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Molecular Architecture and Regulation of the Budding Yeast Kinetochore-Microtubule Interface

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Abstract

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Equal partitioning of replicated chromosomes during mitosis is vital. The genetic information stored in chromosomes provide the template for cells to produce RNA and protein molecules, which carry out cellular functions. Failure to equally divide chromosomes between two daughter cells means a disturbance in this central dogma of biology. Chromosome division is mediated by a bipolar structure specific to mitosis, called the spindle. Cytoskeletal microtubules anchored at each pole grow towards the other pole, resulting in the separation of two poles. During this process, the sister chromatids (replicated pair of chromosomes connected to each other) bind to spindle microtubules. This is made possible by the kinetochore, which assembles on the centromeric DNA region of each sister chromatid. During metaphase, sister kinetochores establish bioriented attachments on dynamic spindle microtubules. In anaphase, microtubule depolymerization is coupled with the segregation of sister chromatids towards their respective poles. Thus, the main function of the kinetochore is to make strong and processive attachments to microtubules.

In the budding yeast *S. cerevisiae* outer kinetochore, the essential Ndc80 and Dam1 complexes are the main microtubule binding components. I show that these two protein complexes have a tripartite interaction and disrupting any one interaction site results in chromosome segregation error. Through these three interactions, the elongated Ndc80 complex bridges two Dam1 complex rings. In addition, I detail the mechanisms by which the Aurora B kinase and Cyclin dependent kinase 1 (Cdk1) regulate the kinetochore-microtubule interface. The Aurora B kinase phosphorylates the Dam1 complex to disrupt each interaction site between the Dam1 and Ndc80 complexes. Further phosphorylation of the Dam1 complex results in the inhibition of both cooperative and single molecule microtubule-binding ability. In contrast, the Cdk1 phosphorylates the Dam1 complex to enhance the cooperative binding onto microtubules. Together, I describe how the Ndc80 and Dam1 complexes are organized at the kinetochore-microtubule interface and the intricate mechanisms regulating these interactions.

The Spc105 and MIND complexes are also essential and conserved components of the outer kinetochore. I show for the first time a recombinant expression and purification method for the Spc105 complex. I detected coiled-coil interactions between the two proteins that compose the Spc105 complex, Spc105p and Kre28p. Purification of the Spc105 complex from budding yeast co-purifies both MIND and Ndc80 complexes. These three protein complexes represent the highly conserved KMN network. Future studies will further utilize these tools for investigating how the Spc105 complex is organized with other outer kinetochore components.

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Chapter 1

Introduction

Mitosis and its spindle

The cell cycle is the process by which one mother cell grows, duplicates its genome, and undergoes mitosis to divide into two daughter cells. The goal of mitosis is to equally partition the duplicated chromosomes between the daughter cells. During human development many generations of the cell cycle result in one zygote becoming an adult, who is made up of about $\sim 3.7 \times 10^{13}$ cells (Bianconi et al., 2013). Ensuring the maintenance of genetic integrity during this process is of paramount importance. Gametic aneuploidy can result in genetic monosomy and trisomy disorders (Oromendia & Amon, 2014). Additionally, aneuploidy is a hallmark of cancer cell genomes and is implicated to drive tumorigenesis (Gordon et al., 2012). These possible adverse effects of aneuploidy throughout an individual's lifetime calls for a deep understanding of chromosome segregation mechanisms during mitosis.

Highly conserved in evolution, chromosome segregation is a microtubule-based process. Part of the cytoskeleton, microtubules are composed of α -tubulin and β -tubulin heterodimers. The tubulin dimers polymerize into a polar and linear protofilament in a GTP-dependent manner, as the tubulin dimer is a GTPase (Desai & Mitchison, 1997). 13 protofilaments serve as the microtubule wall, forming a round hollow tube with a diameter of 25 nm (Evans et al., 1985; Ledbetter & Porter, 1963; Tilney et al., 1973). Microtubules have an inherent polarity with a plus and a minus-end. These microtubule ends alternate between two states of growth (polymerization) and shrinkage (depolymerization); this process is termed dynamic instability and is dependent on the tubulin dimer concentration (Mitchison & Kirschner, 1984; Walker et al., 1988).

Mediated by microtubule polymerization, development of the mitotic spindle is the major structural feature of a mitotic cell. At the start of mitosis, microtubules are nucleated and grow from two cellular structures called the microtubule-organizing center (MTOC; centrosome in humans and spindle pole body in budding yeast). Microtubules growing from one centrosome to another are antiparallel. Bipolar kinesins with two plus-end directed motors cross-link these antiparallel microtubules before walking towards the plus ends (Kapitein et al., 2005; Kashina et al., 1996). This results in centrosome separation, spindle elongation, and spindle bipolarity. Establishment of the mitotic spindle serves to set up the structural environment for sister chromatid congression and segregation during metaphase and anaphase, respectively (Wordeman, 2010).

During the establishment of the mitotic spindle, the duplicated sister chromatids bind and become incorporated into the microtubule network. Prior to the onset of mitosis, DNA replication duplicates each chromosome and forms sister chromatids connected by cohesin (Haering et al., 2008). Each sister chromatid's centromeric DNA has an assembled kinetochore, which attaches the sister chromatids to microtubules (Torrás-Llort et al., 2009). Initially, one kinetochore interacts with the lateral wall of a microtubule originating from one centrosome. Once laterally bound, the chromatids are transported towards the spindle pole independently of microtubule depolymerization (Rieder & Alexander, 1990). This is mediated by dynein in mammalian cells and Kar3p, a kinesin-14 family member, in budding yeast (Tanaka et al., 2005; Yang et al., 2007). During this process, the plus end of the attached microtubule undergoes depolymerization and often catches up to the laterally-bound kinetochore. This results in the conversion from lateral to end-on attachment between the kinetochore and microtubule, and the sister chromatids tracking with the dynamic microtubule tip (Tanaka, 2010).

Next, the sister kinetochores attempt to make bioriented attachments to the spindle microtubules. Biorientation is established when each kinetochore is attached to microtubule tips originating from a unique centrosome. Some sister kinetochores initially make bioriented

attachments on the spindle. Monooriented sister kinetochores, bound to microtubules originating from only one centrosome, localized at a spindle pole have shown to congress towards the spindle midzone prior to biorientation. This is mediated by CENP-E (kinesin 7), a plus-end directed motor that transports these sister kinetochores along microtubules that have already established biorientation (Kapoor et al., 2006). Congression towards the spindle midzone might increase the probability that monooriented sister kinetochores become bioriented by moving to an area with a higher density of microtubules originating from the opposite spindle pole. Establishment of biorientation in all sister kinetochores silences the mitotic checkpoint and triggers the transition from metaphase to anaphase, when sister chromatids segregate towards their respective spindle poles.

Aurora B kinase mediated error correction

Attempts by the sister kinetochores to make bioriented attachments on the mitotic spindle often lead to incorrect attachments, including monotelic, syntelic, and merotelic attachments. Monotelic and syntelic attachments have one and both kinetochores bound to microtubule originating from one centrosome, respectively. Merotelic attachments have one kinetochore bound to microtubules originating from one centrosome and other kinetochore bound to microtubules from both centrosomes. Progression from metaphase to anaphase without correcting these errors can lead to aneuploidy and give rise to chromosomal instability in cancer cells (Gregan et al., 2011). A mitotic cell has two central mechanisms for preventing these events.

First, the error correction pathway detects and corrects aberrant kinetochore-microtubule attachments. The highly conserved Aurora B kinase has emerged as the key regulator in this pathway (Carmena & Earnshaw, 2003). Initial budding yeast genetics experiments showed that temperature sensitive (*ts*) mutants in Aurora B kinase result in chromosome segregation defects (Biggins et al., 1999; Chan & Botstein, 1993). In mammalian cells, inhibiting its activity with a

small molecule yields monooriented chromosomes (Hauf et al., 2003). Aurora B kinase phosphorylates the main microtubule-binding components of the kinetochore, resulting in decreased affinity of these proteins for microtubules *in vitro* (Alushin et al., 2012; Gestaut et al., 2008; Umbreit et al., 2012). Phosphomimetic mutations of these Aurora B kinase phosphorylation sites result in chromosome segregation defects *in vivo* (Cheeseman et al., 2002; Welburn et al., 2010). These studies suggest that the Aurora B kinase targets and destabilizes erroneous kinetochore-microtubule attachments, allowing further attempts for sister kinetochores to establish biorientation.

Tension across bioriented sister kinetochores is implicated as the main regulator of Aurora B kinase activity. Biorientation results in tension across sister kinetochores, which is opposed by the cohesion of sister chromatids by the cohesin complex (Haering et al., 2008; Tanaka et al., 2000). Evidence for tension suppressing Aurora B kinase activity come from studies in budding yeast. Inhibiting cohesin or DNA replication by genetic manipulation hypothetically abolishes tension at the kinetochores. Phenotypes associated with these genetic manipulations can be rescued by an Aurora B kinase *ts* mutant *ipl1-321* at the restrictive temperature (Biggins & Murray, 2001; Tanaka et al., 2002). Aurora B kinase's sensitivity to the absence of tension fits with other studies showing that tension directly stabilizes kinetochore-microtubule attachments (Akiyoshi et al., 2010; Nicklas & Ward, 1994). Under this model, the absence of tension increases Aurora B kinase activity and destabilizes kinetochore-microtubule attachments. In the presence of tension, Aurora B kinase activity is suppressed and kinetochore-microtubule attachments are stabilized.

The spindle assembly checkpoint

The second mechanism for preventing missegregated chromosomes is the spindle assembly checkpoint (SAC). The SAC surveys the attachment state of kinetochores and prevents anaphase onset until all kinetochore-microtubule attachments are satisfied. The Mps1

kinase localizes to kinetochores lacking microtubule attachments, specifically binding to the Ndc80 complex (Hiruma et al., 2015; Ji et al., 2015; Kemmler et al., 2009; Nijenhuis et al., 2013). This allows the Mps1 kinase to phosphorylate the MELT motifs of the nearby kinetochore protein KNL1 (Spc105p in budding yeast), providing a scaffold for recruiting Bub proteins (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012). Next, phosphorylation of Bub1 by Mps1 kinase forms a scaffold for the Mad1-Mad2 dimer to bind (Kim et al., 2012; London & Biggins, 2014); this Mad2 is in the “closed” conformation (C-Mad2). The free and monomeric Mad2 in the “open” conformation (O-Mad2) is then catalytically converted to C-Mad2 by the kinetochore-anchored C-Mad2 (Skinner et al., 2008). This ultimately leads to the association of free C-Mad2 with Cdc20 and formation of the mitotic checkpoint complex (MCC), the SAC effector molecule (Musacchio, 2015).

Sequestering Cdc20 into the MCC results in SAC activation by inhibiting the anaphase promoting complex (APC/C). Cdc20 is a co-activator of APC/C, a ubiquitin ligase that targets securin and cyclin B for proteasome degradation (Oliveira et al., 2010). Securin inhibits the proteolytic cleavage of cohesin, a protein complex responsible for sister chromatid cohesion by trapping them inside the cohesin ring structure (Haering et al., 2008; Jallepalli et al., 2001). Therefore, absence of kinetochore-microtubule attachments leads to the activation of the SAC and persistence of securin and cohesin. This ensures that the sister kinetochores do not segregate until all kinetochore-microtubule attachments are satisfied.

Cyclin B is another important target of APC/C. Cyclin dependent kinases (Cdk) are the master regulators of the cell cycle checkpoints, including transitions into G₁, S, G₂, and M phases of the cell cycle. Inferred by their names, Cdk's are activated by various cyclins during the cell cycle. Although Cdk levels remain relatively constant, the different cyclin levels fluctuate during various parts of the cell cycle. Cyclin B (Clb2 in budding yeast) binds and activates Cdk1 (Cdc28 in budding yeast) to promote the transition from G₂ to M phase (King et al., 1994). Satisfying all kinetochore-microtubule attachments in yeast silences the SAC and leads to the

APC/C-Cdc20 mediated destruction of Clb2, which results in mitotic exit from telophase and progression into cytokinesis (Surana et al., 1993). These mechanistic details show the tight regulation that has evolved to prevent chromosome missegregation and explain why presence of even a single unattached kinetochore creates a strong enough downstream signal to activate the SAC (Li & Nicklas, 1995; Rieder et al., 1995).

The kinetochore-microtubule interface

The process and regulation of sister chromatid segregation discussed above reveal the kinetochore's extensive involvement in the equal partitioning of the duplicated genetic material. The budding yeast kinetochore exhibits a high level of conservation with the human kinetochore (Lampert & Westermann, 2011). Compared to the human kinetochore, which binds ~20-25 microtubules per kinetochore, each budding yeast kinetochore binds only one microtubule (Winey et al., 1995). The power of yeast genetics combined with these advantages make budding yeast an optimal model organism for studying the kinetochore's role in equal chromosome segregation.

The budding yeast kinetochore is a protein super-complex composed of >40 proteins organized into multiple protein complexes. Centromeric DNA on each chromosome is the platform on which the kinetochore assembles, and budding yeast have a short point centromere of ~125 bp (Bloom & Carbon, 1982). This DNA region is uniquely marked by the presence of centromeric histone H3 (CenH3) protein instead of the canonical histone H3 protein; the budding yeast CenH3 is called Cse4p (Carroll & Straight, 2006). Largely, the kinetochore is divided into two spatial categories: inner and outer kinetochores. Inner kinetochore components assemble on or near the centromeric region while outer kinetochore components extend from the inner kinetochore and bind microtubules.

To date, many inner kinetochore proteins have been identified. These include the CBF3 complex, Ctf19 complex, Cbf1p, Mif2p, and Cnn1p (Biggins, 2013). Of these, only the CBF3

complex, Mif2p, Okp1p, and Ame1p (Ctf19 complex components) are essential for viability, implying redundant functionality among inner kinetochore proteins. The heterotetrameric CBF3 complex (Ndc10p, Cep3p, Ctf13p, Skp1p) is thought to nucleate the kinetochore. A temperature-sensitive allele of *NDC10*, *ndc10-1*, prevents kinetochore assembly at the restrictive temperature (Goh & Kilmartin, 1993; He et al., 2000). Mif2p might be the next assembled essential kinetochore protein since Cse4p is co-purified with Mif2p and Mif2p localization to the centromere is dependent on both Cse4p and Ndc10p (Meluh & Koshland, 1997; Westermann et al., 2003). Finally, both Okp1/Ame1p dimer and Mif2p bind MIND complex *in vitro*, the main connection to the outer kinetochore (Dimitrova et al., 2016; Hornung et al., 2014). In this hierarchical manner, the essential inner kinetochore proteins assemble near the centromeric DNA to provide a scaffold for outer kinetochore proteins.

All outer kinetochore proteins are essential and organize into four separate protein complexes: Spc105, MIND, Ndc80, and Dam1 complexes. The Spc105 and Ndc80 complexes connect to the inner kinetochore through the MIND complex. These three protein complexes form the highly conserved KMN (Spc105/KNL1, MIND/Mis12, Ndc80) network (Cheeseman et al., 2006; Kudalkar et al., 2015; Petrovic et al., 2014; Petrovic et al., 2010). Finally, the Dam1 complex is a fungal-specific kinetochore component that is recruited to the kinetochore by the Ndc80 complex (Janke et al., 2002); together, these two protein complexes form the main microtubule-binding components of the budding yeast kinetochore.

Processive kinetochore-microtubule attachments are crucial for preventing sister chromatid missegregation. Upon establishing biorientation, the sister kinetochores experience oscillations of microtubule assembly and disassembly during metaphase. This is termed “directional instability” (Skibbens et al., 1993; Wise et al., 1991) and both kinetochores must remain processively attached to these dynamic microtubules for silencing the SAC. Processive attachment is also important in anaphase when microtubule depolymerization is coupled to sister chromatid segregation. The outer kinetochore components are required for this

processive attachment. Temperature sensitive mutants in the Ndc80 or Dam1 complex components show chromosome segregation errors at the restrictive temperatures (Cheeseman et al., 2001; Janke et al., 2001; Li et al., 2002; Wigge et al., 1998). *In vitro*, the Dam1 and Ndc80 complexes independently bind microtubules and interact on microtubules, which enhances microtubule binding of the Ndc80 complex (Gestaut et al., 2008; Powers et al., 2009; Tien et al., 2010). Furthermore, the Dam1 complex tracks with depolymerizing microtubule plus ends and imparts microtubule tip tracking ability to the Ndc80 complex (Asbury et al., 2006; Lampert et al., 2010; Tien et al., 2010; Westermann et al., 2006). *In vivo*, the Dam1 complex is required for kinetochores to make microtubule tip attachments, which are important for establishing biorientation (Shimogawa et al., 2006; Tanaka et al., 2005).

As described thus far, the most important function of the kinetochore is to strongly and processively attach to microtubules. Studies summarized above demonstrate the extensive research into how the Dam1 and Ndc80 complexes contribute to the kinetochore-microtubule interaction. Understanding the interaction regions between these two kinetochore components has implications for how the kinetochore strongly binds to spindle microtubules and the mechanisms by which the mitotic kinases regulate kinetochore-microtubule attachments. This thesis focuses on studying the molecular architecture of the budding yeast kinetochore-microtubule interface and how they are regulated by phosphorylation events.

Chapter 2

The Ndc80 complex bridges two Dam1 complex rings

Introduction

Kinetochores link replicated chromosomes to spindle microtubules. They form attachments flexible and strong enough to stay attached to microtubules during assembly and disassembly. Estimates of the strength of the attachments suggest that they are much stronger than required just to pull a chromosome through the cellular milieu (Fisher et al., 2009; Nicklas, 1965). Yet, incorrect attachments are weak enough to be detached and corrected.

The Dam1 and Ndc80 complexes are the main microtubule-binding components in the budding yeast kinetochore. The Ndc80 complex connects the Dam1 complex to the rest of the kinetochore. The two complexes interact on microtubules, significantly increasing the strength of Ndc80 complex attachment to microtubules (Lampert et al., 2010; Lampert et al., 2013; Tien et al., 2010). How these two complexes interact on microtubules remains uncertain with conflicting results reported in the literature (Lampert et al., 2013; Maure et al., 2011).

