CDK-dependent phosphorylation and nuclear exclusion coordinately control kinetochore assembly state

Karen E. Gascoigne1,2 and Iain M. Cheeseman1,2

1Whitehead Institute for Biomedical Research and 2Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02142

Abstract

Accurate chromosome segregation requires assembly of the multiprotein kinetochore complex. Prior work has identified more than 100 different kinetochore components in human cells. However, little is known about the regulatory processes that specify their assembly upon mitotic entry and disassembly at mitotic exit. In this paper, we used a live-cell imaging–based assay to quantify kinetochore disassembly kinetics and systematically analyze the role of potential regulatory mechanisms in controlling kinetochore assembly state. We find that kinetochore assembly and disassembly was driven primarily by mitotic phosphorylation downstream of cyclin-dependent kinase (CDK). In addition, we demonstrate that nuclear exclusion of the Ndc80 complex helped restrict kinetochore formation to mitosis. Combining constitutive CDK-dependent phosphorylation of CENP-T and forced nuclear localization of the Ndc80 complex partially prevented kinetochore disassembly at mitotic exit and led to chromosome segregation defects in subsequent divisions. In total, we find that the coordinated temporal regulation of outer kinetochore assembly is essential for accurate cell division.

Introduction

Chromosome segregation requires a physical attachment between centromere DNA and spindle microtubule polymers that is mediated by the multiprotein kinetochore (Cheeseman and Desai, 2008). A striking feature of the vertebrate kinetochore is its dramatic cell cycle–coupled reorganization. In vertebrates, a subset of DNA-proximal kinetochore proteins, termed the constitutive associated centromere network, is present at centromeres throughout the cell cycle to provide a platform for outer kinetochore assembly (Perpelescu and Fukagawa, 2011). However, the majority of the other ~100 different kinetochore components only associate with centromeres during mitosis (Gascoigne and Cheeseman, 2011). Recent work has defined two parallel pathways for outer kinetochore assembly via the constitutive centromere proteins CENP-C and CENP-T (Hori et al., 2008; Carroll et al., 2010; Gascoigne et al., 2011; Guse et al., 2011; Nishino et al., 2013). However, how these pathways are regulated to restrict outer kinetochore formation to mitosis and the consequences of uncoupling kinetochore assembly from cell cycle progression remain unknown.

We have previously proposed several mechanisms by which kinetochore assembly could be temporally regulated (Gascoigne and Cheeseman, 2011): (a) Changes in protein stability or targeted degradation, (b) changes in the physical structure or forces that act on kinetochores throughout the cell cycle, such as interactions with microtubules, (c) changes in the subcellular localization of kinetochore proteins, or (d) cell cycle–coupled posttranslational modifications of kinetochore proteins. To define the contributions of these potential regulatory mechanisms to timely kinetochore assembly, we performed a systematic analysis of kinetochore assembly and disassembly in human cells. Using a live-cell imaging–based assay, we quantified the assembly and disassembly kinetics of 10 representative kinetochore proteins and used this assay to systematically evaluate the aforementioned regulatory mechanisms. We find that protein phosphorylation downstream of CDK and controlled nuclear localization coordinately control kinetochore assembly state. Disrupting these regulatory pathways to partially trap mitotic kinetochore assembly leads to defects in subsequent cell divisions.

Correspondence to Iain M. Cheeseman: icheese@wi.mit.edu

Abbreviations used in this paper: APC/C, anaphase-promoting complex/cyclosome; CDK, CDK inhibitor; KMN, KNL1–Mis12 complex–Ndc80 complex; NEBD, nuclear envelope breakdown; Plk1, Plk1 inhibitor.

