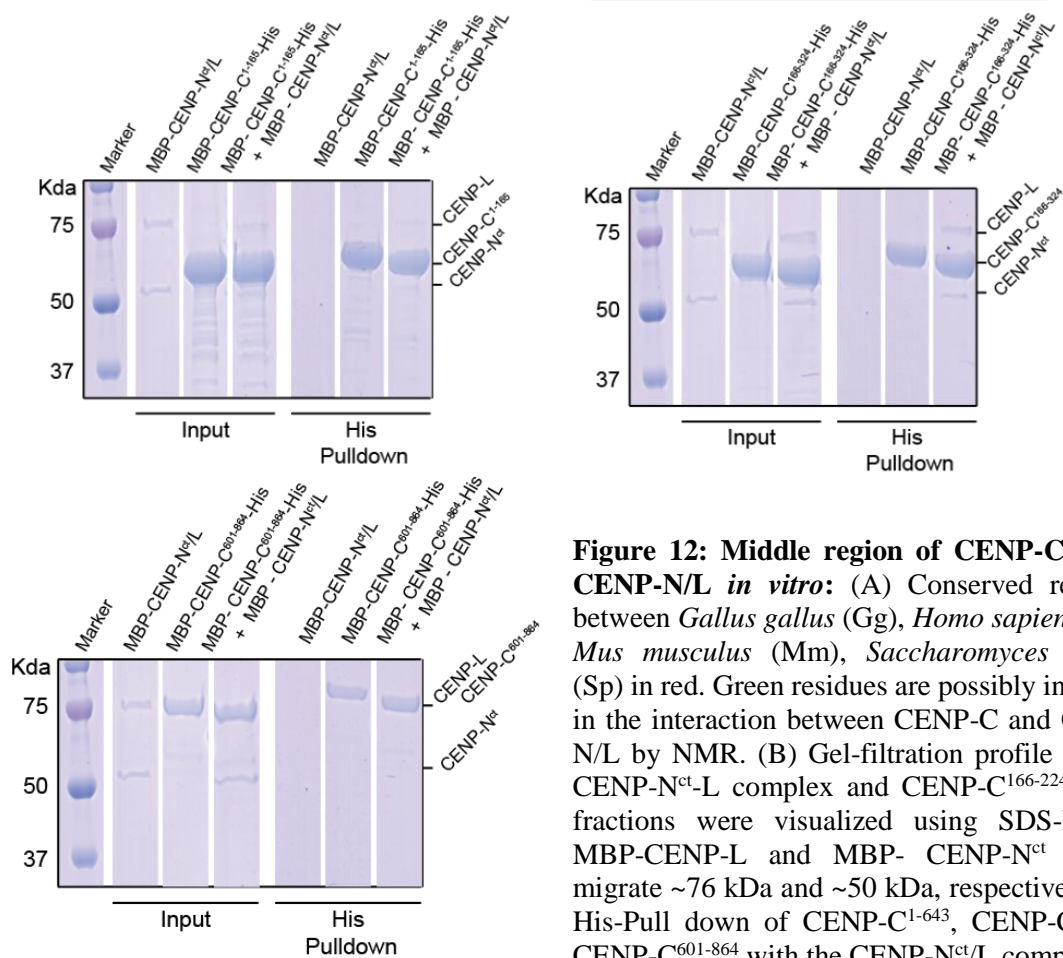
Figure 12: Middle region of CENP-C binds CENP-N/L *in vitro*: (A) Conserved residues between *Gallus gallus* (Gg), *Homo sapiens* (Hs), *Mus musculus* (Mm), *Saccharomyces pombe* (Sp) in red. Green residues are possibly involved in the interaction between CENP-C and CENP-N/L by NMR. (B) Gel-filtration profile for the CENP-N[ct]-L complex and CENP-C[166-224]. Peak fractions were visualized using SDS-PAGE. MBP-CENP-L and MBP-CENP-N[ct] should migrate ~76 kDa and ~50 kDa, respectively. (C) His-Pull down of CENP-C[1-643], CENP-C[166-324], CENP-C[601-864] with the CENP-N[ct]/L complex.

A
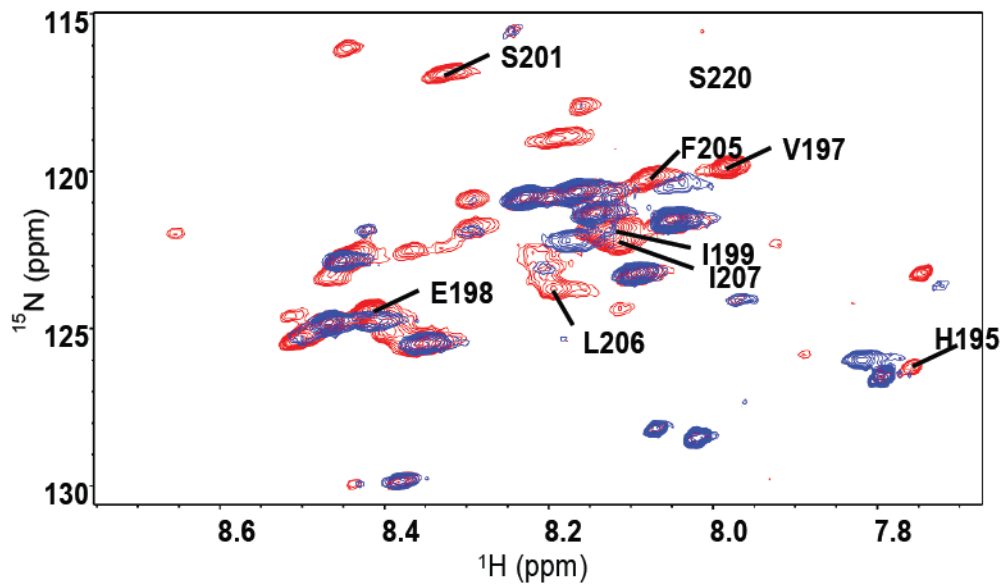
B

Residues where signal disappeared on addition of CENP-N^{ct}/L

GRSPLLVEEA KTPVQTRTVS IAPSSPSPVK DHNVEIESDC EFLIDESGCL
SFKSWFSIPR PHHHHHH

Residues where signal shifted on addition of CENP-N^{ct}/L

GRSPLLVEEA KTPVQTRTVS IAPSSPSPVK DHNVEIESDC EFLIDESGCL
SFKSWFSIPR PHHHHHH

Residues where signal strength increased on addition of CENP-N^{ct}/L

GRSPLLVEEA KTPVQTRTVS IAPSSPSPVK DHNVEIESDC EFLIDESGCL
SFKSWFSIPR PHHHHHH

Figure 13: 3-D NMR shows direct interaction between CENP-C$^{166-224}$ and CENP-N^{ct}-L: (A) ^{1}H-^{15}N HSQC spectrum of free CENP-C$^{166-224}$ (red) overlaid with that of CENP-C$^{166-224}$ in complex with CENP-N^{ct}-L (blue). Residues that showed shifted or abolished peaks are zoomed in and labeled. (B) Details of changed residues seen between free and bound CENP-C$^{166-224}$ by NMR. For e.g. peak for H195 highlighted in blue can be seen in fig 13A at (7.8, 126) in red (i.e. free CENP-C$^{166-224}$) but not in blue (complexed with CENP-N^{ct}-L), whereas un highlighted I199 can be seen in fig 13A at (8.15,122) with an overlaid blue and red signal showing no change.
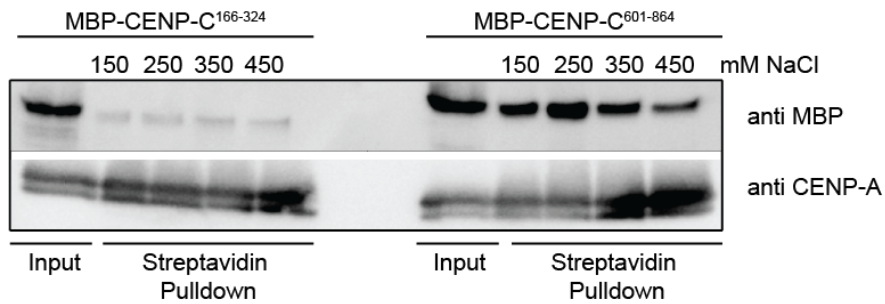
Figure 14: C-terminal of CENP-C binds CENP-A directly: Western blot of streptavidin pull down for CENP-A nucleosomes as per model shown. Pull down was performed at the concentrations indicated.
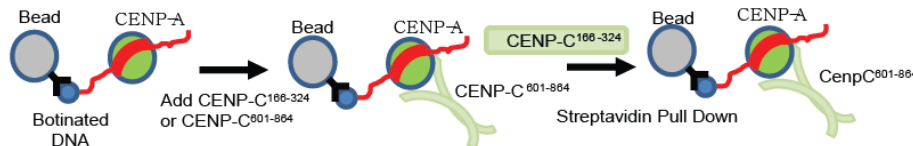
Human CENP-C binds CENP-A (Kato et al. 2013; Falk et al. 2015; Falk et al. 2016) , and our lab have previously demonstrated that the C-terminal dimerization domain can recruit CENP-A to a non-centromeric locus through LacO-LacI experiments (Hori et al. 2013). In addition, my microscopic observation suggested that mitotic centromeric localization of CENP-C$^{601-864}$ depends on CENP-A (Figure 10). I directly tested whether CENP-C fragments bind to reconstituted CENP-A nucleosomes *in vitro*. I prepared a biotinylated CENP-A nucleosome with MBP-fused CENP-C$^{166-324}$ or CENP-C$^{601-864}$ and performed a pull-down experiment using streptavidin beads. As shown in Figure 14, CENP-C$^{601-864}$ bound the CENP-A nucleosome, but CENP-C$^{166-324}$ did not

Based on biochemical and NMR analyses, I can conclude that the middle portion of CENP-C directly binds the CENP-L-N complex and that the C-terminus of CENP-C, directly binds the CENP-A nucleosome.