In addition to providing strong attachments between chromosomes and spindle microtubules, kinetochores also serve as regulatory hubs. The Ndc80 complex and the Dam1 complex are at the center of this regulation. The Ndc80 complex is a scaffold for the spindle assembly checkpoint (Dou et al., 2015; Hiruma et al., 2015; Ji et al., 2015). Both the Dam1 and Ndc80 complexes are targets of Aurora B kinase, which corrects aberrant kinetochore-microtubule attachments to achieve bioriented attachments (Reviewed in Sarangapani & Asbury, 2014). Aurora B kinase phosphorylation of the Dam1 complex components Dam1p, Ask1p, and Spc34p together disrupts the interaction between the Dam1 and Ndc80 complexes (Lampert et al., 2013; Tien et al., 2010); however, which phospho-protein is responsible for disrupting the interaction has yet to be deciphered.

Here, we present data suggesting that the Ndc80 and Dam1 complexes interact through a tripartite interaction. Interactions were detected between the Ndc80p (Ndc80 complex) and Dam1p, Ask1p, and Spc34p (Dam1 complex). These interaction regions correlate with the Aurora B kinase phosphorylation sites on the Dam1 complex, and to previously identified lethal mutations on the Ndc80p. Testing these phosphorylation events and mutations in our functional assay further confirmed the initial results. We suggest that this tripartite Ndc80 and Dam1 complex interaction is more complex than previously thought and is heavily regulated by the Aurora B kinase.

Results

Three Ndc80p regions interact with Dam1p, Ask1p, and Spc34p of the Dam1 complex

We identified where the Dam1 and Ndc80 complexes interact using protein cross-linking and mass spectrometry analysis (Figure 2.1 and 2.2) following protocols developed previously (Hoopmann et al., 2015; Kudalkar et al., 2015; Tien et al., 2014; Zelter et al., 2015). The full decameric Dam1 complex and tetrameric Ndc80 complex were cross-linked in the presence of taxol-stabilized microtubules. The C-terminal regions of Dam1p, Ask1p, and Spc34p formed cross-links to three distinct regions of Ndc80p (Figure 2.1A and Figure 2.2). The corresponding regions of Nuf2p also formed cross-links to these same regions of the Dam1 complex (Figure 2.3). These three C-terminal regions of the Dam1 complex include five sites phosphorylated by Aurora B kinase. Phosphorylation of all five sites fully disrupts the interaction between the Dam1 and Ndc80 complexes (Lampert et al., 2010; Tien et al., 2010). Thus, these sites likely represent primary interaction sites through which the Dam1 complex binds the Ndc80 complex.

To identify the cognate binding site(s) on the Ndc80 complex, we compared the cross-linking data to a map of functional regions on Ndc80p identified in a previous linker-scanning mutagenesis screen (Tien et al., 2013). Three of these regions map near or within the regions that cross-link to Dam1p, Ask1p, and Spc34p (Figure 2.1A). We define these interactions as A,

B, and C, and we will refer to the specific regions on each protein that are involved in these interactions as A^{Dam1p} and A^{Ndc80p}, B^{Ask1p} and B^{Ndc80p}, and C^{Spc34p} and C^{Ndc80p}, respectively (Figure 2.1A and Table 2.1).

We tested if the lethal mutations identified in our screen in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} (Figure 2.1B) interfere with interactions between the Ndc80 and Dam1 complexes on microtubules using single-molecule total internal fluorescence (TIRF) microscopy (Figure 2.1C). Importantly, Ndc80 complexes carrying a mutation in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} demonstrated homogeneous behavior in size exclusion chromatography, similar to the wild type Ndc80 complex (Figure 2.4E). These mutations also did not alter the residence time of the Ndc80 complex alone on microtubules, consistent with previous observations (Figure 2.1D; Tien et al., 2013). As reported previously, the presence of the Dam1 complex significantly increases the residence time of wild-type Ndc80 complex on microtubules (Tien et al., 2010). However, addition of the Dam1 complex only partially increased the residence time of the mutant complexes as compared to the wild-type Ndc80 complex (Figure 2.1D and Figure 2.4A,B). Ndc80-GFP complex containing Ndc80p insertional mutations outside the regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} had similar microtubule and Dam1 complex binding characteristics as the wild-type Ndc80 complex (Figure 2.4C,D). These observations suggest regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} each contribute to the interaction of Ndc80 complex with Dam1 complex; and the insertional mutants in these three regions were previously identified as lethal because they disrupted the interaction between the Dam1 and Ndc80 complexes.

Dam1p, Ask1p, and Spc34p participate in interactions with Ndc80 complex that can be separately regulated by Aurora B kinase

We then asked if the corresponding regions in the Dam1 complex also contribute to the interaction between Dam1 and Ndc80 complexes. Aurora B kinase phosphorylates the Dam1 complex at six different sites: Dam1p S20, S257, S265, S292; Ask1p S200; Spc34p T199

(Cheeseman et al., 2002). Phosphorylation of Dam1p S20 significantly decreases Dam1 complex oligomerization, while phosphorylation of the three C-terminal sites in Dam1p slightly inhibits direct binding of the Dam1 complex to microtubules (Gestaut et al., 2008; Zelter et al., 2015). In our prior work, we showed that phosphorylating all five sites besides S20 fully disrupts interaction between the Dam1 and Ndc80 complexes (Tien et al., 2010). As shown in Figure 2.1A, these five sites fall into the three interaction regions defined above, A^{Dam1p}, B^{Ask1p}, and C^{Spc34p} (Table 2.1). We therefore systematically phosphorylated combinations of these five sites to dissect their contributions to the interaction between the Dam1 and Ndc80 complexes.

We first purified recombinant mutant Dam1 complexes containing different combinations of Ser/Thr to Ala mutations, together with the S20A mutation in all cases. These mutants were treated with Aurora B kinase to produce a series of Dam1 complexes, each with a unique subset of phosphorylated sites. Each phosphorylated Dam1 complex was then tested for its ability to bind the wild-type Ndc80 complex in the single-molecule TIRF assay.

Simultaneously phosphorylating all three regions, A^{Dam1p}, B^{Ask1p}, and C^{Spc34p} completely disrupted the interaction between the Dam1 and Ndc80 complexes, as reported previously (Figure 2.5A and Figure 2.6A; Tien et al., 2010). Individually phosphorylating regions A^{Dam1p}, B^{Ask1p}, or C^{Spc34p} caused only partial disruption. Phosphorylating region B^{Ask1p} together with C^{Spc34p} also only partially disrupted the interaction, but phosphorylating A^{Dam1p} in combination with either B^{Ask1p} or C^{Spc34p} was sufficient to fully disrupt the interaction (Figure 2.5B and Figure 2.6B,C). Neither the alanine mutations themselves, nor phosphorylation of a mutant Dam1 complex with all six sites mutated to alanine had any effect, confirming that the observed disruptions were due to phosphorylation at the known sites (Figure 2.5A, Figure 2.6A, and Figure 2.7). Together these data demonstrate that each of these regions contributes to the interaction between the Dam1 and Ndc80 complexes and is regulated by phosphorylation.

The Dam1 and Ndc80 complexes interact at three different sites

We next mixed and matched phosphorylation of the Dam1 complex with the mutations in Ndc80p. Full disruption by phosphorylation of the Dam1 complex was recapitulated by substituting a Dam1 complex phosphorylation with its corresponding mutation in Ndc80p. For example, phosphorylation of regions A^{Dam1p} and C^{Spc34p} fully disrupted the interaction between the two complexes (Figure 2.5B). Similarly, phosphorylation at region A^{Dam1p} plus mutation in region C^{Ndc80p} fully disrupted the interaction between the two complexes (Figure 2.5C and Figure 2.6E, F).

Phosphorylation combinations that partially disrupted the interaction between the Dam1 and Ndc80 complexes were also recapitulated by substituting a phosphorylation event with mutation in the corresponding binding region of Ndc80p. Phosphorylation at regions B^{Ask1p} and C^{Spc34p} partially disrupted the interaction between the two complexes (Figure 2.5B). Similarly, mutation in region B^{Ndc80p} plus phosphorylation at region C^{Spc34p}, or phosphorylation at region B^{Ask1p} plus mutation in C^{Ndc80p} partially disrupted the interaction between the two complexes (Figure 2.5C and Figure 2.6D). Together, our results demonstrate that the Dam1 and Ndc80 complexes interact at three different sites, each of which is phospho-regulated by Aurora B kinase.

The Ndc80 complex binds two Dam1 complex rings

The Ndc80 complex is predicted to be a long fibril. The coiled-coiled length between regions A^{Ndc80p} and C^{Ndc80p} is predicted to be 29 nm (Lupas & Gruber, 2005) (Figure 2.8). The width of a Dam1 complex ring is only ~7 nm (V. H. Ramey et al., 2011). Assuming that the Ndc80 complex adopts a conformation roughly parallel to the microtubule axis (Alushin et al., 2010; Joglekar, Bloom, & Salmon, 2009), a single ring could not span this distance. Thus we considered that multiple Dam1 complex rings might bind to the Ndc80 complex. Using negative

stain electron microscopy, we imaged the Dam1 complex on microtubules in the absence and presence of the wild-type Ndc80 complex.

The Dam1 complex formed rings on microtubules as previously reported (Figure 2.8A and B; Miranda et al., 2005; Westermann et al., 2005). In the absence of the Ndc80 complex, the Dam1 complex rings tended to bind in doublets of rings, at an inter-ring distance of 13.3 ± 2.4 nm (avg $\pm$ s.d.) (Figure 2.8A-C and Figure 2.9A). This suggests that Dam1 complex rings have an affinity for each other along the longitudinal axis of microtubules and have a tendency to stack together. The stacked double-ring organization may be analogous to the paired ring helices previously reported to form at higher concentrations of Dam1 complex (Miranda et al., 2005). The percent of Dam1 ring pairs found in closely spaced doublets was higher in the presence of the Ndc80 complex, 49.1 % (estimated by Gaussian fitting to the peak, see Methods), as compared to without the Ndc80 complex, 18.8%. In addition, the two rings in each doublet were consistently held apart by a distance of 33.1 ± 3.8 nm, similar to the predicted coiled-coil length between regions A^{Ndc80p} and C^{Ndc80p} (Figure 2.8 and Figure 2.8B). This suggests that one Ndc80 complex bridges two Dam1 rings by binding one ring at region A^{Ndc80p} and a second ring at region C^{Ndc80p}.

To further test this hypothesis, 10 coiled-coil heptad repeats were inserted into the Ndc80p and Nuf2p coiled-coil domains to increase the length of the coiled-coil domain between the interaction regions by a predicted 10.5 nm (Figure 2.8D). In the presence of the 10-heptad mutant Ndc80 (Ndc80^{10-hep}) complex, Dam1 ring doublets were consistently separated by 42.1 ± 2.1 nm, 9 nm farther apart than in the presence of wild-type Ndc80 complex (Figure 2.8 and Figure 2.9C). Finally, mutation in regions A, B or C of Ndc80p abolished its ability to bind two rings as the Dam1 inter-ring distance in these cases was the same in the absence of Ndc80 complex (Figure 2.8D-F and Figure 2.10). These results strongly support the hypothesis that the Ndc80 complex bridges two Dam1 complex rings *in vitro*.

The Ndc80 10-heptad insertion complex is lethal in vivo

We next explored the possibility that Ndc80 complex might also bridge across two Dam1 complex rings *in vivo*. In particular, our observations raise the question of whether the specific 33 nm inter-ring distance, delimited by the Ndc80 complex, is important. To test this we constructed yeast expression plasmids for *ndc80*^{10-hep} and *nuf2*^{10-hep}, encoding Ndc80p and Nuf2p each with 10-heptad repeats inserted. Using a plasmid shuffle assay, we found that even when introduced together, *ndc80*^{10-hep} and *nuf2*^{10-hep} could not support growth, even though the encoded proteins were expressed (Figure 2.11A, B). The Ndc80^{10hep} complex is competent to assemble *in vitro*, to bind microtubules, to interact with the Dam1 complex on microtubules, and to bind two Dam1 complex rings (Figure 2.8; Figure 2.10C). The only known difference is that the inter-ring distance is increased by 9 nm. These results suggest that the Ndc80^{10hep} complex cannot support viability due to an altered Dam1 complex inter-ring distance.

The Ndc80 mutants exhibit biorientation and attachment defects

The Ndc80p mutants in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} cannot bind two Dam1 complex rings, and consequently have decreased interaction with the Dam1 complex on microtubules. To determine if these Ndc80p mutants confer defects in chromosome biorientation and attachment, we used an auxin-inducible degron system for degrading the wild-type Ndc80-AID (Nishimura et al., 2009). Non-degradable wild-type or mutant *NDC80* was integrated at the *URA3* locus. Ndc80-AID was efficiently degraded in the presence of auxin (Figure 2.13). Budding index analyses revealed that cells carrying wild-type *NDC80* progressed through mitosis as expected. Cells without *NDC80* also budded and divided on schedule, despite major defects in kinetochore attachment (see below), as expected since activation of the SAC requires the Ndc80 complex (Dou et al., 2015; Hiruma et al., 2015; Ji et al., 2015). In contrast, cells carrying Ndc80p mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} arrested as

large-budded cells (Figure 2.14) suggesting defects in kinetochore attachment and indicating that the checkpoint remained functional.

We performed a detailed analysis of the phenotype conferred by the *NDC80* mutant alleles by imaging wild-type, depleted, and mutant cells with *CEN3* and spindle pole bodies (SPB) tagged with GFP and mCherry, respectively. Cultures were synchronized at G1, released from synchrony and then imaged after 60 minutes. In the majority of wild-type *NDC80* cells, *CEN3* was bioriented with both *CEN3*-GFP puncta on the spindle axis; 78% were in metaphase and 22 % were in anaphase as judged by the spindle length. In the majority of the Ndc80-depleted cells, *CEN3*-GFP was off of the spindle axis, consistent with complete detachment. A mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}, resulted in 61, 59, and 75% of the cells with monooriented *CEN3* and 34, 39, and 21% of cells with detached *CEN3*, respectively. (Figure 5A, B). In addition, in the majority of cells depleted of Ndc80 or with a mutation in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} the SPBs were separated by more than 2 μ m (Figure 2.12C). These long spindles and the high frequency of detached *CEN3* suggest that few if any of the kinetochores were bioriented. Thus, we conclude that Ndc80p carrying a mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} disrupts kinetochore biorientation and attachment *in vivo*.

The Ndc80 mutants have defects in recruiting the Dam1 complex to the kinetochore

The Ndc80 complex is responsible for recruiting the Dam1 complex to the kinetochore (Janke et al., 2002). Since Ndc80 complexes carrying a mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} are defective in binding the Dam1 complex *in vitro*, we asked if Dam1 complex kinetochore localization is also compromised *in vivo*. We again used the auxin-inducible degron system for degrading the wild-type Ndc80-AID, while wild-type or mutant *NDC80* was integrated at the *URA3* locus; Mtw1-YFP, Dad4-CFP, and Spc110-mCherry marked the kinetochore, Dam1 complex, and SPB, respectively.

Cells expressing wild-type *NDC80* had two distinct clusters of Mtw1-YFP between two SPBs, as expected for bioriented sister kinetochores. Consistent with previous studies (Cheeseman et al., 2001; Hofmann et al., 1998; Li et al., 2002), the Dam1 complex localized along the spindle but also showed two distinct clusters that colocalized with the Mtw1-YFP. (Figure 2.15A, B). None of the mutants showed the organized kinetochores seen in wild-type cells. Instead, in the milder mutants (mutation in region B^{Ndc80p}; C^{Ndc80p}; or B^{Ndc80p} and C^{Ndc80p}) kinetochores were spread along the spindle and a few were detached. In the more severe mutants (mutation in region A^{Ndc80p}; A^{Ndc80p} and B^{Ndc80p}; A^{Ndc80p} and C^{Ndc80p}; A^{Ndc80p}, B^{Ndc80p} and C^{Ndc80p}; or Ndc80-depleted) few kinetochores were associated with the spindle. Instead, most were detached from the microtubules between the two SPBs (Figure 2.16).

Unlike wild-type *NDC80*, Dad4-CFP in mutant Ndc80 cells decorated the spindle but concentrated poorly at the Mtw1-YFP clusters that did occur (Figure 2.15B and Figure 2.16). In wild-type *NDC80* cells, an increase or decrease in Mtw1-YFP fluorescence correlated with an increase or decrease in Dad4-CFP fluorescence, respectively; however, Ndc80-depleted cells showed poor correlation. One or multiple mutations in region A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} resulted in significantly decreased correlation between Mtw1-YFP and Dad4-CFP (Figure 2.15C). Single mutations in region B^{Ndc80p} or C^{Ndc80p} resulted in less severe defects. These observations suggest that mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} had little effect on binding of Dam1 complex to the microtubules, but disrupted the ability of Ndc80 to recruit Dam1 complex to the kinetochore *in vivo*, thereby causing severe defects in kinetochore attachment and biorientation.

Discussion

The discovery that the Dam1 complex forms oligomers and rings around microtubules galvanized the mitosis field by providing a molecular explanation for how kinetochores are able to maintain processive attachment to the flared ends of a depolymerizing microtubule. Rings are not required for the Dam1 complex to track depolymerizing microtubules in the absence of

tension (Gestaut et al., 2008; Grishchuk et al., 2008). However, oligomerization of the Dam1 complex is required to maintain attachments under tension as would be experienced during metaphase (Umbreit et al., 2014; Volkov et al., 2013). Prior models of the kinetochore have all assumed that each kinetochore contains only one Dam1 complex ring. Here we show that the Ndc80 complex bridges two rings of the Dam1 complex *in vitro*. Lethal mutations in Ndc80p that block binding of either one of the two rings result in loss of the Dam1 complex from the kinetochore and failure in biorientation and attachment of kinetochores to the mitotic spindle. These results suggest that faithful microtubule attachments require two Dam1 complex rings per kinetochore *in vivo*.