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Results and discussion

A live-cell assay to monitor kinetochore assembly dynamics

To probe the mechanisms that control kinetochore assembly state, we sought to monitor the kinetics of kinetochore assembly and disassembly in human cells. We first quantified the endogenous levels of representative kinetochore proteins during different cell cycle stages. For these experiments, we co-stained with anti-PCNA antibodies to define the specific cell cycle stage for interphase cells (Fig. 1 A). The constitutive associated centromere network proteins CENP-C, CENP-T, and CENP-I were present at centromeres throughout the cell cycle with levels peaking in G2 and mitosis, consistent with previous observations (McClendall et al., 2007; Eskat et al., 2012). In contrast, components of the KNL1–Mis12 complex–Ndc80 complex (KMN) network, which forms the core of the outer kinetochore–microtubule interface (Cheeseman et al., 2006), were absent from centromeres in G1. The Mis12 complex and KNL1 were recruited to centromeres during S phase, whereas the Ndc80 complex was recruited to kinetochores in late G2, consistent with previous fixed cell analysis (Cheeseman et al., 2008). All components of the KMN network disassembled from kinetochores at anaphase. The localization of the Ska1 complex mirrored that of Ndc80 (Fig. 1 A), consistent with their coordinated functions (Schmidt et al., 2012).

To understand the nature of these differing temporal localization patterns, we next developed a live-cell assay to monitor kinetochore protein localization with high temporal resolution. For this analysis, we generated clonal HeLa cell lines stably expressing GFP fusions for 10 representative inner and outer kinetochore proteins. We imaged each cell line and quantified GFP fluorescence at 2-min intervals starting 30 min before mitotic entry until 30 min after mitotic exit, during which the bulk of kinetochore assembly and disassembly occurs. All analyzed cell lines entered and exited mitosis with apparently normal kinetics during imaging. Using these imaging conditions, cells expressing GFP alone showed a fluorophore bleaching rate of ~5% per hour, which would not significantly affect the assays described here (see Materials and methods) because of the short duration of these experiments (Fig. S1 A).

Quantification of kinetochore-localized GFP signals during mitosis revealed a striking diversity in the assembly and disassembly kinetics of both inner and outer kinetochore proteins (Fig. 1, B–D). Consistent with previous work (Jansen et al., 2007), the centromeric histone H3 variant CENP-A displayed constant levels at kinetochores from mitotic entry to exit (Fig. 1, B and C). Surprisingly, the constitutive centromere proteins CENP-C, CENP-T, and CENP-H showed a distinct assembly and disassembly behavior during mitosis, with levels at kinetochores increasing 50% in the first 10 min after nuclear envelope breakdown (NEBD) and decreasing equivalently beginning 10 min after anaphase onset (Fig. 1, B and D). In contrast, CENP-N displayed inverse assembly kinetics, with levels decreasing by ~40% at NEBD and falling an additional 20% beginning 10 min after anaphase onset (Fig. 1, B and C). This is consistent with previous work indicating that CENP-N levels at centromeres are low during mitosis (McClendall et al., 2007; Hellwig et al., 2011). Thus, although these proteins are present at centromeres constitutively, their levels vary dramatically throughout the cell cycle.

We next analyzed the assembly and disassembly kinetics of the outer kinetochore KMN network. Consistent with our fixed cell analysis, the Mis12 complex components Dsn1 and Mis12 were detectable at centromeres before NEBD (Fig. 1, B and E). However, their levels were only ~30% of those after NEBD, indicating that their assembly occurs primarily upon mitotic entry. In contrast, the Ndc80 complex components Ndc80/Hecl and Spc25 were undetectable at centromeres until prophase. In each case, we observed identical assembly kinetics for distinct subunits of a stably associated complex. The disassembly of KMN network components from centromeres began 6 min before the first visible signs of anaphase chromosome movement, and the levels of all KMN network proteins were undetectable 20 min after anaphase onset (Fig. 1, B and E). The timing of KMN network disassembly precisely coincided with the recruitment of the CENP-A targeting factor Mis18-α to kinetochores (Fig. 1 E). We note that these results are consistent with similar changes in kinetochore assembly observed recently in Drosophila melanogaster (Venkei et al., 2012). Collectively, these data indicate that kinetochore protein localization changes dramatically throughout the cell cycle, particularly at time points immediately surrounding mitotic entry and exit.

Kinetochore disassembly occurs independently of protein degradation

Previously, we proposed several potential regulatory mechanisms that could control kinetochore assembly and disassembly (see Introduction; Gascoigne and Cheeseman, 2011). To identify the contribution of these molecular mechanisms to timely kinetochore formation, we sought to systematically assess the effects of perturbations to each potential mechanism on the kinetics of kinetochore assembly and disassembly.