2.6   Loss of CENP-H recruitment in CENP-C$^{\Delta166\text{-}224}$ results in loss of CENP-O at the centromere

Our lab has shown that the CENP-O group localizes to centromeres downstream of the CENP-H group (Section 1.5, Figure 5) (Hori et al. 2008b; Eskat et al. 2012). DT40 cells with knockout of the CENP-O group are viable; however, they have reduced growth rates (Hori et al. 2008b). In contrast, deficiency of CENP-U (a member of the CENP-O group) causes cell death in mouse ES cells, which indicates abnormal mitotic behavior (Kagawa et al. 2014). The precise function of the CENP-O group is not well understood, but it has been shown to play a role in recovery from spindle damage in chicken DT40 cells (Hori et al. 2008b). This activity requires PLK1 phosphorylation of CENP-U (Kang et al. 2011; Park et al. 2015). It has also been speculated that the CENP-O group plays a role in molecular control of kinetochore-microtubule dynamics and chromosome oscillations (Amaro et al. 2010). Since the CENP-O group is downstream of the CENP-H group, I speculated that if the CENP-C middle region was responsible for recruiting CENP-N-L and thus the CENP-H group it would also be required to recruit the CENP-O group.

To confirm this I expressed CENP-C$^{\Delta166\text{-}224}$ in CENP-C Knockout cells and found an ~80% reduction in both CENP-O and CENP-U which was consistent with CENP-H knockout cells (Hori et al. 2008b) (Figure 15)
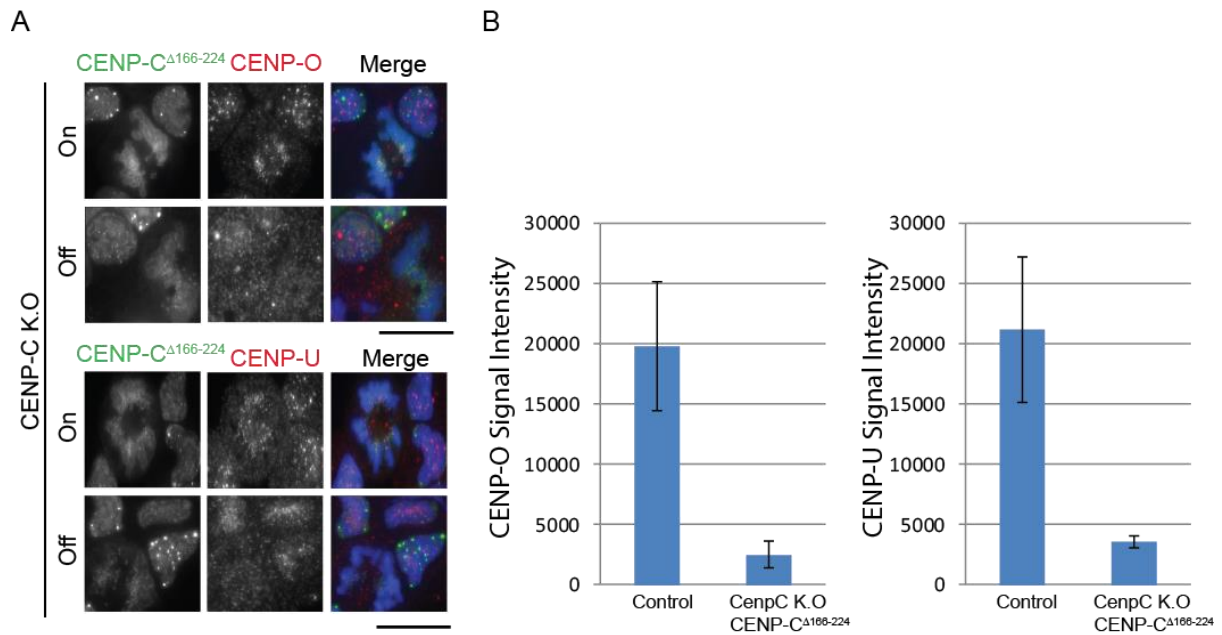
Figure 15: CENP-C$^{\Delta 166\text{-}224}$ shows loss of CENP-O and CENP-U at centromere: (A) Representative images showing loss of CENP-O and CENP-U on CENP-C depletion (36 hrs after addition of Tetracycline). (B) Quantification of loss of CENP-O and CENP-U on CENP-C depletion. Results were plotted as an average of the signal intensities of 20 kinetochores per cell (n > 7 cells). Error bars indicate standard deviation (S.D.).

2.7   Conclusion and Discussion

In recent years, multiple centromeric proteins have been identified (Cheeseman and Desai 2008; Fukagawa and Earnshaw 2014). This still left an important question, namely how these proteins are organized to form a functional kinetochore. It has been shown that there are two distinct pathways (CENP-C and CENP-T pathways) (Hori et al. 2013) for binding of the Ndc80 complex, which is a crucial microtubule protein complex in kinetochores, it remains unclear how these two pathways are organized in centromeric chromatin. In particular, the relationship between the CENP H-I-K-L-M-N group and CENP-C was unclear. Our lab has previously shown that the localization dependency of CENP-C on the CENP H-I-K-L-M-N

group appears to vary during the progression of the cell cycle (Kwon et al. 2007), the mechanism behind this dependency remained unclear.

I showed that the middle portion of chicken CENP-C (CENP-C$^{166-324}$) is sufficient for centromeric localization in interphase through association with CENP-N-L. The C-terminus of CENP-C (CENP-C$^{601-864}$) is essential for centromeric localization during mitosis, through binding to the CENP-A nucleosomes. Based on these findings and other recent reports, I propose a model for CCAN organization during progression of the cell cycle (Figure 16). In interphase, the N-terminus of CENP-N preferentially binds to CENP-A nucleosomes (Fang et al. 2015) and the complex of the CENP-N C-terminus (CENP-N^{ct}) and CENP-L bind to the middle portion of CENP-C (Figure 12). In interphase, the CENP-C–CENP-A interaction must be weak, because centromeric localization of CENP-C during interphase depends on CENP-N, CENP-H, and CENP-K, and because the C-terminus of CENP-C does not localize to interphase centromeres (Figure 8). However, during mitosis CENP-C binds to CENP-A nucleosomes through its C-terminal dimerization domain. Because the middle portion of CENP-C does not efficiently localize to mitotic centromeres, the middle portion is not required for mitotic CENP-C localization. At mitotic phase, CENP-N may not associate with CENP-A nucleosomes, because it does not bind to condensed chromatin (Fang et al. 2015). In fact, live-cell imaging of CENP-N suggested that CENP-N levels at mitotic kinetochores were low (Hellwig et al. 2011). In addition, CENP-N knockout does not cause a CENP-C reduction during mitosis, because CENP-C tightly binds CENP-A nucleosomes, consistent with our previous observation (Kwon et al. 2007). Recent work also shows that CENP-N and CENP-C co-operate to stabilize the CENP-A nucleosome and enhance binding to the centromeric DNA (Guo et al. 2017)
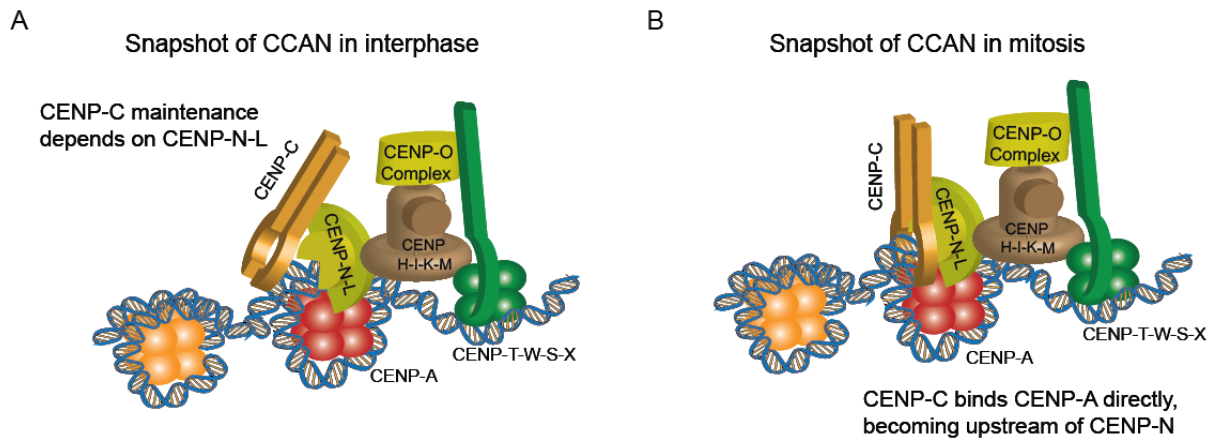
Figure 16: Model of cell cycle dependent modifications to CENP-C centromere binding: A model showing cell cycle-dependent kinetochore organization. (A) In interphase, the N-terminus of CENP-N preferentially binds to CENP-A nucleosomes and the C-terminus of CENP-N and CENP-L bind to the middle portion of CENP-C. The CENP-C–CENP-A interaction must be weak during interphase. (B) In contrast, CENP-C binds to CENP-A nucleosomes through its C-terminal dimerization domain during mitosis. CENP-N may not associate with CENP-A nucleosomes, because CENP-L does not bind to compact chromatin. CENP-N may also stabilize the binding of CENP-C to CENP-A during mitosis (Guo et al. 2017).