Counting of kinetochore components by fluorescence microscopy has yielded conflicting results for the number of Dam1 complexes at the yeast kinetochore depending on the standard used. Assuming two Cse4 histone molecules per kinetochore, Joglekar and coworkers report enough Dam1 complexes to form one ring at a kinetochore during metaphase and a partial ring during anaphase (Aravamudhan, Felzer-Kim, & Joglekar, 2013; Joglekar, Bouck, Molk, Bloom, & Salmon, 2006). Lawrimore and coworkers measured 5.5 Cse4 histone molecules per kinetochore, giving enough Dam1 complex for two rings at a kinetochore during anaphase (Lawrimore et al., 2011); whereas Coffman and coworkers measured 8 Cse4 histone molecules per kinetochore (Coffman et al., 2011). Our results show that the Ndc80 complex can bind two Dam1 rings *in vitro*. Given the results of the latter two groups, we propose that there are two Dam1 complex rings at the kinetochore.

The region before the hairpin and the loop region of Ndc80p have both been identified as binding sites for the Dam1 complex (Lampert et al., 2013; Maure et al., 2011). Our results indicate that the interaction between the Ndc80 and Dam1 complexes is more extensive forming a tripartite network. The C-terminal domains of Dam1p, Ask1p, and Spc34p interact with three distinct regions of Ndc80p, one of which is the hairpin consistent with the previous report (Lampert et al., 2013). Our results are also consistent with a previous study showing an

interaction between Ndc80p and the C-terminal region of Dam1p (Kalantzaki et al., 2015).

However, we did not find any evidence for an interaction between the Ndc80p 'loop' region and Dam1p (Maure et al., 2011). The inability of the loop deletion mutant to assemble the Dam1 complex into the kinetochore might be due to an indirect effect.

Comparing the predicted distances between regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p}, and the 33 nm Dam1 complex inter-ring distance from EM experiments suggests that regions A and B contribute to the Ndc80 complex interaction with the ring closer to the N-terminus of Ndc80p/Nuf2p, while region C contributes to the Ndc80 complex interaction with the second ring (Figure 2.17). Our TIRF microscopy and live cell imaging results suggest that disrupting interaction A is more severe than disrupting interaction B or C. A recent computational model of the structure of the Dam1 complex bound to microtubules positions region A^{Dam1p} and B^{Ask1p} near the microtubule surface, while region C^{Spc34p} is positioned away from the microtubule surface (Zelter et al., 2015). Accordingly, our model of the interaction between Dam1 and Ndc80 complexes shows the Ndc80 complex positioned under the first Dam1 ring and above the second Dam1 complex ring further from Ndc80p N-terminus (Figure 2.17).

Aberrant kinetochore-microtubule attachments are phosphorylated and destabilized by Aurora B kinase (Biggins et al., 1999; Cheeseman et al., 2002; Hauf et al., 2003; Pinsky et al., 2006; Tanaka et al., 2002). Aurora B kinase phosphorylation of the Dam1p, Ask1p, and Spc34p C-terminal sites together fully disrupts the interaction between the Dam1 and Ndc80 complexes (Tien et al., 2010). This regulation mechanism has been thought of as binary, either full or no disruption of the interaction. We show that phosphorylation of certain combinations results in only partial disruption and full disruption does not require phosphorylation of all three proteins. Our results suggest how the Aurora B kinase might fine tune the interaction between Dam1 and Ndc80 complexes, resulting in a range of disruption.

The inability of *ndc80*^{10hep} and *nuf2*^{10hep} together to support growth *in vivo* might be explained in several ways. The addition of extra heptad repeats in the coiled-coil domains might

destabilize formation of the heterotetrameric Ndc80 complex, for example. However, we do not currently favor this hypothesis because the extra-long mutant version of Ndc80p is expressed *in vivo*, and because the Ndc80^{10hep} complex is stable and binds to both microtubules and to the Dam1 complex *in vitro*, similar to wild-type Ndc80 complex. Unnatural orientation of the Ndc80p/Nuf2p globular head in the Ndc80^{10hep} complex, due to the addition of extra heptad repeats, might also disrupt the specific geometry that the Ndc80 complex takes in relation to other kinetochore proteins. However given the presence of the flexible loop in Ndc80p, it seems unlikely that the two ends of the complex are held in a rigid orientation relative to each other. Compared to the wild-type Ndc80 complex, the Ndc80^{10hep} complex further separates the Dam1 complex rings *in vitro*. We propose that the specific 33 nm Dam1 complex inter-ring distance is vital. The transition from 13 nm, in the absence of the Ndc80 complex, to 33 nm Dam1 complex inter-ring distance could signal the establishment of kinetochore-microtubule attachment or biorientation. Deviation from the specific 33 nm distance might disrupt a yet unknown mechanism for detecting kinetochore-microtubule attachments, or bioriented attachments, or both. Future work will focus on characterizing the specific effects of the extra-long Ndc80 complex and the role of the Dam1 complex inter-ring distance *in vivo*.

Materials and methods

Protein expression and purification

The *S. cerevisiae* Ndc80 and Dam1 complexes were independently expressed in *E. coli* using polycistronic vectors, as previously described (Gestaut et al., 2008; Miranda et al., 2005; Powers et al., 2009; Tien et al., 2010; Wei et al., 2005). The Dam1 complex component Spc34p C-terminus was tagged with Hisx6 and the Ndc80 complex component Spc24 N-terminus was tagged with FLAG or Hisx6. Each complex was affinity-purified before further purification

through gel filtration. For TIRF microscopy, Nuf2p C-terminus of the Ndc80 complex was tagged with GFP.

Dam1 complex phosphorylation

GST-Ipl1 and GST-Sli15 (residues 554-698) were purified as previously described (Gestaut et al., 2008; Tien et al., 2010; Zelter et al., 2015). GST-Ipl1 (pSB196, Sue Biggins, Fred Hutchinsin Cancer Research Center, Seattle, WA) and GST-Sli15 (residues 554-698) (pSB503, Sue Biggins) were expressed at 23°C and 37° C, respectively for 2 hours. GST-Ipl1 was purified using GSTrap HP (GE Healthcare Biosciences) following manufacturer's instructions, except that the elution buffer was 50 mM Tris buffer (pH 8.0), 250 mM KCL, 10 mM glutathione. HiTrap 26/10 Desalting column (GE Healthcare) was used to exchange the buffer to 50 mM HEPES buffer (pH 7.4), 100 mM NaCl. GST-Sli15 was purified with glutathione-Sepharose 4B resin (GE Healthcare) following manufacturer's instructions, except that the elution buffer was 20 mM Tris buffer (pH 8.0), 200 mM NaCl, 1 mM β -mercaptoethanol, 1 mM EDTA, 10 mM glutathione.

4 μ M recombinant Dam1 complex was incubated with 0.5 μ M GST-Ipl1, 0.5 μ M GST-Sli15, 200 mM NaCl, 10 mM ATP, 25 mM $MgCl_2$, and 50 mM HEPES buffer, pH 7.4. Reaction mixtures were incubated for 90 min at 30°C. Under these conditions, we achieve nearly stoichiometric phosphorylation of the complex (Gestaut et al., 2008). Mock treated (non-phosphorylated) controls of the Dam1 complex was carried out by substituting ATP with dH_2O .

TIRF microscopy

Glass slides and functionalized coverslips were used to construct flow chambers, as reported previously (Gestaut et al., 2008; Gestaut et al., 2010; Tien et al., 2010; Zelter et al., 2015). A coverslip was adhered to a glass slide with double-sided tape, forming a flow chamber between two adjacent strips of tape. 'Rigor' kinesin was flowed through the chamber to non-specifically bind to the coverslip. Taxol-stabilized, Alexa-647-labelled microtubules were then

flowed in and incubated for 5 minutes for immobilization. For all TIRF microscopy experiments, Ndc80-GFP complex was incubated at 50 pM for single-molecule imaging. Each phosphorylated or mock-treated Dam1 complex mutant construct was incubated at 2.5 nM. GFP and Alexa-647 fluorescence channels were simultaneously recorded using a custom TIRF imaging system (Gestaut et al., 2010). All TIRF microscopy experiments were carried out in BRB80 (80 mM PIPES buffer (pH. 6.8), 1 mM EGTA, 1 mM MgCl₂) in the presence of 8 mg/ml BSA, 0.04 mg/ml κ-casein, and an oxygen scavenger system (200 μg/ml glucose oxidase, 35 μg/ml catalase, 25 mM glucose, and 5 mM DTT). Each experimental condition was assayed between three and seven times yielding between 401 and 2051 measurements. This sample size adequately identified possible differences between different conditions.

Analysis of the single particle tracking was carried out as previously described (Tien et al., 2010; Umbret et al., 2014; Zelter et al., 2015). Custom analysis software was developed in Labview (National Instruments) and Igor Pro (Wavemetrics). Bootstrapping analysis was used to calculate the mean residence times (Umbreit et al., 2014). Randomly resampling each dataset (residence times from each TIRF microscopy experimental condition) with replacement was repeated 1,000 times. Carrying out this method with each dataset yielded normal distributions. Gaussian fits to these distributions were used to estimate the mean residence times and the width of the fit was used as an estimate of error. Statistical analysis of the TIRF data was performed with pairwise z-tests. P-values were computed from the pairwise z-scores: $z = (\mu_1 - \mu_2) \cdot (\delta_1^2 + \delta_2^2)^{-0.5}$. μ_1 and μ_2 are bootstrap average residence times of Ndc80-GFP complex. δ_1^2 and δ_2^2 are the corresponding estimates of error. Tables of pairwise comparisons can be found in Table 2.2 – Table 2.5 and Table 2.7.

Chemical Cross-linking and Mass Spectrometry Analysis (XL-MS)

XL-MS experiment was performed twice, using EDC and DSS cross-linking agents (biological replicate). The two experiments identified similar interaction regions between the

Dam1 and Ndc80 complexes. XL-MS was performed as described in Zelter et al. 2015. Briefly, 10 µg of taxol stabilized microtubules were mixed with 10 µg Dam1 complex and 10 µg Ndc80 complex in 100 µL BRB80 at 25°C and allowed to stand for 5 mins. For DSS cross-linking, 3 µL 14.5 mM DSS in DMSO was added, and the mixture allowed to cross-link for 2 mins at 25°C before quenching by addition of 10 µL 0.5 M NH_4HCO_3 . For EDC cross-linking 7.5 µL 145 mM EDC plus 3.75 µL 145 mM Sulfo-NHS were added to the reaction, and the mixture allowed to cross-link for 30 mins at 25°C before quenching by addition of 10 µL 0.5 M NH_4HCO_3 . Quenched reactions were spun at 58,000 rpm in a TLA100 rotor for 10 minutes at 37°C. The pellet was resuspended in 100 µL ice cold buffer reduced with 10 mM dithiothreitol (DTT) at 37°C for 30 mins followed by 30 mins alkylation at RT with 15 mM iodoacetamide (IAA). Digestion with trypsin at a substrate to enzyme ratio of 60:1 was performed overnight at room temperature with shaking. Digested samples were acidified with 5 M HCL prior to being stored at -80°C until analysis. Mass spectrometry and data analysis was performed on either a Q-Exactive or Q-Exactive HF (Thermo Fisher Scientific) as previously described (Zelter et al., 2015). Each cross-linked sample was run four times and the data were combined before analysis (technical replicate). Mass spectra were converted into mzML using msconvert from ProteoWizard (Chambers et al., 2012). Standard linear peptide searches were performed using Comet to identify all proteins in the sample (Eng et al., 2013). Cross-linked peptides were identified using the Kojak version 1.4.2 cross-link identification software (Hoopmann et al., 2015) following the author's instructions (<http://www.kojak-ms.org>). Kojak results were exported to Percolator version 2.08 (Käll et al., 2007) to produce a statistically validated set of cross-linked peptide spectrum matches (PSMs) at the desired false discovery rate (FDR) threshold. Percolator (RRID: SCR_005040) is a semi-supervised algorithm that assigns a statistically meaningful q value to each PSM through analysis of the target and decoy PSM distributions. Decoy PSMs derive from peptide sequences known to be false. In the current work the target databases consisted of all proteins identified in the sample analyzed, while the decoy databases

consisted of the corresponding set of reversed protein sequences. Cross-link PSMs were considered to be false if at least one of the peptides was from a decoy protein sequence. The data presented in this paper was filtered to show only hits to the target proteins that had a Percolator assigned peptide level q value ≤ 0.05 . The complete, unfiltered list of all PSMs and their Percolator assigned q values, are available on the ProXL web application (Riffle et al., 2016) at: http://proxl.yeastrc.org/proxl/viewProject.do?project_id=24 along with the raw MS spectra and search parameters used.

Electron Microscopy

Cleared tubulin was polymerized in a total volume of 40 μ l BRB80 (80 mM PIPES buffer (pH 6.8), 1 mM EGTA, 1 mM $MgCl_2$) containing 1.75 mM GTP, 1 mM $MgCl_2$, and 3.5% DMSO at 37°C for 30 min. Microtubules were pelleted and resuspended in BRB80 containing 10 μ M taxol. All samples were prepared in BRB80 + 10 μ M taxol by mixing 20 nM microtubules, and 25 nM Dam1 in the absence or presence of 25 nM Ndc80 complex. Samples were incubated at room temperature for 15 minutes. Carbon-coated copper grids were negatively discharged in a glow discharge device. A 5 μ l volume of sample was applied on a discharged grid for 20 s before being blotted. 6 μ l 2% uranyl acetate was then applied on the grid and incubated for 1 min. The grid was blotted and air dried. EM samples were viewed on a transmission electron microscope (Morgagni; FEI) operating at 100 kV. Images were recorded on a bottom-mounted Orius (Gatan) camera at 22,000x magnification. ImageJ was used to measure the Dam1 complex inter-ring distances. Distances from middle of a ring to the middle of the next closest ring, on both sides, were measured.

The EM microscopy experiments were performed with two independent copper grids. Similarity between the two replicates allowed us to combine the data. Statistical analysis of the EM data was performed with pairwise z-tests. P-values were computed from the pairwise z-scores: $z = (\mu_1 - \mu_2) \cdot (\delta_1^2 + \delta_2^2)^{-0.5}$. μ_1 and μ_2 are the center of the Gaussian fits around the single

large cluster in each sample and δ_1^2 and δ_2^2 are the corresponding estimates of error. A table of pairwise comparisons can be found in Table 2.5.

Yeast strain construction and validation

Strains used in this study are derivatives of W303 (Table 2.9). We have previously verified the genotype of the W303 by whole-genome tiling as described (Gresham et al., 2006). For strains constructed for this study, *NDC80* alleles were integrated at the *URA3* locus as previously described (Widlund & Davis, 2005). Plasmids containing the *NDC80* alleles were sequenced before transformation. Successful integration was verified by PCR. Genes were tagged with genes encoding fluorescent proteins as described (Wach et al., 1997) and verified by PCR.

Test for function of Ndc80^{10hep} complex

To test if the Ndc80^{10hep} complex is functional *in vivo*, we used a red/white plasmid shuffle assay (Davis, 1992; E. G. Muller, 1996) using strain JTY5-8B (*ade2-1oc ade3Δ-100 leu2-3,112 ura3-1 ndc80Δ:: natMX*), harboring pJT12 (*NDC80 ADE3* in a 2-μm vector). JTY5-8B was transformed with pJOK13 (*URA3 ndc80^{10hep}*) and selected for growth on SD-ura low adenine plates. Transformation with pJOK13 yielded non-sectored red colonies (strain JOKY3) demonstrating that the *ndc80^{10hep}* is not viable when paired with wild-type *NUF2* (data now shown). JOKY3 was then grown in SD-lys-ura media before being transformed with pJOK018 (*LEU2 nuf2^{10hep}*), pJOK019 (*LEU2 NDC80*), or pRS315 (*LEU2*) and plated on SD-ura-leu plates. Four colonies from each transformation were streaked onto SD-ura low-ade plates to screen for sectoring colonies (technical replicate). Two transformations were performed for each condition (biological replicate), both trials gave the same result.

Yeast live-cell imaging and analysis

Strains for live-cell imaging were constructed using previously described strains: *SPC110-mCherry*, *pCUP1-GFP-LacI12* and *CEN3::33LacO* (Wargacki et al., 2010); *NDC80-3V5-IAA7* and *pGPD1-TIR1* (Miller et al., 2016). Sue Biggins kindly provided the Ndc80-AID strain. Strains containing *NDC80*, *ndc80-314* (ins A^{Ndc80p}), *ndc80-383* (ins B^{Ndc80p}), or *ndc80-563* (ins C^{Ndc80p}) were constructed through an integration method previously described (Widlund & Davis, 2005). YFP and CFP were integrated into Mtw1 and Dad4, respectively, through homologous recombination with PCR products amplified with primers containing homologous sequence of its target locus.

Asynchronously growing cells (30 Klett units) were arrested with 6 μ M α -factor for 2 hours. Auxin (IAA (0.5 mM) was then added and the incubation continued for an additional hour. The cells were released from α -factor into YPD + 0.5 mM IAA medium and imaged at 15 minute time-intervals from time 0 to 120 min. At each time point, cells were also fixed in formaldehyde for budding index analysis. Budding index was determined twice (from two different colonies) and yielded the same conclusion for each of the five yeast strains shown in Figure 2.12. Data is shown for one of the two experiments. Cells were placed on agar pads prior to imaging, as previously described (Muller et al., 2005). Cells were imaged using a DeltaVision system (Applied Precision) equipped with IX70 inverted microscope (Olympus), a Coolsnap HQ digital camera (Photometrics), and a U Plan Apo 100X objective (1.35 NA).

For CEN3 tracking biorientation assay, *CEN3-LacI* was visualized with *GFP-LacO* and the spindle pole bodies were visualized with *SPC110-mCherry*. Both GFP and mCherry were imaged with 0.4 s exposures with 16, 0.2 μ m z-sections. Images were binned 2 x 2 with a final resolution of 512 x 512. Biorientation analysis was done manually for those cells with 2 mCherry puncta using Imaris software (Bitplane) (RRID: SCR_007370). For all the strains tested, the 60 min time point was chosen for data analysis. The biorientation assay was performed twice for

each strain, each time with a different colony and on a different day. The combination of two experiments yielded sample sizes between 190 and 260 cells.