We first considered the contribution of protein degradation to kinetochore disassembly. Anaphase-promoting complex/cyclosome (APC/C)–mediated ubiquitination– and proteasome-dependent degradation of mitotic substrates is a key regulatory mechanism controlling the transition from metaphase to anaphase (Peters, 2006). Kinetochore disassembly begins at anaphase onset when APC/C activity is high. To test the requirements for kinetochore protein degradation to direct kinetochore disassembly, we focused on two outer kinetochore proteins for which ubiquitination has been reported previously (Kim et al., 2011): the Ndc80 complex subunit Ndc80/Hecl and the Mis12 complex subunit Dsn1. Ndc80 contains a destruction box (D box) at residues 372–380 similar to those targeting substrates for APC/C—Cdc20-mediated degradation (Glotzer et al., 1991). A previous study indicated that removal of this sequence altered the function of Ndc80 (Li et al., 2011). However, we found that the kinetochore levels of either Ndc80-GFP or Ndc80-GFP mutated for the D box sequence (Ndc80
dbox; residues 372 and 375—RxxL to AxxA) disassembled from kinetochores during anaphase with similar kinetics (Fig. 2 A). Similarly, the cellular levels of both Ndc80-GFP and Ndc80
dbox-GFP remained...
Figure 1. Kinetochore proteins show distinct assembly and disassembly behaviors. (A, left) Quantification of centromere intensity in immunofluorescence images for the indicated proteins. prom., prometaphase; met., metaphase; ana., anaphase; telo., telophase. (right) Representative immunofluorescence images of kinetochore protein localization after detergent preextraction. (B) Images from time-lapse videos of cells expressing GFP-tagged proteins. Numbering indicates minutes relative to nuclear envelope breakdown (NEBD). (C–E) Quantification of centromeric GFP signals in time-lapse images. n ≥ 8 cells per protein. Error bars indicate SEM. Bars, 5 µM.
unchanged from metaphase to G1 based on fluorescence imaging, indicating that APC-dependent degradation of Ndc80 does not drive its disassembly from kinetochores.

We next tested the role of Mis12 complex degradation in timely kinetochore disassembly. The localization of the Mis12 complex to kinetochores has been reported to depend on the balanced action of the Hsp90 chaperone to stabilize the complex and the SCF (Skp1, Cullin, and F-box) ubiquitin ligase to target it for degradation (Davies and Kaplan, 2010). When the SCF complex component Skp1 was depleted by RNAi, we observed a subtle increase in levels of the Mis12 complex component Dsn1 at kinetochores, but no significant changes in its disassembly kinetics compared with controls (Fig. 2 B and Fig. S1 C). Similarly, a Dsn1 mutant in which all lysine residues were changed to arginine (Dsn1K-R), preventing its ubiquitination, localized to kinetochores (Fig. S1 C) and showed similar kinetochore disassembly kinetics to wild-type Dsn1 (Fig. 2 B).

Finally, to assess the global requirement for protein degradation for timely kinetochore disassembly, we monitored the levels of Ndc80-GFP and GFP-Mis12 at kinetochores in the presence of the proteasome inhibitor MG132 (Fig. 2 C and Fig. S2 B). To initiate mitotic exit in the absence of proteasome activity, we treated cells with the CDK inhibitor (CDKi) flavopiridol. The disassembly kinetics of Ndc80 and Mis12 from kinetochores were identical in the presence or absence of MG132, indicating that global proteasome-mediated protein degradation is not required for kinetochore disassembly (Fig. 2 C and Fig. S2 B). Collectively, these data strongly suggest that kinetochore disassembly is not driven by protein degradation at the metaphase to anaphase transition in human cells.