In this model, I propose that CENP-T associates with centromeric DNA, but this association must be facilitated by the CENP-H group, because CENP-H knockout causes reduction of CENP-T (Hori et al. 2008a) (Figure 10). Recent reports proposed that CENP-C is upstream of other CCAN proteins in human cells (Basilico et al. 2014; Klare et al. 2015). I propose a model, in which CENP-C may largely contribute to localization of the CENP-H group to the mitotic centromere through association of CENP-C to CENP-A (Figure 16). In addition, although I did not demonstrate CENP-H-CENP-C interactions using chicken proteins, it is possible that in addition to CENP-N-L, CENP-H-I-K-M may directly bind to CENP-C, as is suggested with human proteins (Klare et al. 2015; McKinley et al. 2015). Klare et al. demonstrated that mutation of hydrophobic residues of human CENP-C to Alanine (3A mutation), which correspond to responsive residues of chicken CENP-C for binding to CENP-

N-L based on our NMR experiments, caused disruption of the interaction with the CENP-H complex. However, I observed ~60% CENP-H at mitotic chromosomes in CENP-C-deficient chicken DT40 cells (Kwon et al. 2007; Hori et al. 2008a). Thus, as CENP-T and CENP-H are unaffected by CENP-C knockout (Hori et al. 2008a), I suggest that chromatin association of CENP-T also contributes to CCAN assembly in chicken cells and that CENP-C deficiency does not cause a strong reduction in CENP-H proteins through the contribution of CENP-T.

I still do not know why the previously mentioned protein dependency between the CCAN members are slightly different between chicken and human cells. For example, I demonstrate that the second CENP-A binding domain of CENP-C (aa 444–537 in human CENP-C, which correspond chicken CENP-C aa 325–643) is not essential in chicken cells, but this region is required for human cells (Kato et al. 2013; Falk et al. 2015; Falk et al. 2016; Guo et al. 2017) . Of course, I cannot conclude that chicken and human systems are entirely conserved without completing all analyses using both chicken and human cells. However, I believe that essential architecture of kinetochore is similar between chicken and human cells.

Our model suggests that, during interphase, CENP-C localization occurs downstream of CENP-H-I-K-L-M-N group but upstream of CENP-H-I-K-L-M-N group in metaphase, which is inconsistent with the model that CENP-C is upstream of other CCAN proteins throughout the cell cycle (Basilico et al. 2014; Klare et al. 2015). More recent study of CENP-C dependency through the progression of the cell-cycle in human cells shows a similar dependency between CENP-C and the CENP-H group (McKinley et al. 2015).Thus, the architecture of CCAN organization must be altered during progression of the cell cycle in human cells. I propose that these dynamic changes in CCAN organization are essential for formation of a plastic kinetochore structure.

2.8   Experimental Procedure

Molecular Biology, Cell Culture and Transfections

For expression of CENP-C deletion mutants in DT40 cells, Ecogpt gene or Blasticidin S resistance cassette under control of the chicken β-actin promoter was inserted as selection marker. GFP-CENP-C$^{1-643}$, GFP-CENP-C$^{601-864}$, GFP-CENP-C$^{\Delta325-643}$, GFP-CENP-C$^{\Delta166-224}$, and GFP-CENP-C$^{\Delta1-324}$ were cloned in pEGFP vectors. GFP-CENP-C$^{166-324}$-LacI was cloned into pKG194 vector (in house pEGFP based vector with LacI added to C-terminal of MCS (Hori et al. 2013)). A Gene Pulser II electroporator (Bio-Rad) was used for all transfections into DT40 cells. All molecular biology experiments, including Southern and Western Blot analyses, were followed by standard methods. All Chicken DT40 cells were cultured at 38.5°C in Dulbecco`s modified medium supplemented with 10% fetal bovine serum, 1% chicken Serum, β-mercapto-ethanol, penicillin and streptomycin.

Immunofluorescence

Chicken DT40 cells were prepared by the cytospin-method and fixed in 3% paraformaldehyde for 10 min at room temperature. Immunofluorescent staining of DT40 cells was performed using anti-CENP-T (a rabbit antibody against recombinant full-length chicken CENP-T), anti-CENP-C (a rabbit antibody against recombinant chicken CENP-C 1-330 aa), anti-CENP-H (a rabbit antibody against recombinant chicken CENP-H). Several cell lines expressing GFP or mRFP fusions were also used to examine protein localization. Images were

collected with a cooled-coupled device camera (Cool Snap HQ, Roper Scientific) mounted on an inverted microscope (IX71, Olympus) with a 100 X objective lens together with a filter wheel. All subsequent analysis and processing of images were performed using MetaMorph software (Molecular Devices). High-resolution images were also collected using a confocal scanner box (Cell Voyager CV1000, Yokogawa) with an oil immersion objective lens (100 X). Laser sources where 405nm, 488nm, 561nm. Images where passed through appropriate emission filters (BP447/60, BP525/50, BP617/73) and collected using a back-illuminated EMCCD (Yokogawa).

Protein Preparation

Chicken MBP-CENP-C$^{1-165}$-His, MBP-CENP-C$^{166-324}$-His, MBP-CENP-C$^{166-224}$-His, MBP-CENP-C$^{601-864}$-His fragments and chicken MBP-CENP-N^{ct}/L were cloned into pMal co-expression vector. Proteins were expressed in BL21(DE3)Star-pRARE2LysS by addition of 0.2 mM IPTG for 12 hrs. at 16°C. The Proteins were purified using Ni-Sepharose, Glutathione Sepharose, and Amylose resin and a Superdex 200 column. Isotope-labeled proteins were prepared by growing cultures in M9 media containing ^{15}N ammonium chloride and ^{15}N ammonium sulphate (Cambridge Isotope Laboratory) and expressed by addition of 0.2 mM IPTG for 14 hrs. at 16°C. Cell pellets were re-suspended in Lysis Buffer (25 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 µg/ml Lysozymes) and lyzed by sonication. The samples were cleared by centrifugation at 18,000 rpm at 4°C for 30 min. The cleared supernatant was filtered through a 0.4 µm filter to remove residual debris and large aggregates. The lysate was then passed through either a 5ml His-Trap FF (GE Healthcare) or 5 ml MBP-Trap (GE Healthcare) pre-equilibrated with His-running buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 20

53

mM Imidazole) and MBP-running buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT) respectively depending on requirement. The samples were eluted in appropriate running buffer supplemented with 500 mM Imidazole or 10 mM Maltose. All protein fractions were concentrated and dialyzed into MBP-running buffer and loaded onto a Superdex 200 10/300 or HiLoad Superdex 200 16/600 pre- equilibrated with MBP-running buffer. Fractions containing protein were concentrated and stored at 4°C. For NMR samples proteins were treated with TEV protease to cleave MBP which was removed by passing through a Ni-Sepharose column. For smTIRF experiments, all samples were used within 1 week from purification.

His affinity pull-down assay

Purified MBP-CENP-C$^{1-165}$-His, MBP-CENP-C$^{166-324}$-His, MBP-CENP-C$^{166-224}$-His or MBP-CENP-C$^{601-864}$-His fragments and MBP-CENP-N^{ct}-L were mixed in 1:1 molar ratio and incubated at 4°C for 2 hrs. in wash buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 20 mM Imidazole). The mixture of each CENP-C fragment and CENP-N^{ct}-L were added to 30 μl Ni-Affinity beads (Roche) and made up to 100 μl with wash buffer and incubated for 1 hrs. at 4°C with rotating. Beads were washed 3 times with 1ml wash buffer and then eluted with 40 μl elution buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 500 mM Imidazole). The eluate was collected and boiled with SDS sample Buffer and run on 10-20% SDS-PAGE gel. Bands were visualized with coomassie brilliant blue staining.

NMR analysis

NMR experiments were performed on AVANCE DRX600 (Bruker BioSpin) at 298 K using samples dissolved in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM DTT, and 5% D_2O. Sequential assignments of the backbone ^1H, ^{13}C and ^{15}N chemical shifts of CENP-C$^{166-224}$ were obtained from the standard triple-resonance NMR spectra HNCO, HN(CA)CO, HNCA, HN(CO)CA, CBCACONH, and HNCACB (Ikura et al., 1990). Data were processed and analyzed using the program NMRPipe (Delaglio et al., 1995) and the program Kujira (Kobayashi et al., 2007). For the chemical shift perturbation experiment, CENP-N^{ct-}L was added to the CENP-C$^{166-224}$ at the protein ratio of 1:1 and a ^1H–^{15}N HSQC spectrum of the complex was measured.