For Dam1 complex kinetochore localization assay, kinetochore, Dam1 complex, and spindle pole bodies were visualized with Mtw1-YFP, Dad4-CFP, and Spc110-mCherry respectively. All three proteins were imaged with single focal plane 0.4 s exposures. Images were not binned with a final resolution of 1024 x 1024. Line scans analyses were obtained of Mtw1-YFP and Dad4-CFP fluorescence using Imaris software (Bitplane) (RRID: SCR_007370) and extended from one SPB to the other. For each line scan, a correlation of the direction of pixel intensity changes across the line between the Mtw1 and Dad4 channels was calculated to examine co-localization along the spindle. This was calculated as: $R = N_{\text{agree}} - N_{\text{disagree}} / N - 1$, where N_{agree} is the number of pixels that changed in the same direction, N_{disagree} is the number that changed in opposite direction and N is the length of the line scan. For each strain, a mean R was calculated from all line scans. Statistical analysis of the Mtw1-YFP and Dad4-CFP mean R -scores was performed with Student's t-test. A table of the pairwise comparisons can be found in Table 2.8.

Western blot analysis

During the yeast live-cell imaging, samples were collected for western blot analysis. After the addition of auxin, cell samples were collected at various time points. 0.7 ml cells and 0.7 ml 20% trichloroacetic acid were incubated at 4°C for 1 hour. Samples were pelleted for at 20,000xg for 10 min at 4°C. The pellet was washed by resuspending in 1 ml 4°C ethanol. The sample was then pelleted and resuspended once more before a final pelleting step. Supernatant was discarded and sample was air dried before resuspending in 50 µl 0.1N NaOH, SDS-PAGE sample buffer. Western blot analysis was performed, probing for Ndc80p (gift from Arshad Desai, Ludwig Cancer Research Center, University of California San Diego) and β-Actin (Abcam). Protein levels analysis was carried out using Image Studio Lite (Li-Cor) (RRID:

SCR_014211). Each western blot was performed twice, yielding the same conclusion for each experiment.

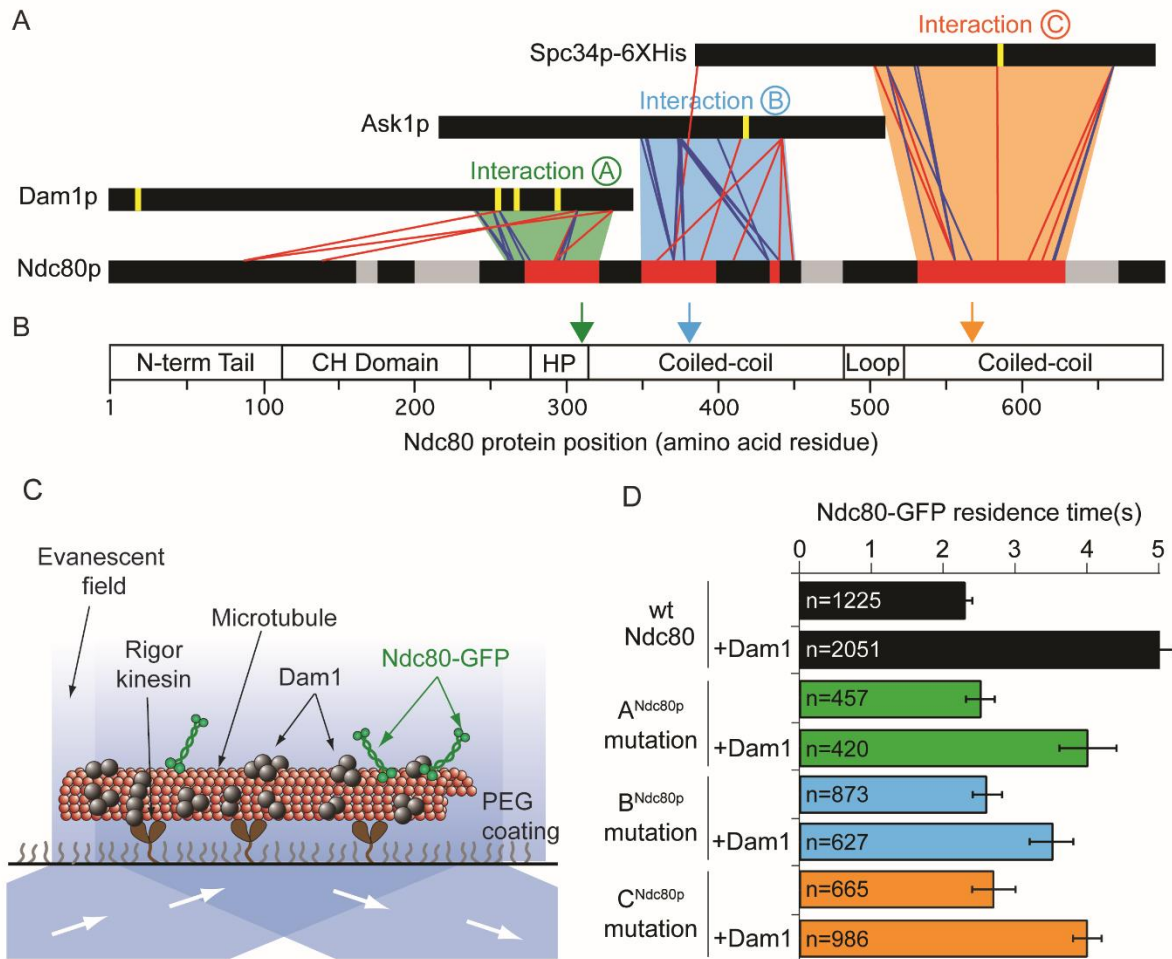


Figure 2.1

Dam1p, Ask1p, and Spc34p form cross-links to three distinct Ndc80p regions.

(A) Cross-links between the Dam1p, Ask1p, and Spc34p of the Dam1 complex and Ndc80p of the Ndc80 complex. Dam1 and Ndc80 complexes were cross-linked in the presence of microtubules. Horizontal black bars represent proteins and the six vertical yellow lines indicate Aurora B kinase phosphorylation sites on the Dam1 complex. Red and blue lines show cross-links formed with DSS and EDC cross-linkers, respectively. For clarity, only the cross-links between the Dam1 complex proteins and Ndc80p are displayed. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.05 . Red bars on Ndc80p indicate regions where clusters of lethal mutations mapped (from Tien et al., 2013) to cross-linked regions. Grey bars on Ndc80p indicate clusters of lethal insertions outside of cross-linked regions. Green, blue, and orange trapezoids represent putative interactions (A, B, and C) between the Dam1 and Ndc80 complexes. (B) Bar diagram of Ndc80p with structural features. Green, blue and orange arrows indicate the positions of lethal mutations in interaction regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} used in this study. (CH: calponin homology; HP: hairpin) (C) Diagram showing the setup of TIRF microscopy experiments. Single molecule Ndc80-GFP complex binding on microtubules was visualized in the presence or absence of the Dam1 complex. (D) Lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} partially disrupts the Ndc80 complex's interaction with Dam1 complex. Average residence time of Ndc80-GFP mutant and wild-type complexes on microtubules in the presence or absence of Dam1 complex. Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Methods for additional details). Refer to Table 2.2 for statistical analysis of data in part (D).

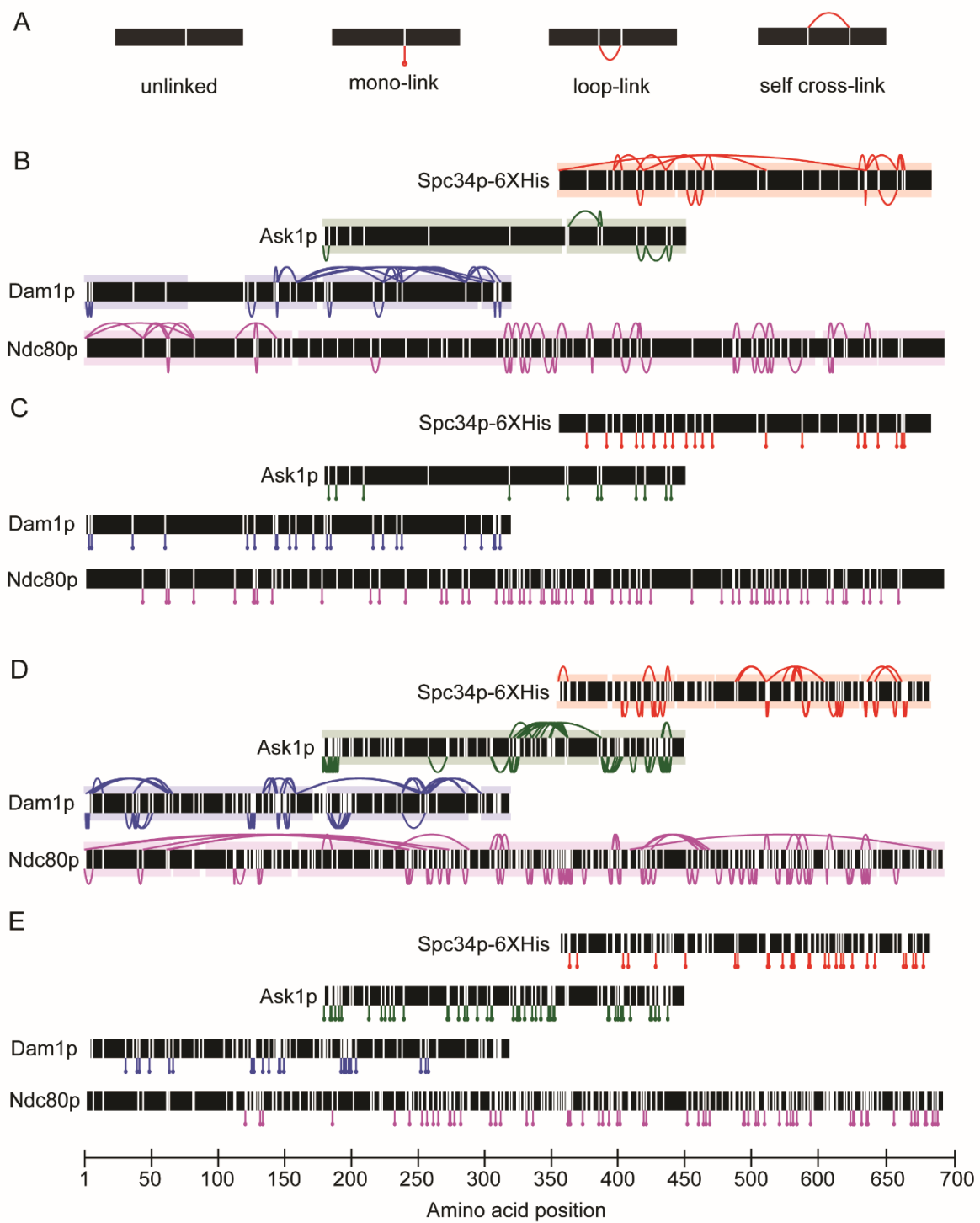


Figure 2.2

Dam1 and Ndc80 complexes robustly react with DSS and EDC cross-linking agents.

(A) Four types of peptides identified by mass spectrometry after cross-linking and trypsin digestion. Horizontal black bars represent protein sequence and vertical white lines represent residues able to react with the cross-linking agent. Unlinked: peptide that did not react with cross-linking agent; mono-link: only one end of the cross-linking reagent reacted with a residue; loop-link: intra-protein cross-link without a tryptic site in between the two cross-linked residues; self cross-link: intra-protein cross-link with a tryptic site in between the two cross-linked residues. Dam1 and Ndc80 complexes were cross-linked in the presence of microtubules using DSS (panels B and C) or EDC (panels D and E). (B, D) Cross-links above and below the black bars represent self-crosslinks and loop-links, respectively. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.05 . Highlighted regions above and below black bars represent sequence coverage from mass spectrometry. (C, E) Lines below the black bars represent mono-links.

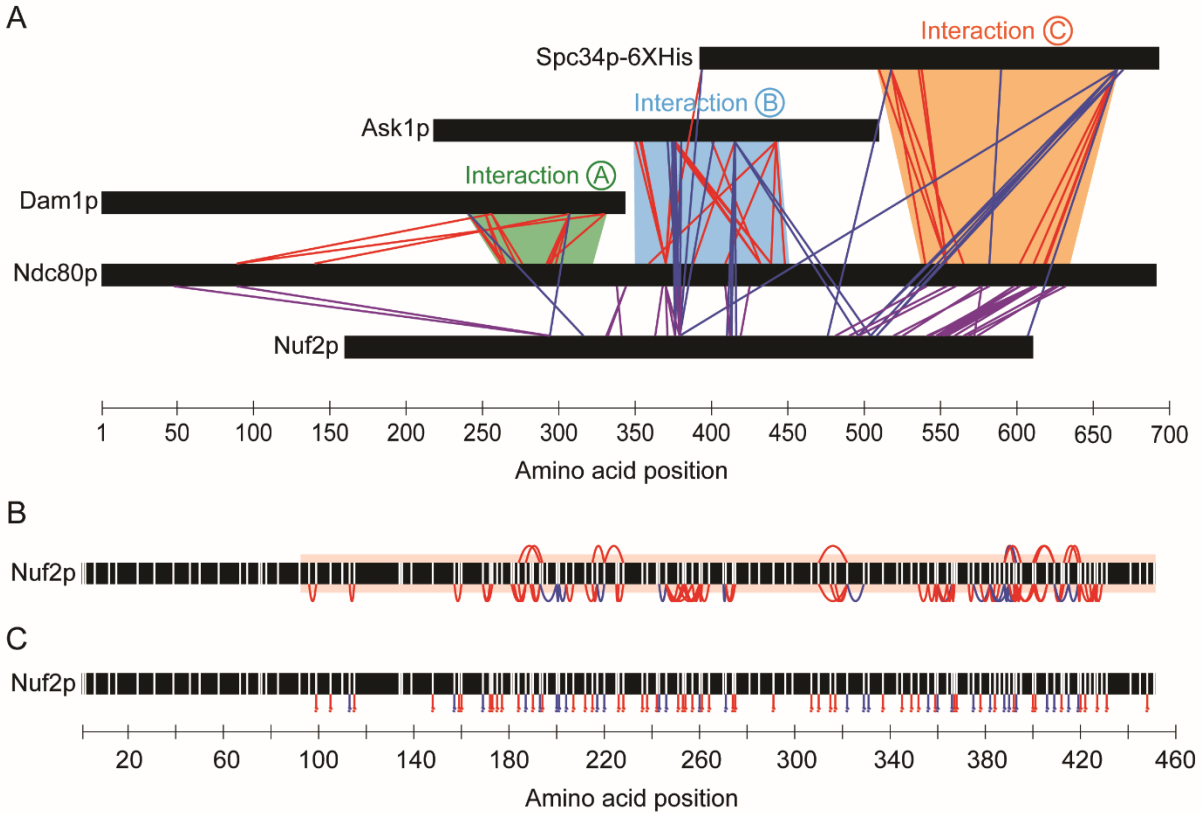


Figure 2.3
Dam1p, Ask1p, and Spc34p cross-link to Nuf2p, in agreement to the coiled-coil structure of Ndc80p and Nuf2p.
 (A) Cross-links between the Dam1p, Ask1p, and Spc34p of the Dam1 complex and Ndc80p and Nuf2p of the Ndc80 complex. For clarity, cross-links between the Dam1 complex proteins are not shown. Red lines represent cross-links between Ndc80p and Dam1p, Ask1p, or Spc34p. Blue lines represent cross-links between Nuf2p and Dam1p, Ask1p, or Spc34p. Purple lines represent cross-links between Ndc80p and Nuf2p. (B) Cross-links above and below the black bar represent self-crosslinks and loop-links, respectively, for Nuf2p. (C) Lines below the black bar represent mono-links for Nuf2p. Data from cross-linking experiments using DSS and EDC cross-linking agents are both shown. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.05 .

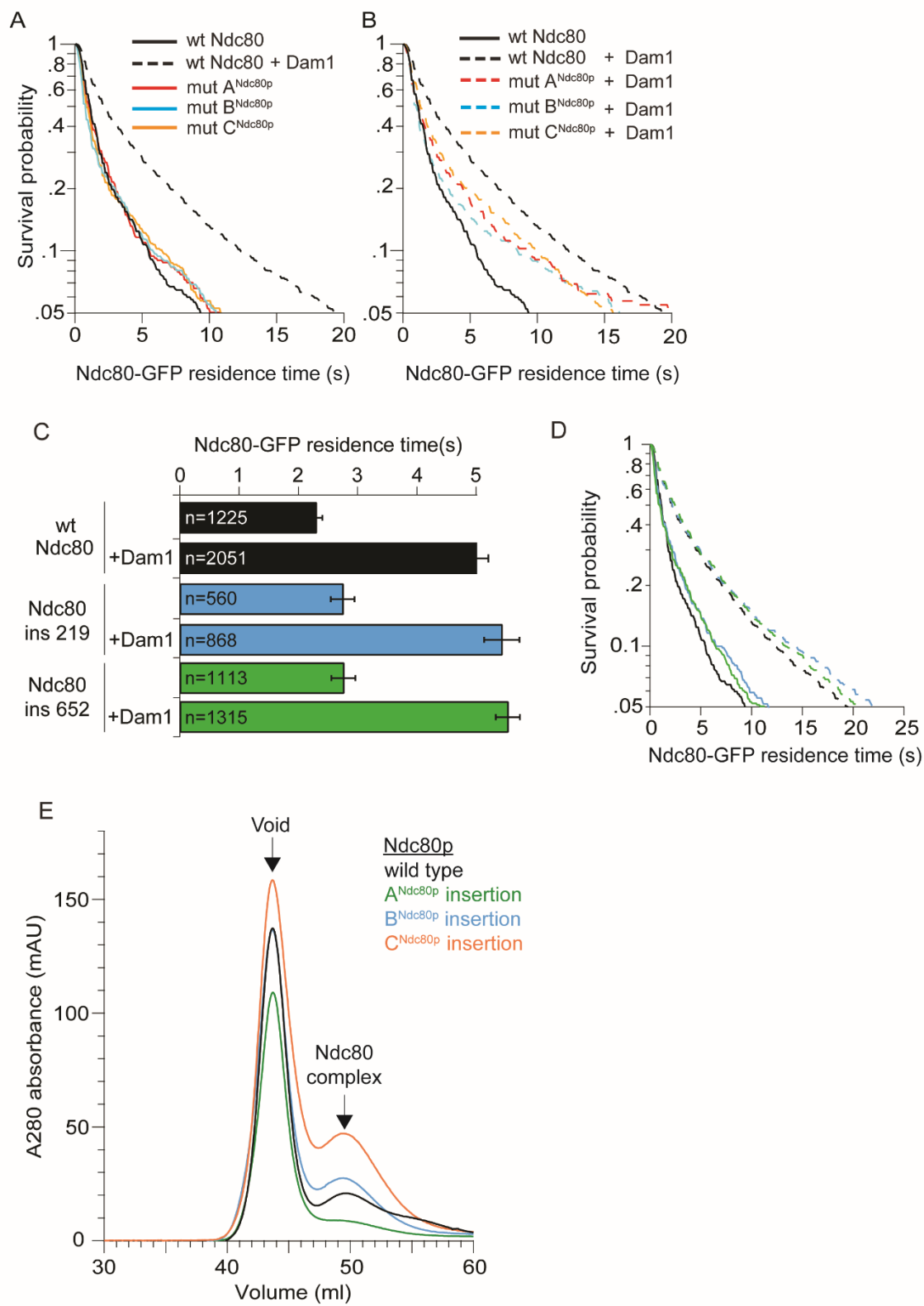


Figure 2.4

Ndc80p mutants A, B , and C have partially disrupted interaction with the Dam1 complex, while mutants ins 219 and ins 652 are not affected.