**Kinetochore disassembly can occur independently of cytoskeletal changes at anaphase**

We next sought to assess the contribution of changes in microtubule dynamics and cytokinesis to kinetochore disassembly. When kinetochore–microtubule interactions were prevented by treatment with the microtubule-depolymerizing drug nocodazole, Ndc80 disassembly from kinetochores occurred with normal kinetics in cells induced to exit mitosis by inhibition of the Mps1 kinase. We next monitored outer kinetochore components after inhibition of CDK activity (Fig. S2 A). To broadly assess the role of mitotic phosphorylation in kinetochore assembly, we monitored kinetochore protein localization after individual inhibition of the mitotic kinases Aurora B, Plk1, or Mps1, or CDK1 (Fig. S2 A). Inhibition of Aurora B, Plk1, or Mps1 caused only minor effects on the microtubule localization of CENP-T, CENP-C, and CENP-I, Dsn1, KNL1, Ndc80, or Ska3 (Fig. S2 A). However, we observed a strong reduction in the localization of multiple kinetochore components after inhibition of CDK activity (Fig. S2 A).

To test the role of CDK activity in kinetochore assembly and disassembly, we next monitored outer kinetochore disassembly kinetics after inhibition of CDK activity using the CDKi flavopiridol in mitotic HeLa cells expressing Ndc80-GFP or GFP-Mis12. Disassembly of Ndc80 or Mis12 from kinetochores during mitosis, indicating that additional factors act immediately before mitosis to contribute to maximal kinetochore assembly. To test the role of Ndc80 complex nuclear exclusion in timely kinetochore assembly, we artificially targeted Ndc80-GFP to the nucleus throughout the cell cycle using a fusion to the SV40 NLS (Fig. 3 C). Ndc80-NLS-GFP localized to kinetochores during mitosis, indicating that it was correctly incorporated into the Ndc80 complex. Both Ndc80-GFP and Ndc80-NLS-GFP were absent from centromeres in G1 (Fig. 3 C). However, Ndc80-NLS-GFP localized prematurely to centromeres during G2, indicating that the centromere is competent to recruit Ndc80 at this time. Thus, nuclear exclusion prevents the premature loading of the Ndc80 complex onto kinetochores.
kinetochores occurred within 5 min of treatment with 5 μM flavopiridol and occurred with similar kinetics to cells exiting an unperturbed mitosis. This indicates that inhibition of CDK activity alone is sufficient for outer kinetochore disassembly (Fig. 4, A and B). Interestingly, when CDK activity was only partially inhibited by treatment with a low dose of flavopiridol (2 μM), Ndc80 disassembly occurred with slower kinetics than in unperturbed cells (Fig. S2 B). Similarly, when we induced mitotic exit by CDK inhibition in the presence of the general phosphatase inhibitor okadaic acid, the disassembly kinetics of Ndc80 and Mis12 were also slowed, indicating a high sensitivity to the levels of CDK-dependent phosphorylation (Fig. S2 B). When interphase cells were treated with okadaic acid, we observed a rapid assembly of GFP-Mis12 and Ndc80-NLS-GFP at kinetochores in G2 cells (Fig. 4 C and Fig. S2 C). This okadaic acid–induced assembly was prevented by pretreatment with CDKi.

The observation that outer kinetochore assembly is acutely sensitive to CDK activity suggests that the action of counteracting phosphatases may be crucial for timely disassembly of these proteins at mitotic exit. The PP2A (protein phosphatase 2A) and its regulatory subunit B55-α have recently been identified as critical factors for the dephosphorylation of CDK1 substrates during anaphase (Schmitz et al., 2010). When we induced mitotic exit by CDK inhibition in cells depleted of B55-α, the kinetics of Ndc80 disassembly from kinetochores were significantly reduced (Fig. 4 A and Fig. S2 D), indicating a crucial role for PP2A–B55-α in kinetochore disassembly. In contrast, Mis12 complex disassembly was only subtly affected by B55-α depletion (Fig. 4 B). These data hint at differing sensitivities to the CDK kinase/phosphatase balance for the assembly/disassembly of different kinetochore subunits. Consistent with this, treatment of HeLa cells with diverse phosphatase inhibitory compounds had differing effects on the disassembly of kinetochore subcomplexes (Fig. 4, D and E). Collectively, these data indicate that the kinetochore assembly state is highly sensitive to the balance of CDK kinase and phosphatase activity within the cell.
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Phosphorylation of CENP-T by CDK controls assembly of a functional pool of Ndc80 at kinetochores