CENP-A Streptavidin pull-down assay

Dynabeads M280 Streptavidin (Invitrogen) were washed in the nucleosome binding buffer (20 mM Tris-HCl ph 7.5, 1 mM DTT, 250 mM KCl). CENP-A nucleosomes were prepared as described previously (Tachiwana et al. 2015). 100 μl of biotinylated CENP-A nucleosome (0.1 μg/μl) was added to 100 μl of washed beads and incubated for 2 hrs. at 4°C while rotating. Beads were washed 3 times in binding buffer (20 mM HEPES, 20% glycerol, 1 mM DTT, .2 mM EDTA, 150-450 mM NaCl). 30 μl of beads were incubated with purified MBP-CENP-C$^{166-324}$-His or MBP-CENP-C$^{601-864}$-His for 1 hrs. at 4°C with rotating. The supernatant was removed and beads were washed 3 times with binding buffer. Beads were subsequently boiled in SDS sample Buffer and run on 10-20% SDS-PAGE gel. The gel was transferred to a PVDF membrane using a Semi-Dry transfer system (Bio-Rad). The bands were visualized by Western blot analysis with anti-MBP antibody (NEB) and anti-CENP-A antibody.

Chapter 3

Regulation of the outer kinetochore-microtubule binding by the inner kinetochore

Abstract

The kinetochore uses large protein sub-complexes to form the load bearing structure necessary for microtubule attachment. The 16-subunit protein complex known as the constitutive centromere associated network (CCAN) forms the foundation for kinetochore assembly on the centromeric chromatin. The outer kinetochore acts as the linker connecting the microtubules to the inner chromatin binding elements in the kinetochore. It remains unclear whether the inner kinetochore acts only as a platform for recruiting the outer kinetochore or whether it influences kinetochore microtubule attachments. To address this question, I show that the outer kinetochore Mis12 complex is insufficient to form a functional kinetochore. Using a single molecule approach, I demonstrate that the CENP-C and CENP-T pathways are both able to enhance the microtubule binding of the Ndc80 complex by five-fold. Thus, I show that a mechanism exists to regulate outer kinetochore microtubule binding through the CCAN.

3.1 Introduction

In chapter 2, I showed that the inner kinetochore (CCAN proteins) components are interdependent in their recruitment to the kinetochore and not simply recruited by a hierarchy. Once the inner kinetochore is established (section 1-5,6,7) it would act as the platform for recruitment of the outer kinetochore. The outer kinetochore directly connects to the microtubules. Given the interdependent nature of the kinetochore organization, I speculated that the inner kinetochore may play a role beyond only acting as a platform for assembly of the outer kinetochore. In fact, given the highly interactive meshwork within the kinetochore, many weak and transient interactions may exist and such highly dynamic interactions may not be detectable in solution. However, more specialized techniques may reveal such interactions in the formation of a functional kinetochore. In fact, previous studies using outer kinetochore proteins have shown that the Mis12 complex and the Dam1 complex in yeast can enhance binding of the Ndc80 complex to microtubules (Cheeseman et al. 2006; Kudalkar et al. 2015). I tried to address whether the inner kinetochore was able to cause a similar effect.

I focused on the role of the Mis12 complex (which is recruited by CENP-C), and CENP-T on stabilizing the interaction between the Ndc80 complex and microtubules. I show that members of the chicken Mis12 complex do not induce formation of an artificial kinetochore when localized to an ectopic site suggesting that inner kinetochore components are required to form a functional kinetochore. In this chapter, I demonstrate that both the Mis12 complex and CENP-T can enhance the microtubule binding ability of the Ndc80 complex using single molecule total internal reflection microscopy (smTIRF), Finally, I propose that a mechanism exists to regulate the binding between the kinetochore and microtubules using inner kinetochore components.

3.2 Artificial tethering of Mis12 complex components do not induce an artificial kinetochore

Our lab has previously shown that CENP-C and CENP-T can induce formation of an artificial kinetochore when they are localized to an ectopic site on chromosomes whereas the Ndc80 complex which is the main microtubule binding complex does not induce an artificial kinetochore (Hori et al. 2013). This suggests that the presence of a microtubule binding protein at the centromere is insufficient to form a functional kinetochore. Previous works have also shown that the Mis12 complex is able to stabilize the interaction between Ndc80 and microtubules (Cheeseman et al. 2006; Kudalkar et al. 2015). I speculated that tethering the Mis12 complex using a LacO-LacI tethering system (Hori et al. 2013) might induce the formation of an artificial functional kinetochore at ectopic sites. As the original system was unable to recruit Mis12-LacI to the ectopic LacO array, I modified the tethering system using a GFP binding protein – LacI intermediate (GBP-LacI) to recruit GFP-Mis12 to the ectopic LacO site (Figure 17D). I found that the indicated GFP-Mis12 complex components could localize to the ectopic site using this system (Figure 17A).

Upon examining the survival rates of clones expressing the GFP-Mis12, GFP-NNF1, and GFP-DSN1 after removal of the endogenous centromere, I did not detect difference in the rates of theses clones as compared to control cells expressing GFP alone (Figure 17B). Immunostaining showed that none of the ectopically localized Mis12 components could recruit the full Mis12 complex (Figure 17C). Based on this analysis, the tested Mis12 complex components are insufficient for the formation of a functional kinetochore.
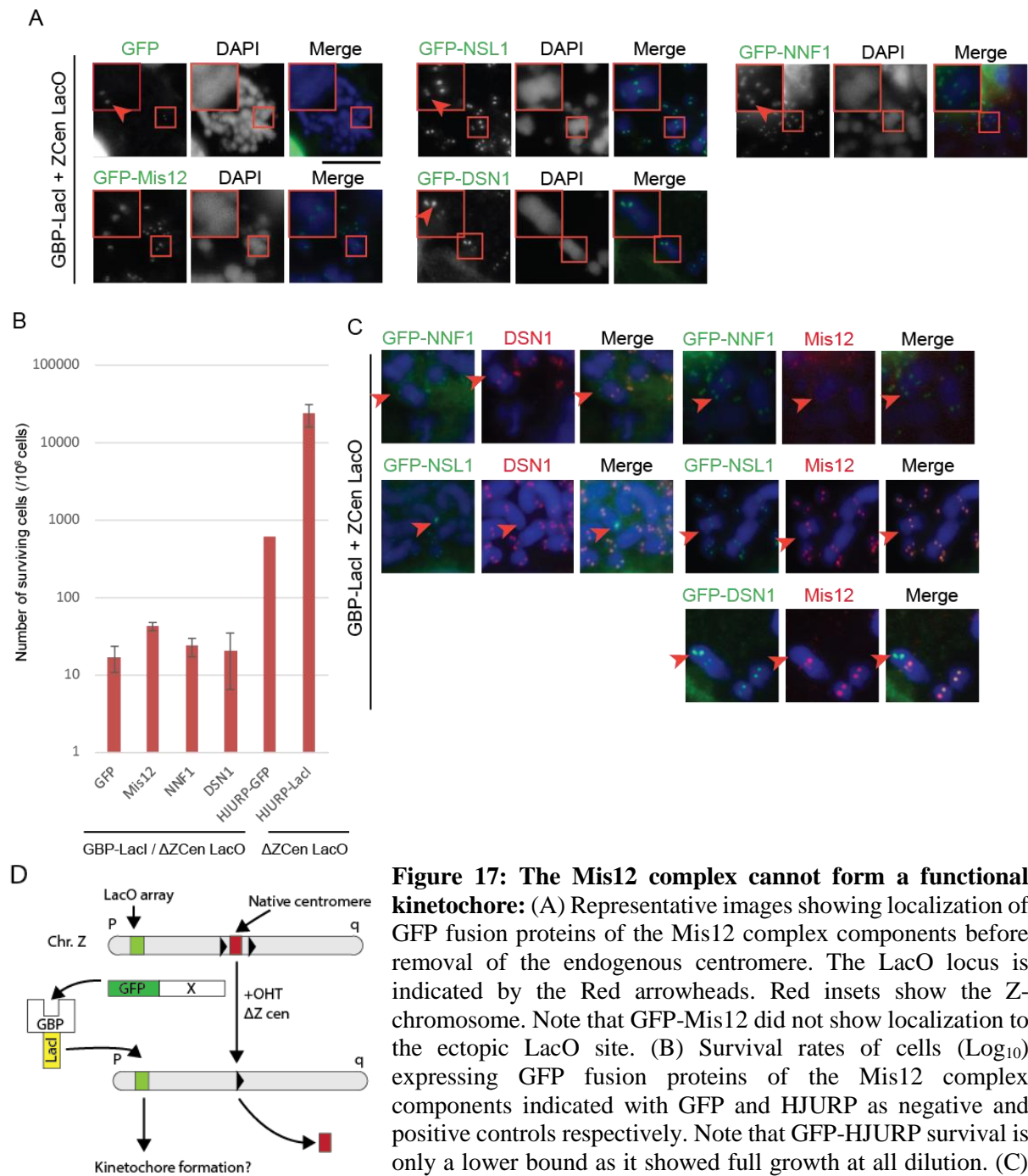
Figure 17: The Mis12 complex cannot form a functional kinetochore: (A) Representative images showing localization of GFP fusion proteins of the Mis12 complex components before removal of the endogenous centromere. The LacO locus is indicated by the Red arrowheads. Red insets show the Z-chromosome. Note that GFP-Mis12 did not show localization to the ectopic LacO site. (B) Survival rates of cells (Log$_{10}$) expressing GFP fusion proteins of the Mis12 complex components indicated with GFP and HJURP as negative and positive controls respectively. Note that GFP-HJURP survival is only a lower bound as it showed full growth at all dilution. (C) Representative images showing no formation of the full Mis12 complex with the GFP fusion proteins of the indicated Mis12 components (green) at the LacO array (red arrowheads). Other Mis12 components where visualized by immunostaining with the appropriate antibody (red). (D) Schematic showing the adapted experimental design to examine kinetochore formation at a neocentromeric locus after removal of endogenous centromere. Adapted from (Hori et al. 2013).