(A), (B) Survival probability of the data shown in Figure 2.1D: microtubule residence times of Ndc80-GFP complex with a lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the absence (A) and presence (B) of wild-type Dam1 complex. Data here have a range of n= 420 to 2051. (C) Average microtubule residence times of Ndc80-GFP complexes with wild-type Ndc80p, or lethal five amino acid insertional mutation at position 219 or 652. Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Methods for additional details). Refer to Table 2.3 for statistical analysis. (D) Survival probability curves of the data shown in (C). (E) Superdex 200 size exclusion chromatography of the Ndc80 complex with wild-type Ndc80p or insertional mutation in A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}.

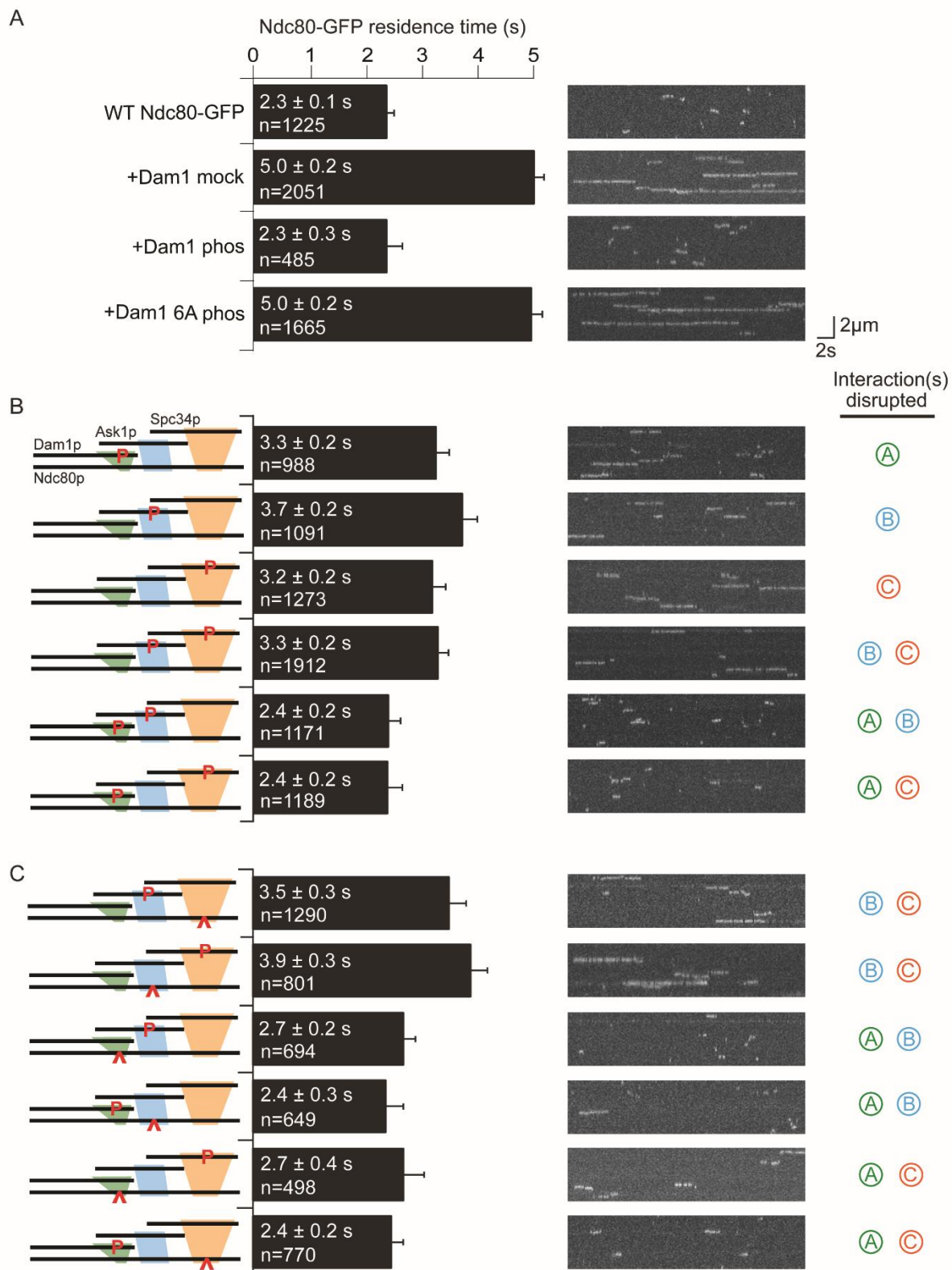


Figure 2.5

Dam1 and Ndc80 complexes interact through three distinct sites.

(A) Average microtubule residence time of the wild-type Ndc80-GFP complex alone, in the presence of mock treated, phosphorylated Dam1 complex, or phosphorylated Dam1 6A mutant complex. Dam1 6A: all six Dam1 complex phosphorylation sites mutated to Ala. (B) Average microtubule residence time of wild-type Ndc80-GFP complex in the presence of selectively phosphorylated Dam1 complex. Diagram on the left indicates phosphorylated proteins. (C) Average microtubule residence time of Ndc80-GFP complex with lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the presence of selectively phosphorylated Dam1 complex. Diagrams on the left indicate phosphorylated protein (P) and Ndc80p region with a lethal mutation (^). On the right are representative kymographs for each experiment. Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Methods or additional details). Refer to Table 2.4 for statistical analysis.

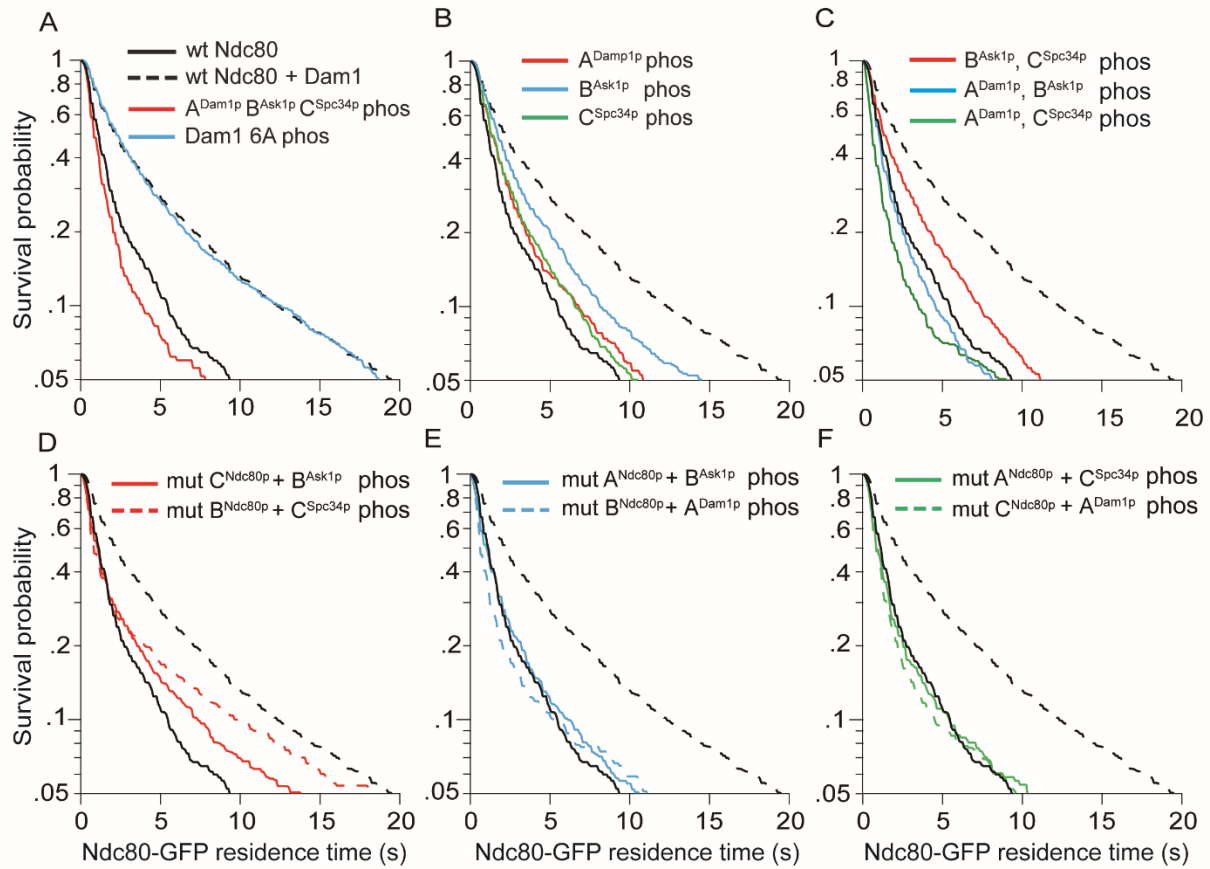


Figure 2.6

Microtubule residence time survival probability plots of experiments in Figure 2.

For all graphs, solid and dashed black lines represent curves for wild-type Ndc80 complex in the absence and presence of wild-type Dam1 complex, respectively. (A)-(C) Wild-type Ndc80-GFP complex microtubule residence time in the presence of variably phosphorylated Dam1 complex. Dam1 6A phos: all Dam1 complex phosphorylation sites mutated to Ala and treated with Aurora B kinase. (D)-(F) Microtubule residence time of Ndc80-GFP complex with a lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the presence of variably phosphorylated Dam1 complex. Data here have a range of n= 485 to 2051.

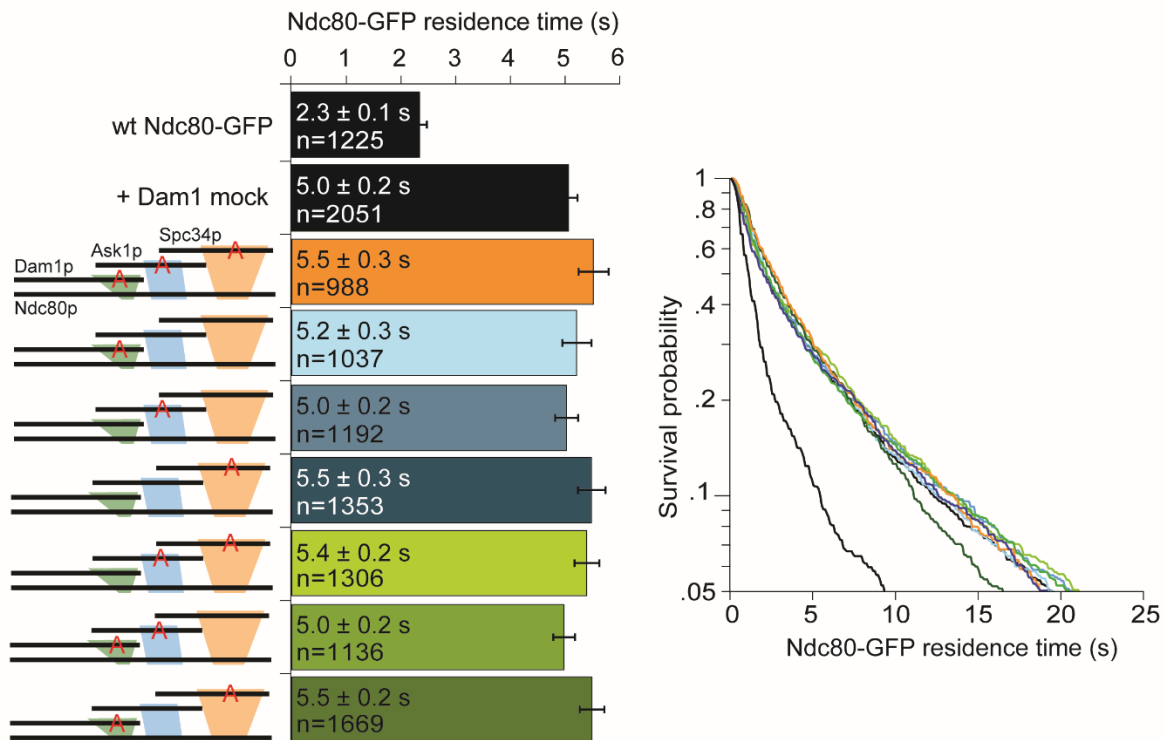


Figure 2.7
Mutating Dam1 complex Aurora B kinase phosphorylation sites to Ala does not affect the interaction between Dam1 and Ndc80 complexes.
 Average microtubule residence time of wild-type Ndc80-GFP complex in the presence of mock treated phosphorylation-blocking Dam1 complex mutant constructs. A red "A" identifies the phosphorylation sites that are mutated to alanine. Mock treatment was carried out by substituting ATP with H₂O. Survival probability plots of the average microtubule residence time shown on the right. Refer to Table 2.5 for statistical analysis.

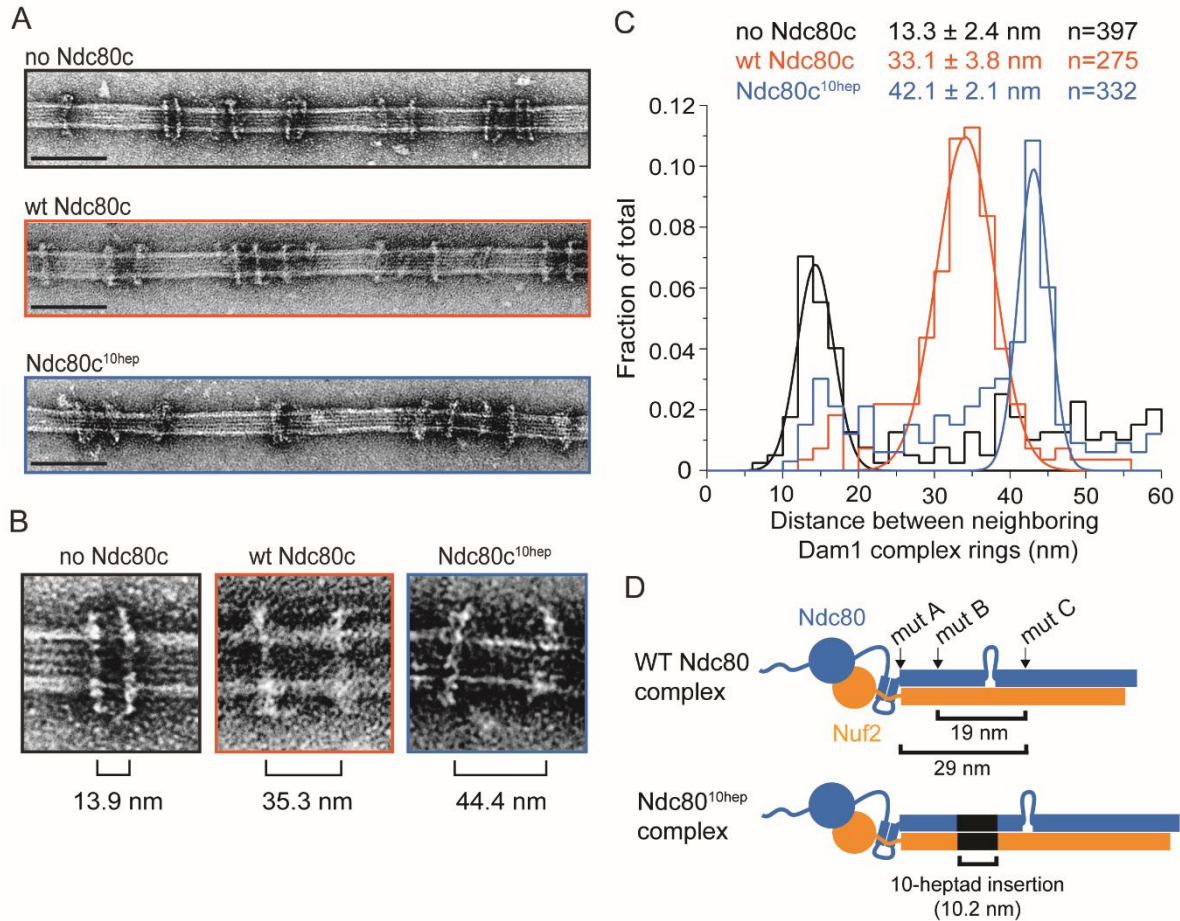


Figure 2.8

The Ndc80 complex binds two Dam1 complex rings.

(A) Representative EM images of Dam1 complex rings on microtubules in the absence or presence of the Ndc80 complex. Three experiments include no Ndc80 complex (Ndc80c), wild-type Ndc80c, and Ndc80c^{10hep}. Protein concentrations were 20 nM tubulin and 25 nM Dam1 and Ndc80 complexes. Scale bars: 100 nm. (B) Zoomed in images of a Dam1 complex ring doublet for the three experiments. Distances from middle of one ring to that of next ring are indicated. (C) Distribution of closest neighboring inter-ring distances measured for the three different conditions. Measurements made between 0-60 nm are shown. The cluster of distance measurements was fitted with a Gaussian distribution and the vertices ± standard deviations for each fit are listed. (D) Diagram demonstrating the predicted coiled-coil distances between the Ndc80p lethal mutations. Diagram below shows the location of additional 10-heptad insertion between the hypothesized binding sites of two Dam1 complex rings. Refer to Table 2.6 for statistical analysis of data in part (C).