The observation that outer kinetochore disassembly is exquisitely sensitive to CDK activity suggests that kinetochore proteins could be direct CDK substrates, with phosphorylation controlling protein interactions at kinetochores. We have previously reported that CENP-T can interact directly with the Ndc80 complex via its N terminus, an interaction that is enhanced by phosphorylation of CENP-T by CDK (Gascoigne et al., 2011; Nishino et al., 2013). Biochemical and structural analysis of CENP-T has demonstrated that mutation of these phosphorylated residues to aspartic acid effectively mimics phosphorylation of the protein (Nishino et al., 2013). Surprisingly, although preventing phosphorylation of CENP-T blocked recruitment of the Ndc80 complex to kinetochores (Gascoigne et al., 2011), phosphomimetic mutation of 10 CDK sites in CENP-T (CENP-TSD) had only a minor effect on kinetochore disassembly.

Figure 3. Interphase localization of kinetochore proteins contributes to kinetochore assembly dynamics. (A) GFP-tagged protein localization for the indicated proteins and cell cycle stages. (B, bottom left) Images of cells expressing GFP-Mis12 and mCherry-Ran-T24N. (top) Quantification of centromeric GFP-Mis12. (C, top left) Images of Ndc80-GFP or Ndc80-NLS GFP in G2 cells. (bottom) Quantification of centromeric GFP intensity. (B and C, right) Graphs show quantification of centromeric GFP signals in cells entering mitosis. Bars, 10 µm. n ≥ 10 cells. Error bars represent SDs.
kinetochore disassembly. To test this, we prevented the nuclear exclusion of Ndc80 in the presence of constitutively phospho-
ylated CENP-T by coexpression of Ndc80-NLS-GFP and mCherry–
CENP-TSD after RNAi-based depletion of the endogenous pro-
teins. Ndc80-NLS-GFP disassembled from kinetochores with
similar kinetics to Ndc80-GFP in the presence of wild-type
CENP-T. Importantly, in the presence of CENP-TSD, the disassem-
bly of Ndc80-NLS-GFP was greatly slowed and showed bipha-
sic kinetics. In Ndc80-NLS-GFP/CENP-T SD double mutants,
(Fig. 5, A and B). When monitored in our live-cell assay, the
disassembly kinetics of Ndc80-GFP in the presence of CENP-TSD
were similar to control cells, with only a small additional pool
of Ndc80 (20%) persisting 10 min after anaphase onset. Dis-
assembly of the Mis12 complex subunit GFP-Dsn1 was also
unaffected in CENP-T SD mutants (Fig. 5 B).

In addition to CENP-T dephosphorylation, the aforemen-
tioned results suggested that exclusion of Ndc80 from the newly
forming nucleus during anaphase might also be important for
kinetochore disassembly. To test this, we prevented the nuclear exclusion of Ndc80 in the presence of constitutively phosphorylated CENP-T by coexpression of Ndc80-NLS-GFP and mCherry–CENP-TSD after RNAi-based depletion of the endogenous proteins. Ndc80-NLS-GFP disassembled from kinetochores with similar kinetics to Ndc80-GFP in the presence of wild-type CENP-T. Importantly, in the presence of CENP-TSD, the disassembly of Ndc80-NLS-GFP was greatly slowed and showed biphasic kinetics. In Ndc80-NLS-GFP/CENP-T SD double mutants,
40% of Ndc80 remained at kinetochores 40 min after anaphase onset, and Ndc80 was still detectable at centromeres 2 h after anaphase (Fig. 5, B and C). In contrast, under these conditions, disassembly of the Mis12 complex was unaffected (Fig. S3 A), suggesting that there are multiple independent pathways for kinetochore disassembly.