As neither the Ndc80 complex nor the Mis12 complex components (NNF1, Mis12, and DSN1) induce formation of a functional kinetochores unlike CENP-C or CENP-T, I hypothesized that the inner kinetochore may play a role in aiding the formation of the outer kinetochore as well as stabilizing kinetochore-microtubule attachments. However, it is still unknown whether there are regulatory factors or structural factors that facilitate recruitment of the outer kinetochore to form. This is an important question for future study.

3.3   The Ndc80^{bonsai} complex binds to single microtubules based on TIRF imaging.

To examine whether the inner kinetochore acts enhances Ndc80 binding to the microtubules, I decided to use single molecule analysis (Figure 18A). Single molecule TIRF microscopy allows us to study the kinetics of reaction in much more detail than is possible in bulk assays especially in cases of weak, dynamic or localized interactions. Due to the unstable nature of the full length Ndc80 complex, I used the Ndc80^{bonsai} complex (Ciferri et al. 2008) which lacks the coiled-coil region of the full length Ndc80, to study the interaction with microtubules. As the GFP tagged versions of the Ndc80^{bonsai} complex was unstable, I prepared a FITC labelled Ndc80^{bonsai} complex through Sortase mediated ligation of FITC to the c-terminal of Spc25 (a member of the Ndc80 complex) (Figure 18B).
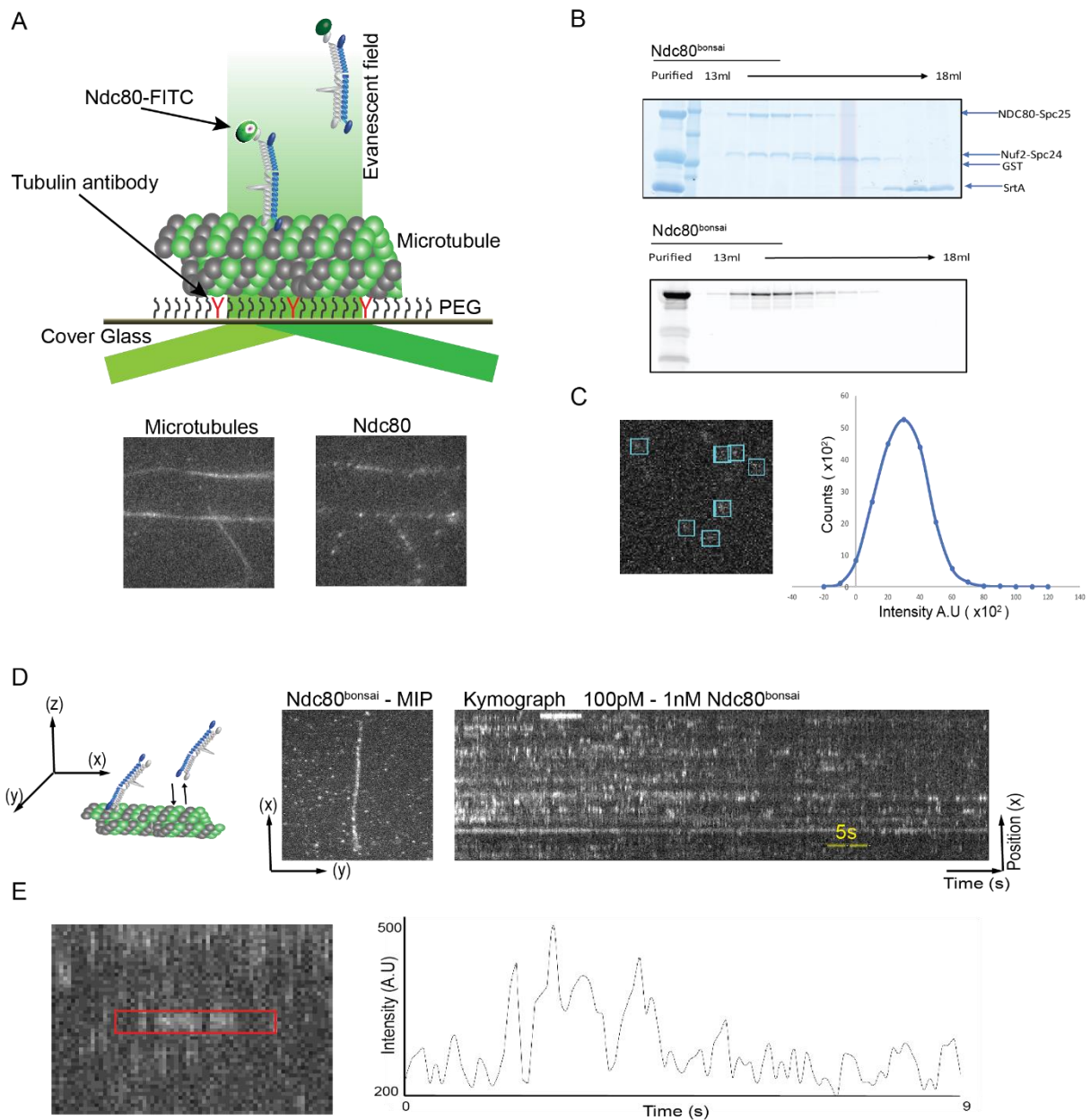
Figure 18: Ndc80^{bonsai} can bind microtubules *in vitro*: (A) Schematic diagram of the TIRF assay. PEGylation of the cover slip surface prevents non-specific adsorption to the surface. Hilyte-647 labelled microtubules are attached to the cover slips by anti-tubulin antibodies which are non-specifically adsorbed onto gaps in the PEGylation. Only Ndc80^{bonsai}-FITC in the illuminated area will show fluorescence (green). Representative images of microtubules and single FITC binding events. (B) SDS-Page showing purified Ndc80^{bonsai}-FITC (above) and the same gel under 488nm illumination showing FITC fluorescence (below). (C) Image of 20pM Ndc80^{bonsai} on non-PEGylated cover slips. Blue boxes show spots detected by image tracker. Graph shows distribution of background corrected integrated fluorescence values for detected spots. (D) Image of Ndc80^{bonsai} bound to microtubules over time by maximum image projection (MIP) of the image stack and as a kymograph (position on microtubule vs time) of the same microtubule. Axis show relative orientations (E) Representative image of a single binding event (highlighted inside the red box) and record of brightness vs time of the same event. Brightness of most particles was consistent over time.

The signal intensities of the Ndc80^{bonsai} complex suggested that the complex was mostly monomeric when adhered non-specifically to the cover slip (Figure18C). Subsequent imaging in the presence of Hilyte-647 labelled microtubules showed clear binding of the Ndc80^{bonsai} complex to microtubules with mostly short-lived binding events (Figure 18D, E).

3.4   Mis12 and CENP-T enhance binding of the Ndc80^{bonsai} complex to microtubules

Previous works using various model organisms have shown that both the Mis12 complex and CENP-T bind to the Ndc80 complex (Cheeseman et al. 2006; Screpanti et al. 2011; Malvezzi et al. 2013; Nishino et al. 2013; Kudalkar et al. 2015). I confirmed this interaction for my constructs (Figure 19A). Work using the yeast Mis12 complex has shown that it enhances binding of the Ndc80 complex to microtubules *in vitro* (Kudalkar et al. 2015). Given that the Mis12 complex forms a part of the CENP-C pathway, I tried to address whether CENP-T also enhances the interaction between Ndc80 and microtubules, and if so, whether its effect is similar to that of the Mis12 complex (CENP-C pathway).