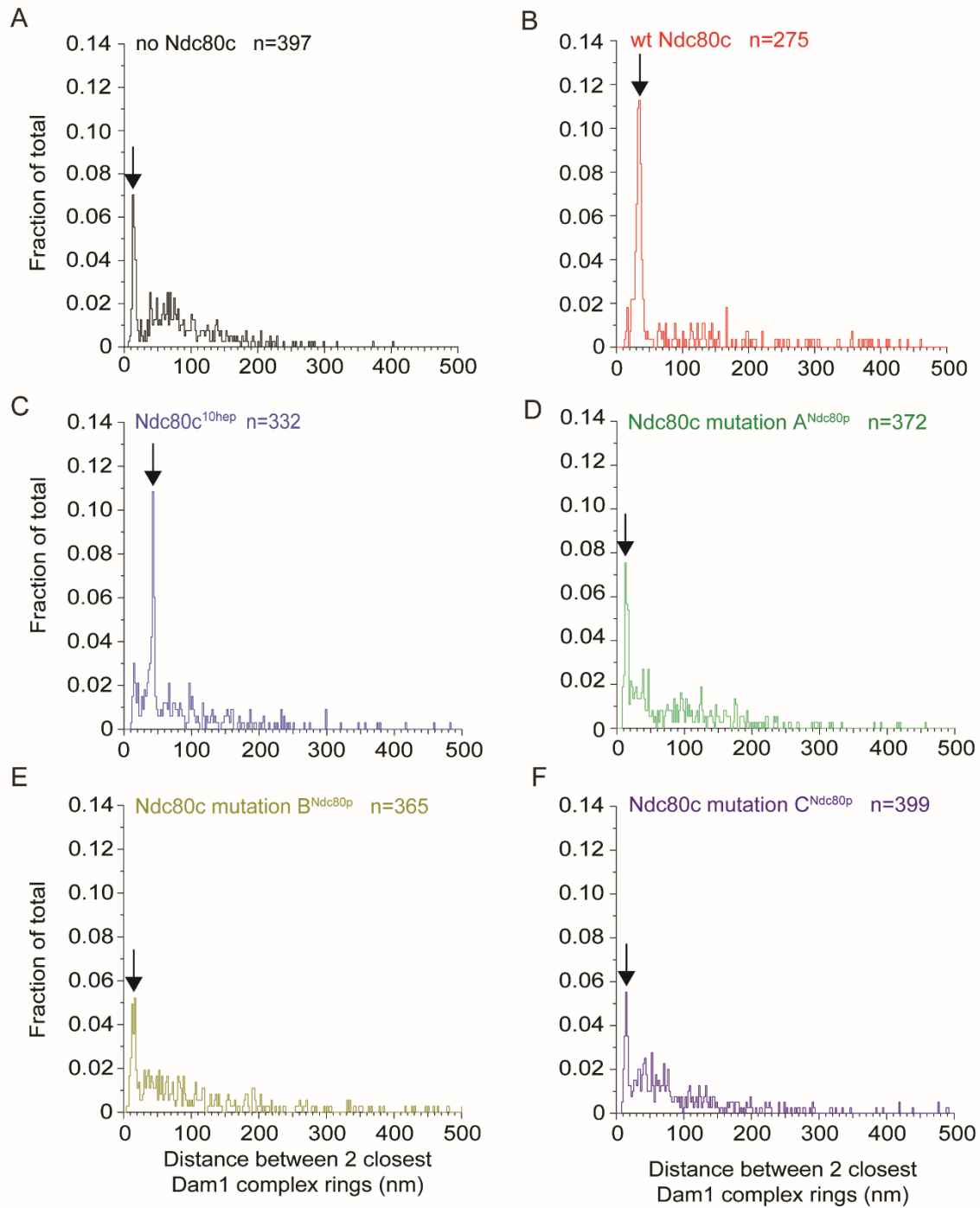


Figure 2.9

Full distributions of Dam1 complex inter-ring measurements for various experiments shown in Figure 2.8.

Six conditions include Dam1 complex rings on microtubules in the absence of Ndc80 complex (Ndc80c) (A), presence of wild-type Ndc80c (B), Ndc80c^{10hep} (C), Ndc80c lethal mutation A^{Ndc80p} (D), B^{Ndc80p} (E), and C^{Ndc80p} (F). The arrows identify the sharp peaks that contain at least 20% of the events.

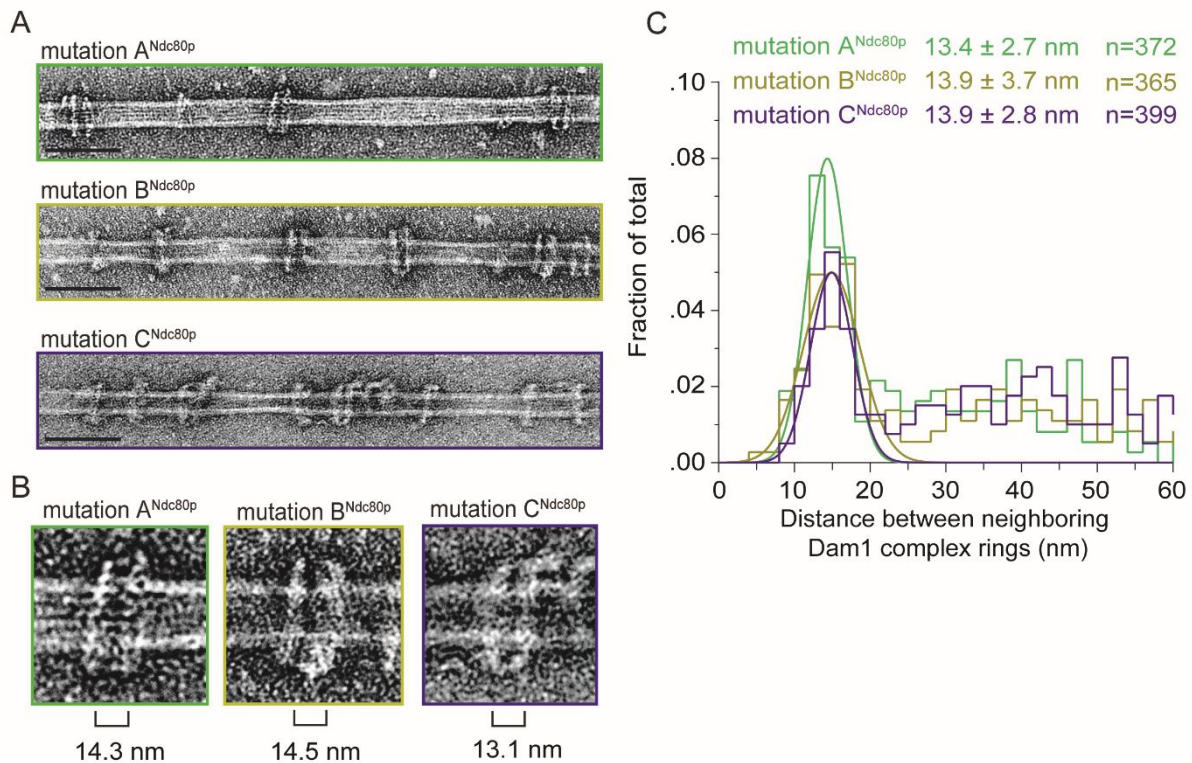


Figure 2.10

Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} disrupts the Ndc80 complex's ability to bind two Dam1 complex rings.

(A) Representative EM images of Dam1 complex rings on microtubules in the presence of the Ndc80 complex with a lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}. Scale bars: 100 nm. Protein concentrations were 20 nM tubulin and 25 nM Dam1 and Ndc80 complexes. (B) Zoomed in images of a Dam1 complex ring doublet for the three experiments. Distances from middle of one ring to those of the closest two rings were measured. (C) Distribution of closest inter-ring distances measured for the three experiments. Measurements made between 0-60 nm are shown. The cluster of distance measurements was fitted with a Gaussian distribution and the vertex ± standard deviation for each fit is listed. Refer to Table 2.6 for statistical analysis of data in part (C).

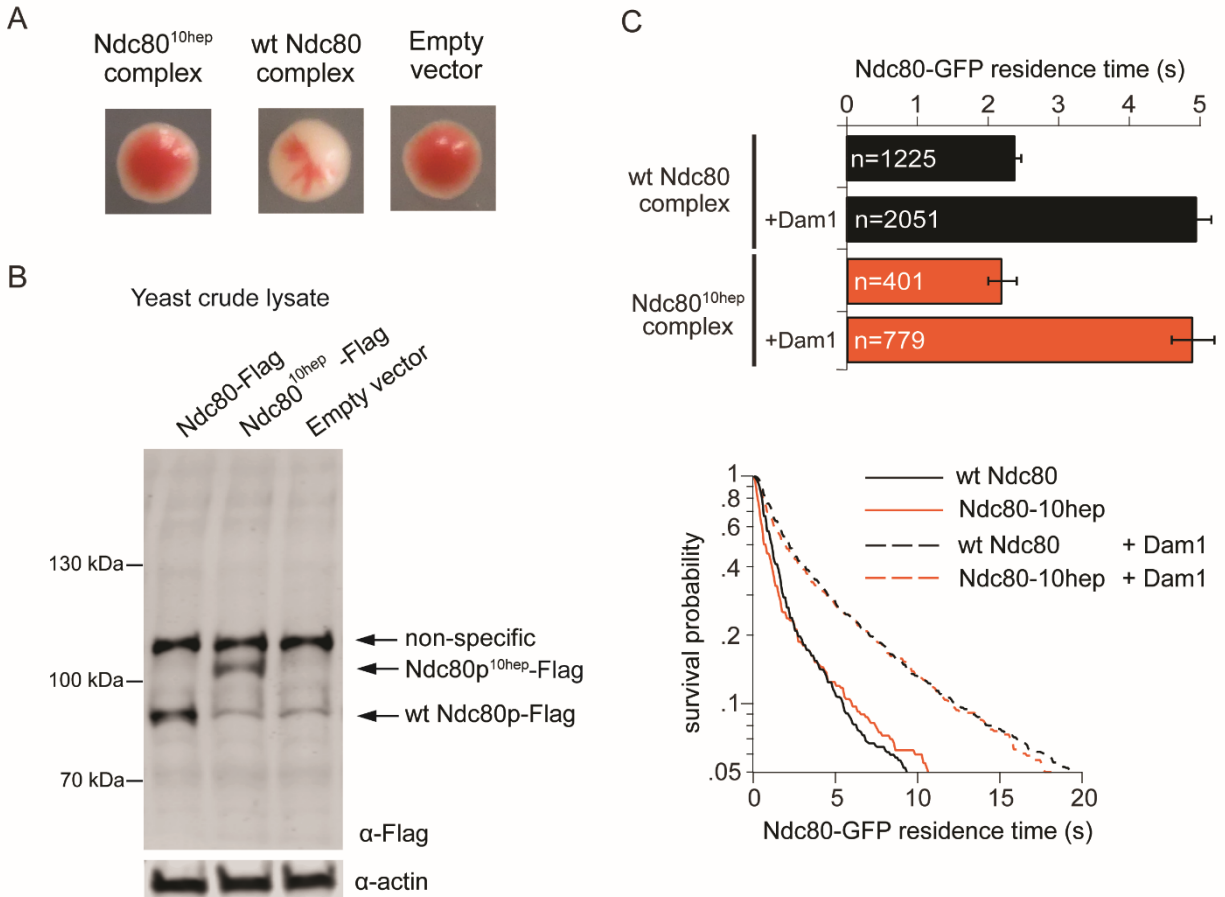


Figure 2.11

The Ndc80^{10hep} complex does not support growth.

(A) Red/white plasmid shuffle assay to test the viability of the Ndc80^{10hep} complex. Solid red colonies indicate the inability of the Ndc80^{10hep} complex and empty vector to support growth. Sectoring white colony indicates the ability of the wild-type Ndc80 complex to support growth. (B) α-FLAG immunoblot of crude lysate of cells expressing either wild-type Ndc80p, Ndc80p^{10hep}, or empty vector. α-actin immunoblot of the same lysates shows equal loading of the lysates. (C) Wild-type or Ndc80^{10hep} complex average residence time (top) on microtubules in the absence and presence of Dam1 complex as derived from the survival probability curves (bottom). Bars represent average residence time ± error of the mean (estimated by bootstrapping analysis; see Methods or additional details). Refer to Table 2.7 for statistical analysis of data.

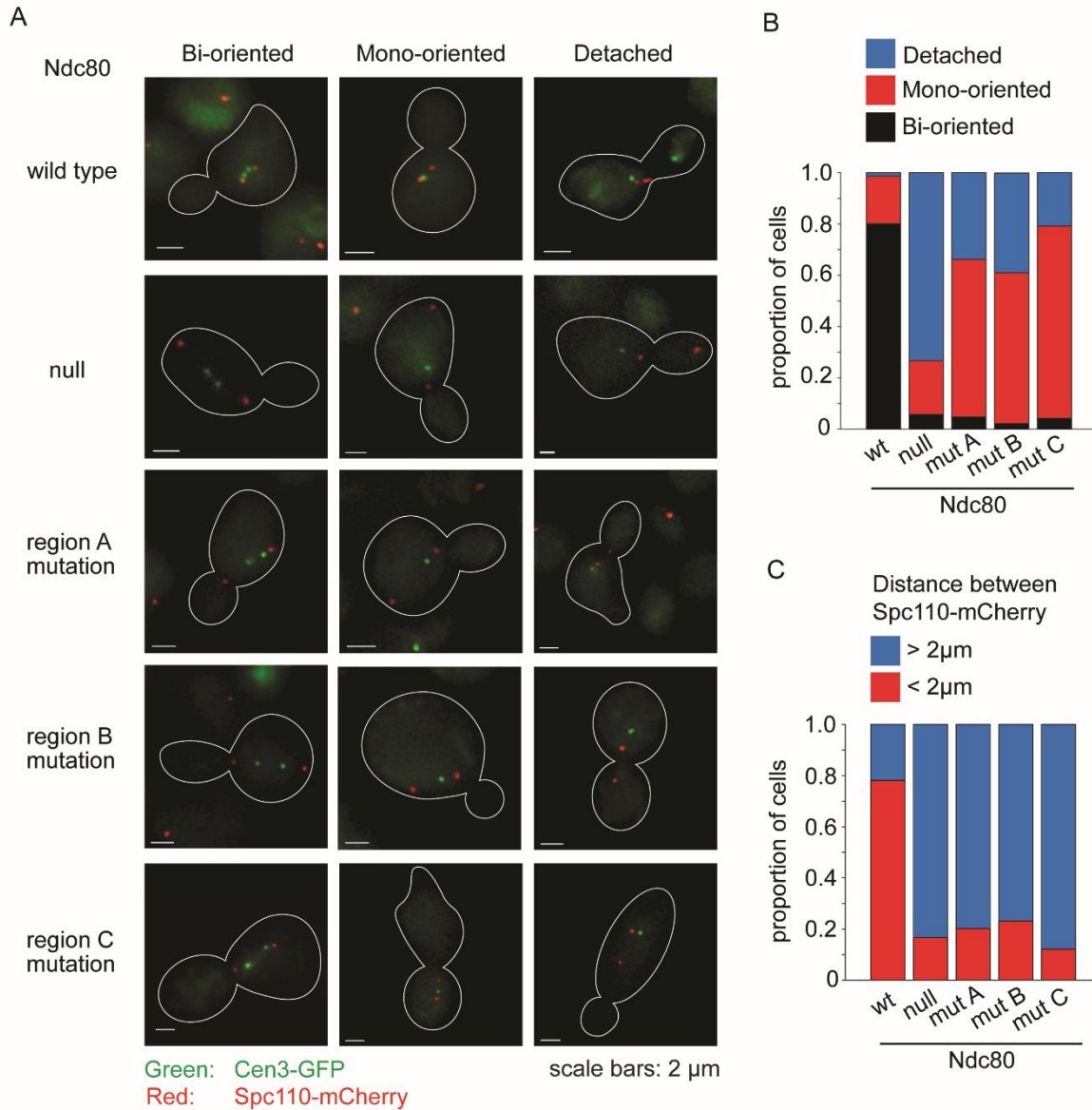
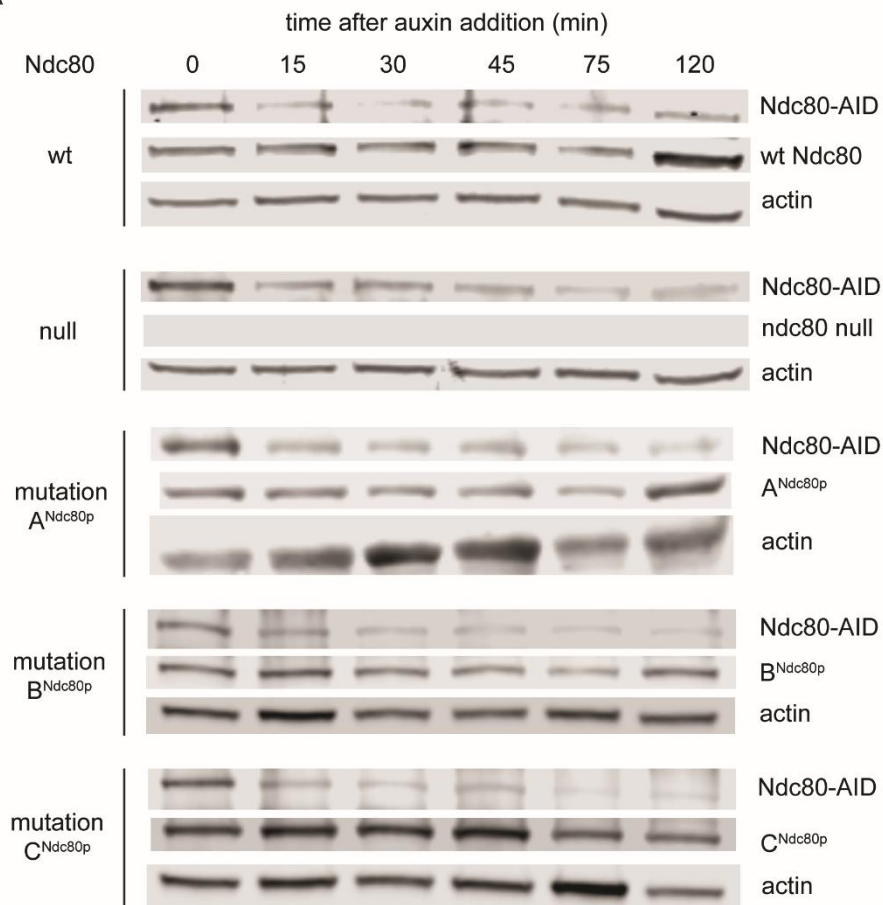


Figure 2.12

Lethal mutations in A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} have biorientation and microtubule attachment defects *in vivo*.