We next sought to test the consequences of this delayed outer kinetochore disassembly. Replacement of CENP-T with GFP-CENP-T<sup>SD</sup> alone or Ndc80 with Ndc80-NLS-GFP alone had no significant effect on chromosome segregation. In addition, when both Ndc80 and CENP-T proteins were replaced with Ndc80-NLS-GFP and mCherry-CENP-T<sup>SD</sup>, no phenotype was observable 48 h after siRNA addition. This is in stark contrast to replacement of CENP-T with GFP-CENP-T<sup>SS</sup> (phosphoinhibitory), in which a highly penetrant chromosome alignment defect was observed 48 h after depletion of the endogenous protein (Gascoigne et al., 2011). However, in subsequent cell divisions (72 h time point), Ndc80-NLS-GFP/mCherry–CENP-T<sup>SD</sup> cells spent twice as long in mitosis as control cells, with 25% of cells displaying chromosome bridges at anaphase (Fig. 5, D and E). In metaphase Ndc80-NLS/CENP-T<sup>SD</sup> cells, the levels of the representative kinetochore proteins CENP-A and Dsn1 were normal in the subsequent cell division after the failure of full kinetochore disassembly (72 h; Fig. S3 C). This suggests that the observed chromosome segregation defects are unlikely to be caused by abnormalities in kinetochore composition. In addition, the failure of Ndc80 to fully disassemble from centromeres did not lead to detectable persistent microtubule interactions at telophase or prevent nuclear envelope reformation (Fig. S3 B). Collectively, these data indicate a crucial role
for CDK-dependent phosphorylation of CENP-T in the direct recruitment of the Ndc80 complex to mitotic kinetochores and suggest that removal of this phosphorylation is essential for full kinetochore disassembly and accurate chromosome segregation in subsequent divisions.

Here, we systematically tested the contributions of multiple cellular activities to timely kinetochore disassembly. We find that protein degradation and cell cycle–coupled cytoskeletal changes do not play critical roles in the regulation of the kinetochore assembly state in human cells. Instead, we find that CDK-dependent phosphorylation of kinetochore substrates, coupled with exclusion of the Ndc80 complex from the nucleus, directs the kinetics of kinetochore assembly and disassembly. Importantly, disrupting timely kinetochore disassembly prevents accurate chromosome segregation in subsequent cell cycles. As kinetochore composition appears normal in these subsequent mitoses, we speculate that chromosome bridges observed in such cells arise from problems with centromere replication in the presence of constitutively localized outer kinetochore components. Although this work defines the basic parameters that control kinetochore assembly state, our results suggest that additional aspects of kinetochore assembly state are also regulated in addition to CENP-T phosphorylation and Ndc80 subcellular localization. For example, the differential sensitivity of the Ndc80 and Mis12 complexes to phosphatase inhibition suggests that multiple regulatory pathways may act to control kinetochore assembly during mitotic entry and exit. Defining the other interactions and properties that coordinateably control kinetochore assembly is an exciting challenge for future work.

Materials and methods

Cell culture and transfection

Cell lines were maintained in DMEM supplemented with 100 U/ml streptomycin, 100 µM penicillin, 2 mM glutamine, and 10% (vol/vol) fetal calf serum. All cells were cultured at 37°C with 5% CO2. HeLa LacZeo/TO cells (a gift from S. Taylor, University of Manchester, Manchester, UK) were maintained in 2 µg/ml Blasticidin and 200 µg/ml Zeocin. Cells expressing tetracycline-inducible GFPγH or mCherryγH fusions were generated by Flp recombinase–mediated integration of a pCDNA5-FRT-TO–based plasmid (Invitrogen). Cells were then maintained in 2 µg/ml Blasticidin and 400 µg/ml Hygromycin B. Clonal cell lines constitutively expressing GFPγH or mCherryγH fusions were generated using retroviral infection of HeLa cells with a pBABE-blast– or pBABE-Puromycin–based vector.

See Table S1 for a list of cell lines used in this study. Transient transfection of HeLa cells with a pBABE-blast– or pBABE-Puromycin–based vector.

Online supplemental material

Fig. S1 assesses factors controlling kinetochore assembly kinetics. Fig. S2 shows that inhibition of CDK, but not other mitotic kinases, affects kinetochore assembly. Fig. S3 shows that inhibition of Ndc80 complex disassembly does not cause immediate mitotic defects. Table S1 shows cell lines used in this study. Table S2 shows antibodies used to visualize kinetochore proteins. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201301006/DC1.

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References


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