As CENP-T binding to Ndc80 is controlled by CDK1 (Nishino et al. 2013), I used a phospho-mimetic version of CENP-T containing its microtubule binding domain (CENP-T$^{1-100}$ T84D, S100D). Both the Mis12 complex and CENP-T complexes associate with microtubules through Ndc80 at various concentration but not alone (Figure 19B). When they are diluted to concentrations where I can distinguish individual Ndc80^{bonsai}-FITC signals as seen in figure 18C. I detect single molecule binding events for these complexes to microtubules. I observed both long (>5s) and short (<5s) binding events (Figure 19C) unlike the Ndc80 complex alone which showed mainly short binding events (<5s) (Figure 18D).
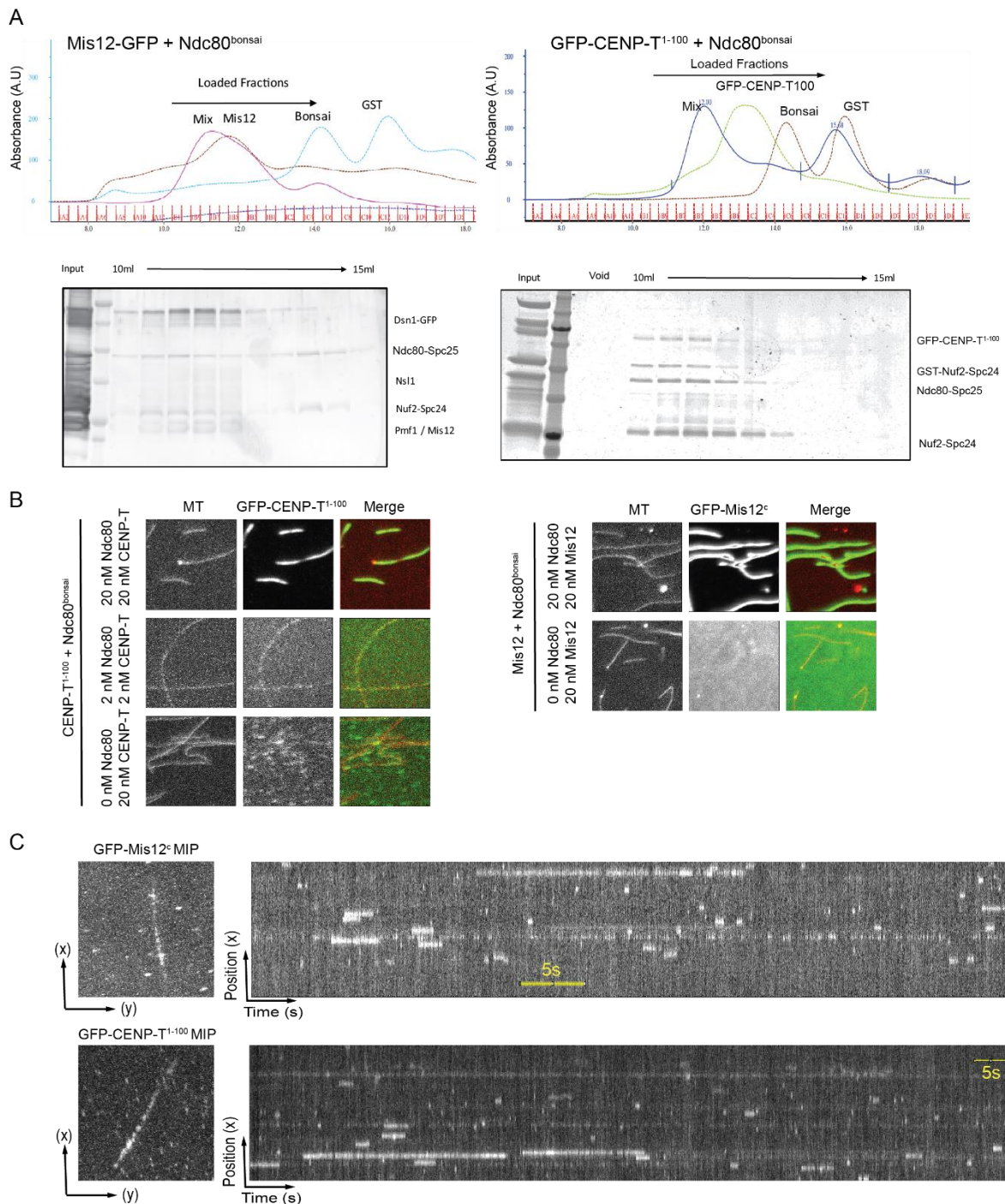
Figure 19: Mis12 and CENP-T$^{1-100}$ can localize to microtubules through Ndc80^{bonsai}: (A) SEC profile of Mis12-GFP and Ndc80^{bonsai} interaction (upper left), and GFP-CENP-T$^{1-100}$ and Ndc80^{bonsai} interaction (upper right). SDS PAGE showing interacting proteins with wells loaded as on the corresponding SEC profile. (B) Representative images of indicated GFP fusion proteins (green) binding to microtubules (red) in the presence and absence of Ndc80^{bonsai} at the indicated concentrations. Strong co-localization can be seen in the presence of Ndc80^{bonsai}. (C) Image of indicated GFP fusion protein and Ndc80^{bonsai} at <1nm concentration bound to microtubules over time by maximum image projection (MIP) and as a kymograph (position on microtubule vs time) of the same microtubule. A mix of long and short binding events can be seen.

Measurement of the Ndc80^{bonsai} complex binding events in BRB80 solution (120 mM K$^+$) showed mostly monomeric binding (Figure 18E) and were fitted to approximate first order decay (Figure 20B). The fitting function indicated a k$_{off}$ value of 1.042 $\pm$ 0.021 s^{-1} which corresponds to a mean residence time of 0.96 $\pm$ 0.02 s. This is consistent with previously reported values using the yeast Ndc80 complex (Powers et al. 2009) but is lower than those of the human full length Ndc80 complex (Umbreit et al. 2012). This is likely due to structural differences between the full length Ndc80 and Ndc80^{bonsai} complexes. I found the both Mis12 and CENP-T$^{1-100}$ tended to associate with the microtubules mostly as monomers through the Ndc80 complex, although a very small number of multimeric bindings where observed (Figure 20A). All multimeric events were discarded from the analysis as I could not differentiate between bleaching and unbinding events in these cases. I subsequently measured binding events for the Ndc80 complex + Mis12 complex and the Ndc80 complex + CENP-T$^{1-100}$ and found that they dramatically enhanced the microtubule binding of the Ndc80 complex. Both showed second order decay with two populations of particles showing slow and fast decay rates. I measured their respective disassociation rate constants by fitting a second order exponential curve resulting in k$_{off}$ values of 0.959 $\pm$ 0.12 s^{-1} (=k$_{off\ fast}$) and 0.142 $\pm$ 0.027 s^{-1} (=k$_{off\ slow}$) for Mis12 + Ndc80 and 0.814 $\pm$ 0.09 s^{-1} (=k$_{off\ fast}$) and 0.151 $\pm$ 0.024 s^{-1} (=k$_{off\ slow}$) for CENP-T$^{1-100}$ + Ndc80 (Figure 20B). Thus, while a population of both complexes shown similar residence times to the Ndc80 complex alone, I also saw a population showing a 6-fold increase in mean residence time vs Ndc80 (Figure 20C). This is likely due to different structural states within the complexes and with tubulin.

Interestingly, while both the Mis12 complex and CENP-T showed a substantial increase in mean residence time compared to the Ndc80 complex alone, both show very similar binding characteristics to each other. As CENP-T and Mis12 are also thought to bind competitively to

Ndc80 (Malvezzi et al. 2013; Nishino et al. 2013), I speculate that both might contribute to similar structural changes in the Ndc80 complex.

Figure 20: Mis12 and CENP-T[1-100] can enhance binding of Ndc80[bonsai] to microtubules: (A) Representative image of a single binding event (central white line in kymograph) and record of brightness vs time of the same event. Brightness of most particles was consistent over time. Blue arrows indicate an event with 3 GFP binding and each either bleaching or unbinding. All such events were not included in counts. (B) Survival probability vs time plotted for each complex measured from individual binding events (n=456 for Ndc80, n=171 for Ndc80 + Mis12, n=134 for Ndc80 + CENP-T[1-100]). Red dotted lines indicate exponential fits used to determine mean residence time (R^2>0.9). (C) Mean residence times calculated from fitted exponential functions. Both Mis12 and CENP-T[1-100] show a 2nd order function indicating 2 population having fast and slow turnover rates. Error bars show S.E.M

65

3.5   Conclusion and Discussion

The recruitment of the outer kinetochore is restricted to mitosis and is tightly regulated. While the effect of the inner kinetochore on this regulation has been well studied (Nishino et al. 2013; Rago et al. 2015; Huisin'T Veld et al. 2016), the role played by the CCAN in regulating outer kinetochore microtubule binding remains unclear. Of the two pathways (CENP-C and CENP-T) linking the inner and outer kinetochore, only CENP-T is a direct Ndc80 complex binding partner, whereas CENP-C binds the Ndc80 complex through the Mis12 complex. Recent studies have suggested that CENP-T may also recruit the Mis12 complex alongside the Ndc80 complex (Rago et al. 2015; Huisin'T Veld et al. 2016) but the role of this ternary complex is unknown.