(A) Representative images from Ndc80-AID degron experiments with cells carrying an extra copy of *NDC80* wild-type, no *NDC80* (null), or a mutation in A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}. Cells were treated with 6 μ M α -factor for two hours prior to treatment with 6 μ M α -factor and 0.5 mM auxin for one additional hour. At time 0, cells were released from α -factor and incubated in YPD medium containing 0.5 mM auxin. Images were taken at 60 min after release from α -factor arrest. Representative images are shown. (B) Stacked bar graphs showing proportion of the cells containing bioriented, monooriented or detached CEN3-GFP. Spindles with two CEN3-GFP puncta positioned between the two SPBs were counted as bioriented. Spindles with one CEN3-GFP puncta positioned between the two SPBs were counted as monooriented and spindles with one (or rarely two) CEN3-GFP puncta positioned off the spindle axis were counted as detached. Only cells with two Spc110-mCherry puncta and at least one CEN3-GFP punctum were included in the analysis. (C) Stacked bar graph showing the distance between SPBs. Wild-type *NDC80* n=236 cells; no *NDC80* n=210 cells; A^{Ndc80p} n=210 cells; B^{Ndc80p} n=190 cells; C^{Ndc80p} n= 260 cells.

A



B

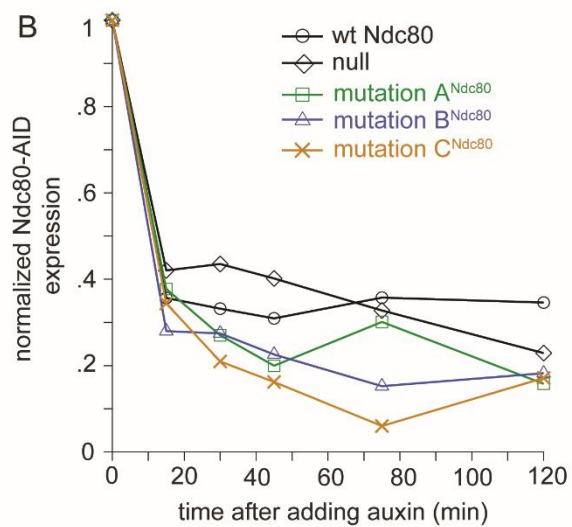


Figure 2.13

Wild-type Ndc80-AID is degraded upon the addition of auxin.

(A) Western blot images from Ndc80-AID degron experiments, blotting for Ndc80-AID and β -actin. Whole cell protein precipitation was carried out for Ndc80-AID cells carrying an extra copy of *NDC80* wild-type, no *NDC80* (null), lethal mutation in A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}. Samples were collected at various time points after adding auxin (Images were taken 120 min after auxin addition). (B) Normalized Ndc80-AID protein levels analyzed by western blot band intensities.

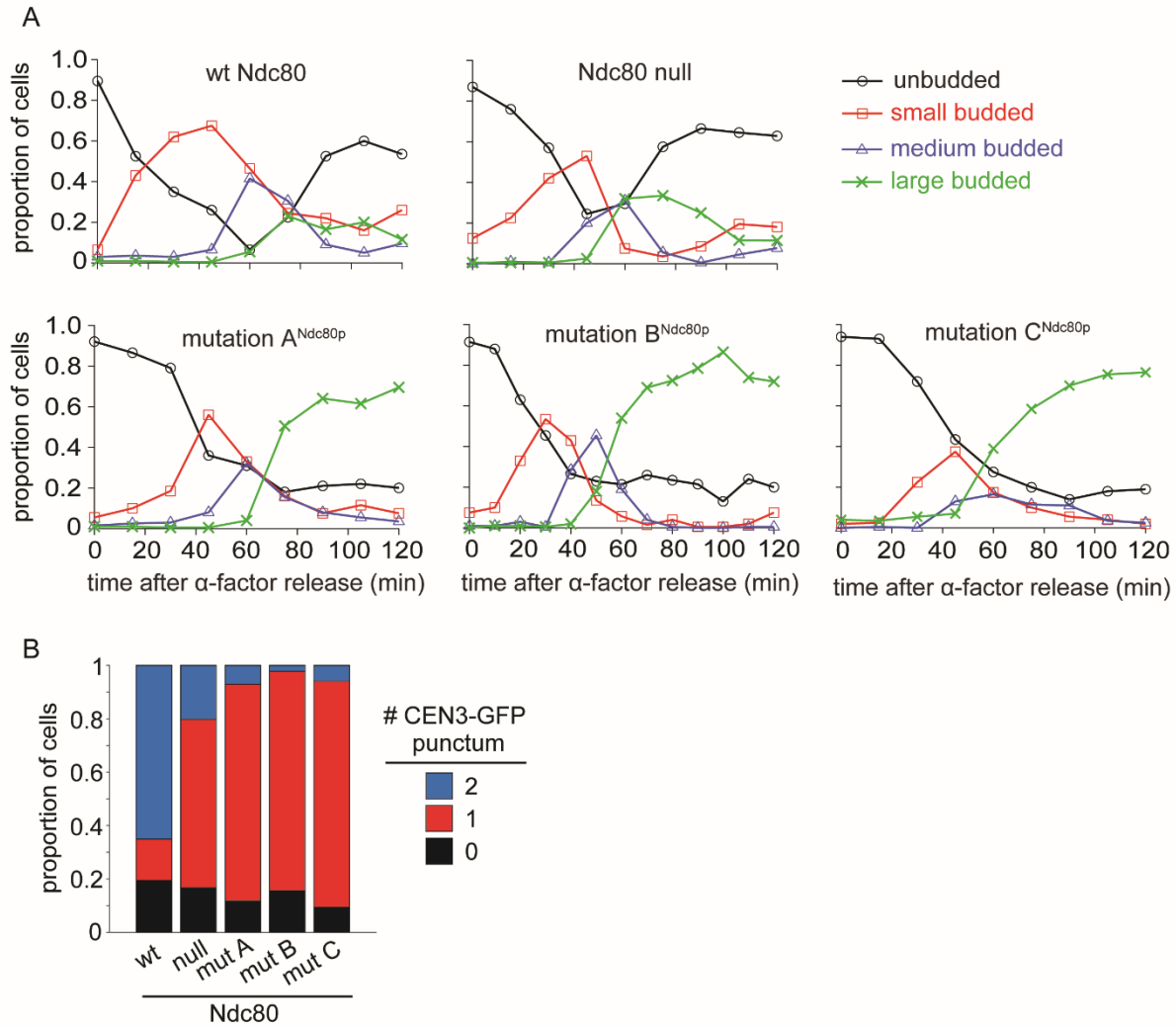


Figure 2.14

Lethal mutation in region A^{*Ndc80p*}, B^{*Ndc80p*}, or C^{*Ndc80p*} causes mitotic arrest.

(A) Budding index analyses of *Ndc80*-AID degen experiments. *Ndc80*-AID cells carrying an addition copy of *NDC80* wild-type, no *NDC80* (null), lethal mutation in region A^{*Ndc80p*}, B^{*Ndc80p*}, or C^{*Ndc80p*} were fixed at various time points after release from α -factor arrest (time 0). (B) Stacked bar graphs showing the percentage of cells with zero, one, or two CEN3-GFP spots for the various cell types.

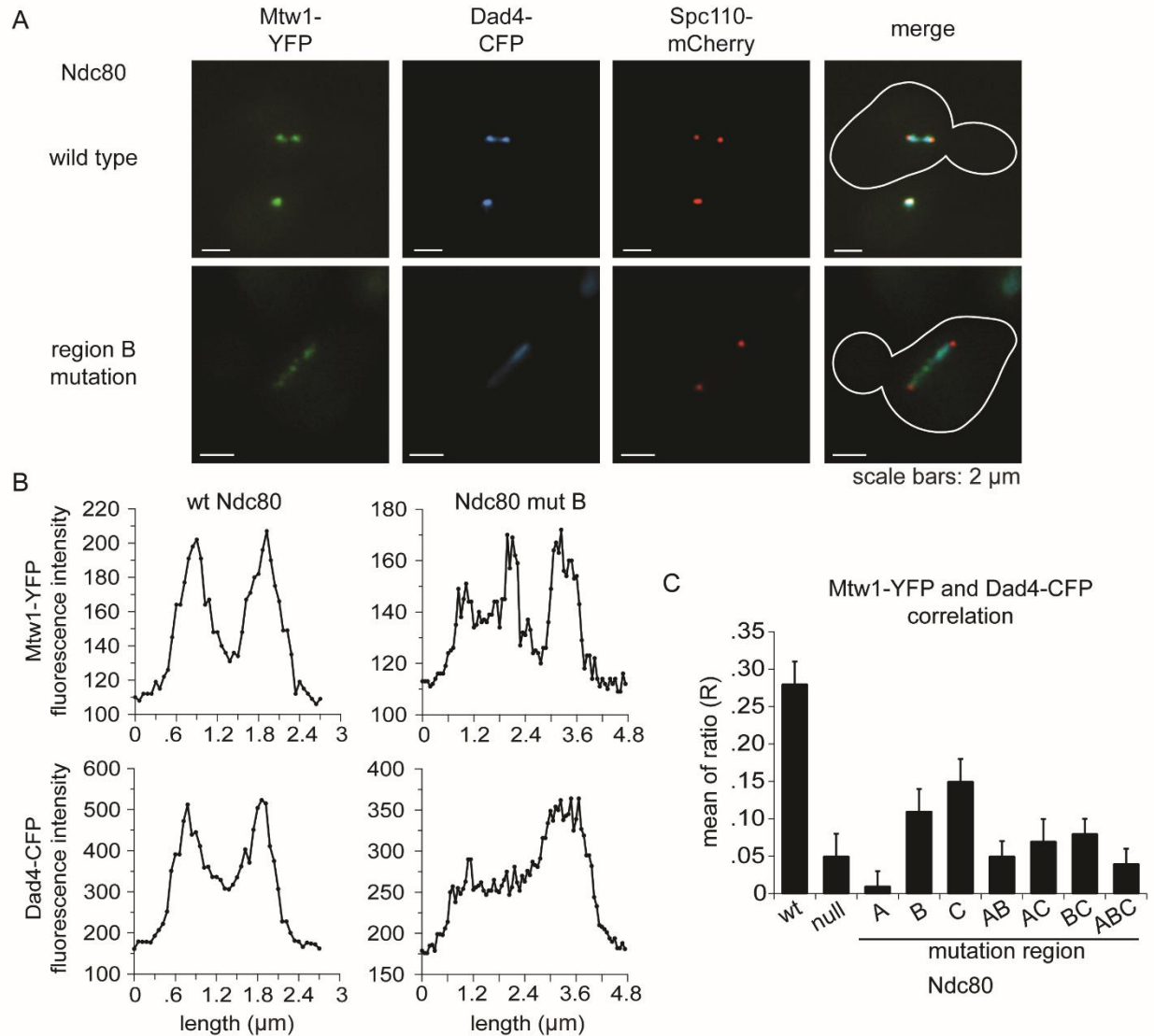


Figure 2.15

Lethal mutations in A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} confer defects in Dam1 complex recruitment to the kinetochore.

(A) Representative images from *NDC80-AID* degreen experiments with cells also containing wild-type *NDC80* or mutation in region B^{Ndc80p}. The inner kinetochore was visualized with Mtw1-YFP, the Dam1 complex with Dad4-CFP, and the SPB with Spc110-mCherry. Cells were treated with 6 μ M α -factor for two hours prior to the addition of 0.5 mM auxin for one additional hour. At time 0, cells were released from α -factor arrest and incubated in YPD medium containing 0.5 mM auxin. Images were taken at 60 min after release from α -factor arrest. (B) Line scan traces of Mtw1-YFP and Dad4-CFP from the images in (A). Only cells with two SPBs in the same plane of focus were selected for analysis. Each line scan extended from one SPB to the other. Each point represents one pixel. (C) Summary of correlation analysis carried out between Mtw1-YFP and Dad4-CFP. For each line scan, a correlation of the direction of pixel intensity changes across the line between the Mtw1-YFP and Dad4-CFP channels was calculated to examine colocalization along the spindle (see materials and methods). Between 18 and 33 cells were analyzed for each mutant. Refer to Table 2.8 for statistical analysis of data.

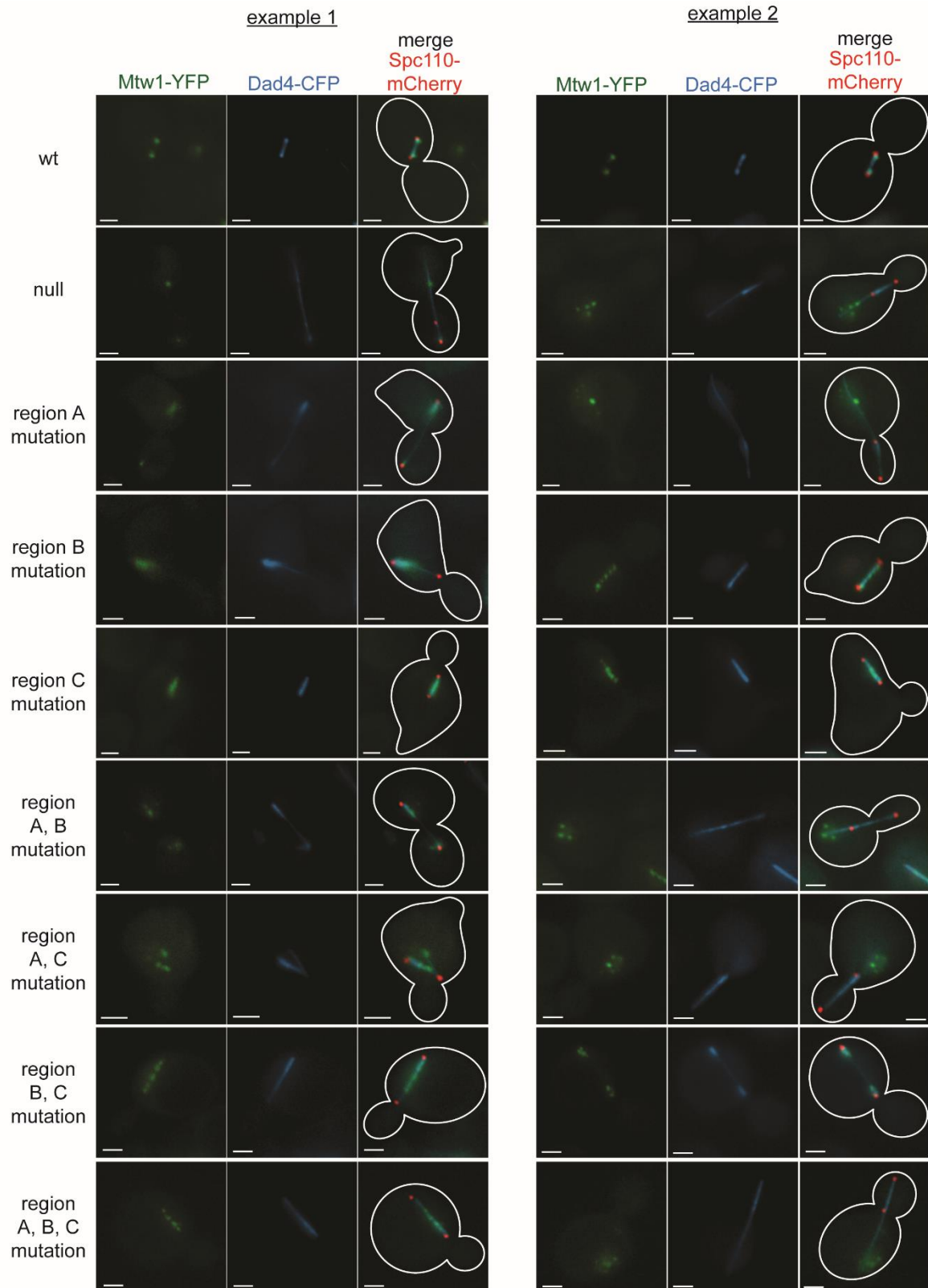


Figure 2.16

Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} results in severe defects in kinetochore biorientation and attachment.