By tethering the Mis12 complex components (NNF1, DSN1, and Mis12) to ectopic sites on the chromosome, I show that it is unable to form the full Mis12 complex and thus a functional artificial kinetochore. This is similar to Spc24 which while forming a full Ndc80 complex cannot form a functional artificial kinetochore even though it is the main microtubule binding component of the full kinetochore (Hori et al. 2013). Thus, I speculate that the CCAN is essential for the function of the outer kinetochore. A recent study has shown that the Mis12 complex in yeast is able to enhance the binding of microtubules to the Ndc80 complex (Kudalkar et al. 2015). I show that the human Mis12 complex is similarly able to enhance the binding of Ndc80 to microtubules. The Mis12–Ndc80 complex shows a 2-step decay which might be due to shifting between a weak and strong binding conformation of Ndc80 to the microtubule lattice. Structural studies show the binding region of Mis12 on Ndc80 to be quite far from the microtubule binding region of Ndc80 (Petrovic et al. 2016a). In addition, I show

66

that Mis12 is unable to bind microtubules on its own. This suggests that Mis12 causes a structural change in Ndc80 resulting in greater microtubule binding.

Single molecule analysis using CENP-T[1-100] also shows a similar enhancement of microtubule binding to the Ndc80 complex. Like Mis12, CENP-T is unable to bind microtubules directly. Intensity measurements and size exclusion chromatography show CENP-T and Ndc80 to bind each other in a 1:1 ratio. This is expected as this portion of CENP-T only contains one of the putative Ndc80 binding domains (Rago et al. 2015) and this binding region is suggested to bind Ndc80 competitively with the Mis12 complex (Malvezzi et al. 2013; Nishino et al. 2013). It likely induces similar structural changes to Ndc80 thus causing the similar enhanced microtubule binding of Ndc80. Using larger fragments of CENP-T and/or adding the Mis12 complex to form a ternary complex would be necessary to further establish the role played by CENP-T in stabilizing the interaction between Ndc80 and microtubules and whether the binding enhancement by CENP-T and Mis12 is additive as is the case with Mis12 and Dam1 (Kudalkar et al. 2015) or whether full length CENP-T recruit multiple Ndc80 as proposed by Huisin'T Veld et al. 2016 to the centromere.

These findings highlight a mechanism to control the binding of microtubules to the outer kinetochore by inner kinetochore components.

3.6   Experimental Procedure

Molecular Biology, Cell Culture and Transfections

For expression of Mis12 components in DT40 cells, Ecogpt gene or Blasticidin S resistance cassette under control of the chicken β-actin promoter was inserted as selection marker. Mis12 components were cloned into pKG194 vector (in house pEGFP based vector with LacI added to C-terminal of MCS(Hori et al. 2008a)). Mis12 components for GBP-LacI cell lines where cloned into pEGFP-N1 vector. A Gene Pulser II electroporator (Bio-Rad) was used for all transfections into DT40 cells. All molecular biology experiments, including Southern and Western Blot analyses, were followed by standard methods. All Chicken DT40 cells were cultured at 38.5°C in Dulbecco`s modified medium supplemented with 10% fetal bovine serum, 1% chicken Serum, β-mercapto-ethanol, penicillin and streptomycin. Ectopic tethering of Mis12-Laci fusion proteins was performed as previously described (Hori et al. 2013).

Immunofluorescence

Chicken DT40 cells were prepared by the cytospin-method and fixed in 3% paraformaldehyde for 10 min at room temperature. Immunofluorescent staining of DT40 cells was performed using anti-Mis12 (a rabbit antibody against recombinant full-length chicken Mis12), anti-DSN1 (a rabbit antibody against recombinant chicken DSN1). Several cell lines expressing GFP fusions were also used to examine protein localization. Images were collected with a cooled-coupled device camera (Cool Snap HQ, Roper Scientific) mounted on an inverted microscope (IX71, Olympus) with a 100 X objective lens together with a filter wheel.

All subsequent analysis and processing of images were performed using MetaMorph software (Molecular Devices).

Protein Preparation

NDC80^{bonsai} and NDC80^{bonsai}-LPETG were cloned into pGEX-2rbs vector (modified pGEX vector, (Ciferri et al. 2008)). Mis12 was cloned into pLC2000 (Cheeseman Lab). GFP-CENP-T$^{1-100}$ was cloned into pMal expression vector. Point mutations of GFP-CENP-T$^{1-100}$ (S84D, S100D) were made by PCR. Proteins were expressed in BL21(DE3)Star-pRARE2LysS by addition of 0.2 mM IPTG for 12 hrs. at 16°C. The Proteins were purified using Ni-Sepharose, Glutathione Sepharose, and a Superdex 200 column. Cell pellets were re-suspended in Lysis Buffer (25 mM Tris-HCl pH 7.5, 2 mM EDTA, 100 μg/ml Lysozymes) and lyzed by sonication. The samples were cleared by centrifugation at 18,000 rpm at 4°C for 30 min. The cleared supernatant was filtered through a 0.4 μm filter to remove residual debris and large aggregates. The lysate was then passed through either a 5ml His-Trap FF (GE Healthcare) or 1ml GST-Trap (GE Healthcare) pre-equilibrated with His-running buffer (10 mM Tris-HCl pH 7.5, 500 mM NaCl, 1 mM DTT, 20 mM Imidazole) and MBP/GST-running buffer (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT) respectively depending on requirement. The samples were eluted in appropriate running buffer supplemented with 500 mM Imidazole or 10mM reduced Glutathione. All protein fractions were concentrated and dialyzed into MBP-running buffer and loaded onto a Superdex 200 10/300 pre-equilibrated with MBP-running buffer. Fractions containing protein were concentrated and stored at 4°C. For smTIRF experiments, NDC80^{Bonsai} was treated with precission protease (GE) to cleave GST. All samples were used within 2 weeks from purification.

Flow Cell Preparation

The cover slips and slide glass used to prepare the flow cells were cleaned using 2% Micro-90 detergent (International Products Co.), milliQ water and 1N KOH each followed by 10 min sonication and water rinse and then dried using a nitrogen stream. They were subsequently amino silanated by incubating in VectaBond (Vecta Labs) for 5 min and washing in milliQ water and drying under a nitrogen stream. Dried cover slips were attached to slides using double sided tape to prepare two side by side chambers per slide. 10 µl mPEG-SVA 5000 (Layson bio) was flowed into each channel at 72.5 mg/ml or 36 mg/ml and incubated for 3 hrs. in a humidified chamber. Flow cells were finally washed in milliQ water, dried under a nitrogen stream and stored in vacuum.

Sortase Mediated Protein Ligation

Approximately 50 µM of Ndc80^{bonsai}-LPETG was mixed with 150 µM SrtA* (plasmid was a gift from Hidde Ploegh (addgene plasmid #51141)), 1mM GGGGK-FITC (Genescript), and 10U precission protease (GE). The reaction was incubated at 4°C for 16 hrs. in 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM DTT. The unligated peptide was removed on a Superdex 200 10/300 SEC (GE). Ligation was confirmed by running on an SDS page and imaging using a Typhoon FLA 9500 (GE).

smTIRF Microscopy

All proteins expressed were purified using size exclusion chromatography as described in section 2.3. Protein interactions were confirmed by mixing in 1:1 ratio and subsequent gel

filtration. Complexed fractions were collected and stored at 4°C and used within 2 weeks. Flow cells were prepared as in section 2.7. Stock HiLyte-647-labeled microtubules were polymerized from purified tubulin (Cytoskeleton, Denver, CO) in BRB80 buffer (80 mM PIPES/KOH, 1 mM EGTA, and 1 mM MgCl$_2$, pH 6.8) supplemented with 1 mM GTP at 37°C for 20 min. Polymerized microtubules were stored at room temperature after addition 200 µM Taxol making a final solution of BRB80 containing 20 µm Taxol and an additional 5 min incubation at 37°C. Microtubules were diluted to 200x dilution of the stock for smTIRF. To secure microtubules to the cover slips, 0.05 mg/ml anti-tubulin antibody (Sigma T9028) was flowed into the flow cells and allowed to adhere to the cover slip surface for 10 min and then washed out with BRB80 buffer (80 mM PIPES, 1 mM EGTA, 1 mM MgCl$_2$) supplemented with 0.5 mg/ml α-casein. Taxol-stabilized Hilyte-647 labelled microtubules were flowed in and allowed to bind for 10 min and washed out with BRB80 plus 20 µM Taxol. Finally, 100 pM-1 nM protein was flowed in in BRB80 containing 20 µM Taxol, 0.5 mg/ml α-casein and an oxygen scavenger (25 mM D-Glucose, 200 µg/ml glucose oxidase, 35 µg/ml catalase and 10 mM DTT). Flow chambers were then sealed using paraffin to limit exposure to atmospheric oxygen.

TIRF imaging was performed using an inverted microscope equipped with a handmaid prism-less TIR system (D. Axelrod et al., 2001). Laser sources for 488 nm and 561 nm excitation light were solid-state CW lasers (SAPPHIRE 488-20 and Compass 561-20, respectively; COHERENT). Lasers were guided to back focal plane of objective lens (CFI Apo TIRF 60X Oil, N.A. 1.49; Nikon) through back port of the microscope. TIR and EPI illumination were switched by tilting an incident angle of the lasers. The separated images were passed through dual band laser split filter sets (Di01-R488/561-25x36, Di02R561-25x36, FF01-525/45-25 and FF01-609/54-25; Semrock) and captured by two EM-CCD cameras

(iXon3 897; Andor) equipped with 4× intermediate magnification lenses (VM Lens C-4 ×; Nikon). Time-lapse images were obtained in 100 ms exposure for 2 min.

TIRF data analysis

TIRF data was analyzed using ImageJ plugins "Particle track and analysis" (PTA), TrackMate (Tinevez et al. 2016) and "Kymograph direct" (Mangeol et al. 2016). The software allows for tracking the position and brightness of individual fluorescent particles. Only particles with a residence time greater than 100 ms were used in calculating diffusion rates. Due to low signal to noise ratio and residence times of NDC80^{Bonsai}-FITC, Kymograph direct was unable to detect binding events so TrackMate was used exclusively to measure residence times by directly tracking fluorescent spots from raw images, PTA was used to measure particle intensities. For all GFP tagged proteins PTA was used to measure particle intensities and Kymograph direct was used to create kymographs for microtubule specific binding. Residence times were subsequently extracted from the kymographs using kymograph direct. k_{off} values were calculated by performing survival analysis from residence times using Prism 7 (GraphPad Software) to form cumulative histograms. These histograms were fitted to one step or two step exponential decay functions so as to maximize R^2 by non-linear regression.

Chapter 4

Discussion

The kinetochore has emerged as a truly elaborate structure, which plays a fundamental role in chromosome segregation. A large body of work conducted over the last 30 years has shed light on the various and numerous members of the kinetochore and on the interactions between them. In this thesis, I aimed to highlight the dynamic and interconnected nature of theses interactions and presents a picture of their effects throughout the cell cycle. However, there are still many questions, inconsistencies, and debates behind the basic mechanisms underlying kinetochore formation. In this chapter, I will discuss some of these questions and possible future directions in the field.

4.1   The kinetochore forms an intricate meshwork of interdependent relationships.

The kinetochore appears to have evolved multiple seemingly redundant yet highly interconnected pathways for its recruitment, maintenance, and function. This leaves the kinetochore with a great deal of robustness but has made elucidating the exact functioning of any one member a very difficult proposition. Most members on their own also seem to perform multiple roles at different cell cycle stages, resulting in cases where entire functional domains can be removed without any apparent affect to the kinetochore function yet most proteins themselves are indispensable. An example of this is can be seen in my work in section 3.1. CENP-C is known to be essential for kinetochore function and is directly downstream of CENP-A during mitosis (McKinley et al. 2015). Depletion of CENP-C by siRNA results in loss of many CCAN members in human cells (Basilico et al. 2014; Klare et al. 2015) by which one can assume CENP-C to be the key determinant of the kinetochore after CENP-A, but other work in both human (McKinley et al. 2015) and chicken (Chapter 2) show CENP-C to be dependent on the CENP-H group for interphase function. Similarly, CENP-N is also a CENP-A binding protein and has been shown to help fasten CENP-A to the DNA (Guo et al. 2017), and also to be involved in the interphase recruitment of CENP-C (McKinley and Cheeseman 2016) but appears downstream of CENP-C in mitosis (Klare et al. 2015). CENP-N is though, mainly found at the centromere during S-phase (Hellwig et al. 2011) possibly due to the inaccessibility of the CENP-N binding region of CENP-A during mitotic chromosome compaction as suggested by Fang et al 2015. This results in a need to change the recruitment hierarchy in the kinetochore where CENP-N is now maintained by CENP-C as CENP-C becomes tightly bound to CENP-A during mitosis (Falk et al. 2015; Guo et al. 2017). Working from the outer kinetochore side, deletion of the Mis12 binding region of CENP-C is on its own

viable, thus CENP-C may be more involved in a regulatory role (Suzuki et al. 2015). Similarly, a putative CENP-A binding site in chicken cells is also dispensable (Section 2.3). Thus the primary factors controlling CCAN recruitment remain unclear. Finding minimal functional version of CENP-C and CENP-N may provide some insight into which regions are the most essential for their functions.

A similar issue can be found for CENP-T-W-S-X which utilizes both direct DNA interactions with its histone fold domain (Nishino et al. 2012) and interactions with the CENP-H group to localize to the kinetochore (Hori et al. 2008a; Basilico et al. 2014; McKinley et al. 2015). The exact nature of these recruitments remains unknown, especially concerning the selectivity of CENP-T-W-S-X for DNA. It also remains unclear what role the CENP-H group plays in recruiting CENP-T as well as in conferring any DNA selectivity. This remains an important question for further study.

A third seemingly redundant aspect of the kinetochore is the two outer kinetochore recruitment pathways, namely the CENP-C and CENP-T pathways. Artificial tethering of CCAN components to ectopic sites on the chromosome show that either CENP-C or CENP-T alone are sufficient to recruit the outer kinetochore independently of the rest of the CCAN (Hori et al. 2013). Thus, it stands to reason that the recruitment of the outer kinetochore is an independent property of CENP-C and CENP-T. Interestingly, both CENP-T and CENP-C can recruit the entire outer kinetochore, even though CENP-T and Mis12 have been speculated to compete for Ndc80 (Malvezzi et al. 2013). This suggests that there exist more interaction sites on CENP-T that can recruit the Mis12 and Ndc80 complex. Recent work has possibly found such a site for recruiting Mis12 directly to CENP-T (Rago et al. 2015; Huisin'T Veld et al. 2016). This leads to many questions as to how these interactions are regulated.

My work on the effect of the Mis12 complex and CENP-T on the interaction between Ndc80 and microtubules show that at least initially both pathways i.e. CENP-T-Ndc80 and Mis12-Ndc80 work by increasing the affinity of Ndc80 for microtubules rather than by simply oligomerizing the outer kinetochore. This result is similar to work done using yeast proteins (Kudalkar et al. 2015). I also show that both pathways appear to increase the affinity of Ndc80 to microtubules to a similar extent, possibly as they confer the same conformational changes to Ndc80. Further work needs to be done using newly found interaction/regulatory sites on CENP-TY and larger *in vitro* complexes to fully study the role of the inner kinetochore on the microtubules.

4.2   How is the inner kinetochore regulated?

While a lot of work has been done to study the regulation of the outer kinetochore and the spindle assembly checkpoint (Foley and Kapoor 2012; Musacchio 2015; Etemad and Kops 2016), regulatory elements within the inner kinetochore remain poorly understood. For e.g. the results shown in Chapter 3 show that CENP-C can change its recruitment hierarchy from being dependent on the CENP-H group during interphase to being directly downstream of CENP-A during metaphase. Yet we do not know the regulatory factors responsible for this change. Some possible causes for these changes are phosphorylation of various CCAN components by Aurora B or the Cyclin dependent kinases. Other possibilities include epigenetic or structural changes to the CENP-A chromatin (Hori et al. 2014; Fang et al. 2015). Similar regulation of the CCAN is especially important for recruiting the outer kinetochore were regulation by Aurora B and CDK1 help recruit the outer kinetochore to CENP-C (Yang et al. 2008; Welburn

et al. 2010; Rago et al. 2015) and CENP-T (Nishino et al. 2013; Rago et al. 2015) respectively. There exist many unknown substrates for various mitotic kinases in the kinetochore complex and their discovery remains an important goal in the field.

4.3   Does the kinetochore form on a single or multiple CENP-A?

In *S. cerevisiae* the entire kinetochore is built onto a single CENP-A nucleosome (Furuyama and Biggins 2007). In organisms with regional centromeres, kinetochore recruitment becomes more complicated as rather than being DNA-sequence specific, centromeres recruit onto a region marked by CENP-A (a histone H3 variant). These regions usually have CENP-A interspersed between canonical H3 nucleosomes, yet it remains unknown how theses CENP-A are arranged (Fukagawa and Earnshaw 2014). My work suggest that the kinetochore maintains a network of interdependent interactions within the kinetochore and each of these interactions are built atop a CENP-A nucleosome, yet it remains unknown if each full kinetochore is required to exist on the same nucleosome as in *S. cerevisiae* or if the kinetochore straddles multiple CENP-A nucleosomes. In fact, as discussed in section 1.7 many regulatory mechanism appear to exist to ensure that the kinetochore is only recruited to areas of high CENP-A concentration either natural or by ectopic recruitment of CENP-A deposition factors (Hori et al. 2013). But this is not the case with single CENP-A nucleosomes where we do not see kinetochore recruitment (Gascoigne et al. 2011). It remains an interesting question how densely CENP-A needs to be distributed for kinetochores to form as well as how this question can be studied. The use of single molecule analysis of *in vitro* reconstituted CENP-A arrays seems like a novel technique to study such a question.

77

4.4   How do neocentromeres form?

Studies have shown that cells are able to induce formation of new centromeres to a small degree if the original centromere is lost (Shang et al. 2013). This can be due to centromeres forming on new sites on the same chromosome or by the fusing of two different chromosomes (Shang et al. 2013). CENP-A is also known to be deposited stochastically to random sites on the chromosome (Bodor et al. 2014) through the CENP-A cloud (Shang et al. 2013). This result especially has medical importance as the ability of ostensibly damaged cells to survive by acquiring new centromeres can lead to various cancers. In normal case the mechanism involved in preventing non-specific CENP-A deposition can prevent random kinetochore formation but damage to the CCAN can induce movement of the centromere (Hori et al. 2017). The formation of dicentric chromosomes also invariably leads to DNA damage. Thus, the study of the factors controlling CENP-A deposition and kinetochore licensing remain essential to the field.

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