Examples of commonly observed phenotypes in experiments with NDC80-AID degron cells carrying an additional copy of *ndc80* containing single or multiple mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p}, or not carrying a second copy of *NDC80* (null). Each example shows Mtw1-YFP, Dad4-CFP, and Spc110-mCherry and the same experiment procedure was carried out as in Figure 2.15. The majority of mutant cells with two SPBs exhibited detached kinetochores or had kinetochores spread along spindle microtubules. All scale bars: 2μm

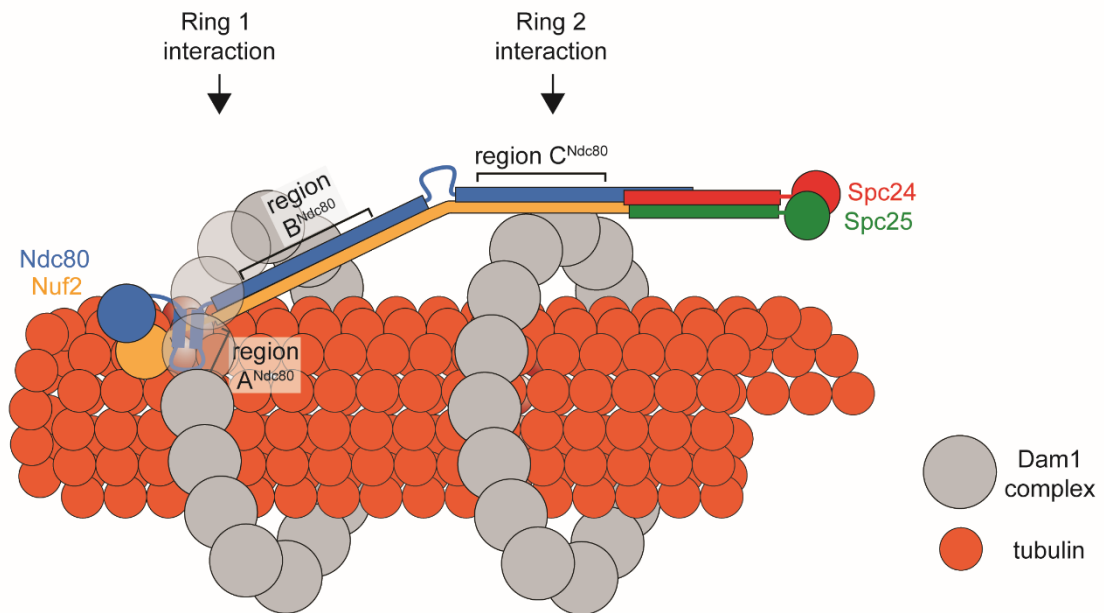


Figure 2.17

Model of the Ndc80 complex bridging two Dam1 complex rings, separated by 33 nm.

Results in this study support ring 1 interaction being comprised of interaction A and B, and ring 2 interaction being comprised of interaction C (refer to Figure 2.1). This image depicts only one Ndc80 complex across two Dam1 complex rings, but multiple Ndc80 complexes would be present *in vivo*.

Table 2.1 The interacting regions in the Dam1 and Ndc80 complexes

Interaction	protein region	amino acids	Phosphorylated residues	Five amino acid mutation (insertion) position
A	A ^{Dam1p}	241 – 330	S257, S265, S292	n/a
	A ^{Ndc80p}	262 – 322	n/a	314
B	B ^{Ask1p}	133 – 225	S200	n/a
	B ^{Ndc80p}	350 – 448	n/a	383
C	C ^{Spc34p}	118 – 274	T199	n/a
	C ^{Ndc80p}	532 – 630	n/a	563

Table 2.2 Pairwise statistical comparisons for Figure 2.1D

Sample 1 (proteins on microtubules)	Sample 2 (proteins on microtubules)	p-value
wt Ndc80c	wt Ndc80c + Dam1c mock	<0.001
wt Ndc80c	Mutant A Ndc80c	0.17
wt Ndc80c	Mutant B Ndc80c	0.089
wt Ndc80c	Mutant C Ndc80c	0.089
Mutant A Ndc80c	Mutant A Ndc80c + Dam1c mock	<0.001
Mutant B Ndc80c	Mutant B Ndc80c + Dam1c mock	0.0016
Mutant C Ndc80c	Mutant C Ndc80c + Dam1c mock	<0.001

Table 2.3 Pairwise statistical comparisons for Figure 2.4C

Sample 1 (proteins on microtubules)	Sample 2 (proteins on microtubules)	p-value
wt Ndc80c	Ndc80c ins 219	0.04
wt Ndc80c	Ndc80c ins 652	0.04
wt Ndc80c + Dam1c mock	Ndc80c ins 219 + Dam1c mock	0.20
wt Ndc80c + Dam1c mock	Ndc80c ins 652 + Dam1c mock	0.074

Table 2.4 Pairwise statistical comparisons for Figure 2.5

Sample 1 (proteins on microtubules)	Sample 2 (proteins on microtubules)	p-value
wt Ndc80c	wt Ndc80c + Dam1c phos	0.48
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c 6A phos	0.48
wt Ndc80c + Dam1c phos	wt Ndc80c + Dam1c 6A phos	<0.001
wt Ndc80c	wt Ndc80c + Dam1c A phos	<0.001
wt Ndc80c	wt Ndc80c + Dam1c B phos	<0.001
wt Ndc80c	wt Ndc80c + Dam1c C phos	<0.001
wt Ndc80c	wt Ndc80c + Dam1c B,C phos	<0.001
wt Ndc80c	wt Ndc80c + Dam1c A,B phos	0.29
wt Ndc80c	wt Ndc80c + Dam1c A,C phos	0.29
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c A phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c B phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c C phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c B,C phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c A,B phos	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c A,C phos	<0.001
wt Ndc80c + Dam1c A phos	wt Ndc80c + Dam1c A,B phos	<0.001
wt Ndc80c + Dam1c A phos	wt Ndc80c + Dam1c A,C phos	<0.001
wt Ndc80c + Dam1c B phos	wt Ndc80c + Dam1c A,B phos	<0.001
wt Ndc80c + Dam1c C phos	wt Ndc80c + Dam1c A,C phos	0.0022
wt Ndc80c + Dam1c B phos	wt Ndc80c + Dam1c B,C phos	0.074
wt Ndc80c + Dam1c C phos	wt Ndc80c + Dam1c B,C phos	0.33
wt Ndc80c + Dam1c B phos	mut C Ndc80c + Dam1c B phos	0.26
wt Ndc80c + Dam1c C phos	mut B Ndc80c + Dam1c C phos	0.026
wt Ndc80c + Dam1c B phos	mut A Ndc80c + Dam1c B phos	<0.001
wt Ndc80c + Dam1c A phos	mut B Ndc80c + Dam1c A phos	0.0054
wt Ndc80c + Dam1c C phos	mut A Ndc80c + Dam1c C phos	0.13
wt Ndc80c + Dam1c A phos	mut C Ndc80c + Dam1c A phos	<0.001
mut C Ndc80c + Dam1c mock	mut C Ndc80c + Dam1c B phos	0.12
mut B Ndc80c + Dam1c mock	mut B Ndc80c + Dam1c C phos	0.48
mut A Ndc80c + Dam1c mock	mut A Ndc80c + Dam1c B phos	0.0016
mut B Ndc80c + Dam1c mock	mut B Ndc80c + Dam1c A phos	0.0011
mut A Ndc80c + Dam1c mock	mut A Ndc80c + Dam1c C phos	0.0094
mut C Ndc80c + Dam1c mock	mut C Ndc80c + Dam1c A phos	<0.001
wt Ndc80c + Dam1c B,C phos	mut C Ndc80c + Dam1c B phos	0.26
wt Ndc80c + Dam1c B,C phos	mut B Ndc80c + Dam1c C phos	0.048

wt Ndc80c + Dam1c A,B phos	mut A Ndc80c + Dam1c B phos	0.13
wt Ndc80c + Dam1c A,B phos	mut B Ndc80c + Dam1c A phos	0.48
wt Ndc80c + Dam1c A,C phos	mut A Ndc80c + Dam1c C phos	0.23
wt Ndc80c + Dam1c A,C phos	mut C Ndc80c + Dam1c A phos	0.48

Table 2.5 Pairwise statistical comparisons for Figure 2.7

Sample 1 (proteins on microtubules)	Sample 2 (proteins on microtubules)	p-value
wt Ndc80c	wt Ndc80c + Dam1c 6A mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site A Ala mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site B Ala mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site C Ala mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site B,C Ala mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site A,B Ala mock	<0.001
wt Ndc80c	wt Ndc80c + Dam1c site A,C Ala mock	<0.001
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c 6A mock	0.074
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c site A Ala mock	0.074
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c site B Ala mock	0.48
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c site C Ala mock	0.040
wt Ndc80c + Dam1c mock	wt Ndc80c + Dam1c site B,C Ala mock	0.26
wt Ndc80c + Dam1 mock	wt Ndc80c + Dam1c site A,B Ala mock	0.48
wt Ndc80c + Dam1 mock	wt Ndc80c + Dam1c site A,C Ala mock	0.0734

Table 2.6 Pairwise statistical comparisons for Figure 2.8 and 2.10

Sample 1	Sample 2	p-value
no Ndc80 complex	wt Ndc80 complex	<0.001
no Ndc80 complex	Ndc80 ^{10hep} complex	<0.001
no Ndc80 complex	Mutant A Ndc80 complex	0.48
no Ndc80 complex	Mutant B Ndc80 complex	0.44
no Ndc80 complex	Mutant C Ndc80 complex	0.44
wt Ndc80 complex	Ndc80 ^{10hep} complex	0.016
wt Ndc80 complex	Mutant A Ndc80 complex	<0.001
wt Ndc80 complex	Mutant B Ndc80 complex	<0.001
wt Ndc80 complex	Mutant C Ndc80 complex	<0.001
Ndc80 ^{10hep} complex	Mutant A Ndc80 complex	<0.001
Ndc80 ^{10hep} complex	Mutant B Ndc80 complex	<0.001
Ndc80 ^{10hep} complex	Mutant C Ndc80 complex	<0.001
Mutant A Ndc80 complex	Mutant B Ndc80 complex	0.44
Mutant A Ndc80 complex	Mutant C Ndc80 complex	0.44
Mutant B Ndc80 complex	Mutant C Ndc80 complex	0.48

Table 2.7 Pairwise statistical comparisons for Figure 2.11

Sample 1 (proteins on microtubules)	Sample 1 (proteins on microtubules)	p-value
Ndc80c ^{10hep}	Ndc80 ^{10hep} + Dam1c mock	<0.001
wt Ndc80c	Ndc80 ^{10hep}	0.29
wt Ndc80c + Dam1c mock	Ndc80 ^{10hep} + Dam1c mock	0.36

Table 2.8 Pairwise statistical comparisons for Figure 2.15

Sample 1 (Ndc80 mutation)	Sample 2	p-value
Ndc80 depleted (null)	Wild-type Ndc80	<0.001
Ndc80 depleted	Region A ^{Ndc80p}	0.26
Ndc80 depleted	Region B ^{Ndc80p}	0.15
Ndc80 depleted	Region C ^{Ndc80p}	0.0070
Ndc80 depleted	Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	0.78
Ndc80 depleted	Region A ^{Ndc80p} B ^{Ndc80p}	0.98
Ndc80 depleted	Region A ^{Ndc80p} C ^{Ndc80p}	0.60
Ndc80 depleted	Region B ^{Ndc80p} C ^{Ndc80p}	0.38
Wild-type Ndc80	Region A ^{Ndc80p}	<0.001
Wild-type Ndc80	Region B ^{Ndc80p}	<0.001
Wild-type Ndc80	Region C ^{Ndc80p}	0.0024
Wild-type Ndc80	Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	<0.001
Wild-type Ndc80	Region A ^{Ndc80p} B ^{Ndc80p}	<0.001
Wild-type Ndc80	Region A ^{Ndc80p} C ^{Ndc80p}	<0.001
Wild-type Ndc80	Region B ^{Ndc80p} C ^{Ndc80p}	<0.001
Region A ^{Ndc80p}	Region B ^{Ndc80p}	0.009
Region A ^{Ndc80p}	Region C ^{Ndc80p}	<0.001
Region A ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	0.40
Region A ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p}	0.26
Region A ^{Ndc80p}	Region A ^{Ndc80p} C ^{Ndc80p}	0.099
Region A ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.03
Region B ^{Ndc80p}	Region C ^{Ndc80p}	0.23
Region B ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	0.081
Region B ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p}	0.14
Region B ^{Ndc80p}	Region A ^{Ndc80p} C ^{Ndc80p}	0.37
Region B ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.54
Region C ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	0.0023
Region C ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p}	0.0047
Region C ^{Ndc80p}	Region A ^{Ndc80p} C ^{Ndc80p}	0.035
Region C ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.054
Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	Region A ^{Ndc80p} B ^{Ndc80p}	0.77
Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	Region A ^{Ndc80p} C ^{Ndc80p}	0.42
Region A ^{Ndc80p} B ^{Ndc80p} C ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.21
Region A ^{Ndc80p} B ^{Ndc80p}	Region A ^{Ndc80p} C ^{Ndc80p}	0.58
Region A ^{Ndc80p} B ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.32
Region A ^{Ndc80p} C ^{Ndc80p}	Region B ^{Ndc80p} C ^{Ndc80p}	0.74

Table 2.9 Table of yeast strains used in this study

Strain	Genotype ^a	Reference
W303	<i>ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	
JTY5-8b	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12]	Tien et al., 2013
JOKY3	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK013]	This study
JOKY34	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJT14]	This study
JOKY35	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pRS316]	This study
JOKY4	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK013] [pJOK018]	This study
JOKY5	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK013] [pJOK017]	This study
JOKY15	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK013] [pRS315]	This study
JOKY16	<i>ade3Δ-100 ura3-1::NDC80::URA3 CEN3::33LacO::kan pCUP1-GFP12LacI12::HIS leu2::pGPD1-OsTIR1::LEU2 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX</i>	This study
JOKY17	<i>ade3Δ-100 ura3-1::ndc80-940::URA3 CEN3::33LacO::kan pCUP1-GFP12LacI12::HIS leu2::pGPD1-OsTIR1::LEU2 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX</i>	This study
JOKY18	<i>ade3Δ-100 ura3-1::ndc80-1148::URA3 CEN3::33LacO::kan pCUP1-GFP12LacI12::HIS leu2::pGPD1-OsTIR1::LEU2 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX</i>	This study
JOKY19	<i>ade3Δ-100 ura3-1::ndc80-1687::URA3 CEN3::33LacO::kan pCUP1-GFP12LacI12::HIS leu2::pGPD1-OsTIR1::LEU2 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX</i>	This study
JOKY20	<i>ade3Δ-100 CEN3::33LacO::Kan pCUP1-GFP12LacI12::HIS leu2::pGPD1-OsTIR1::LEU2 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX</i>	This study
JOKY47	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK035] [pJOK038]	This study
JOKY40	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pRS316] [pJOK038]	This study
JOKY48	<i>ade3Δ-100 cyh2^r lys2Δ::HIS3 ndc80Δ::natMX</i> [pJT12] [pJOK036] [pJOK038]	This study
JOKY51	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::URA3 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX DAD4-CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY52	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::NDC80::URA3 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX DAD4-CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY53	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-A^b::URA3 SPC110-Cherry::hphMX NDC80-3V5-IAA7::kanMX DAD4-CFP::hphMX MTW1-Venus::kanMX</i>	This study

JOKY54	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-B^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY55	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-C^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY56	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-A^bB^bC^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY57	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-A^bB^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY58	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-A^bC^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study
JOKY59	<i>ade3Δ leu2-3::pGPD1-OsTIR1::LEU2 ura3-1::ndc80-B^bC^b::URA3</i> <i>SPC110-Cherry::hphMX NDC80-3V5-IAA7:kanMX DAD4-</i> <i>CFP::hphMX MTW1-Venus::kanMX</i>	This study

^aAll strains have the same markers as W303 except as noted

^b Indicates the region of the mutation. "A," "B," and "C" signify a 15 bp insertion at base pair 940, 1148, and 1687, respectively.

Table 2.10 Yeast plasmids used in this study

Plasmid	Relevant markers	Reference
pRS315	<i>CEN6 ARSH4 LEU2 bla</i>	Sikorski and Hieter 1989
pRS316	<i>CEN6 ARSH4 URA3 bla</i>	Sikorski and Hieter 1989
pJT12	<i>NDC80 ADE3 LYS2 bla</i> in 2 μ m vector	Tien et al, 2013
pJT14	<i>NDC80</i> in pRS316	Tien et al, 2013
pJOK13	<i>ndc80^{10hep}</i> in pRS316	This study
pJOK17	<i>NUF2</i> in pRS315	This study
pJOK18	<i>nuf2^{10hep}</i> in pRS315	This study
pJOK19	<i>NDC80</i> in pRS315	This study
pJOK35	<i>NDC80-FLAG</i> in pRS316	This study
pJOK36	<i>ndc80^{10hep}</i> in pRS316	This study
pJOK38	<i>nuf2^{10hep}</i> in pRS315	This study

Chapter 3

Regulation of the Dam1 complex's interaction with microtubules

Introduction

The kinetochore is a network of protein complexes that assemble on centromeric DNA. During mitosis, kinetochores attach to spindle microtubules and mediate the equal division of duplicated chromosomes into the two daughter cells (Biggins, 2013). The hetero-decameric yeast Dam1 complex is recruited to the kinetochore by the highly conserved Ndc80 complex and is essential to attach kinetochores to microtubules (Janke et al., 2002). In vitro, the Dam1 complex recapitulates many functions of the kinetochore, including the ability to couple cargo to dynamic microtubules, while bearing load (Asbury et al., 2006). In solution, the Dam1 complex exists primarily as a 406-kDa dimer (Wang et al., 2007). In the presence of microtubules, however, it oligomerizes into rings encircling the microtubule (Miranda et al., 2005; Westermann et al., 2005). Such oligomerization is required for the Dam1 complex to form microtubule attachments that are robust against tension (Umbreit et al., 2014).

Kinases regulate mitosis and chromosome segregation. For example, the cyclin dependent kinase 1 (Cdk1) is active during mitosis when bound to cyclin B. This complex is responsible for many mitotic processes including sister chromatid cohesion, spindle pole body separation, spindle elongation, and spindle positioning (Enserink & Kolodner, 2010). In addition, errors in chromosome segregation are devastating to the cell and, in multicellular organisms, are associated with a range of diseases as well as being a hallmark of cancer (Gordon et al., 2012). Intricate error correction mechanisms exist to prevent such errors and delay cell cycle progression until correct kinetochore-microtubule attachments are achieved (Foley & Kapoor, 2013). A major component of these protective pathways is the Aurora B kinase, which

phosphorylates kinetochore proteins to promote detachment of incorrectly attached kinetochores from microtubules (Kalantzaki et al., 2015; Pinsky et al., 2006).

Aurora B kinase phosphorylates the Dam1 complex at six different sites: Dam1p S20, S257, S265, S292, Ask1p S200, and Spc34p T199. The four Dam1p sites have been shown to play a role in regulating the ability of Dam1 complex to bind microtubules and mutations that block phosphorylation at all four sites are lethal. However, either the N-terminal site alone or the three C-terminal sites are sufficient for viability, suggesting functional redundancy (Cheeseman et al., 2002). Moreover, removal of the C-terminus of Dam1p reduces the affinity of the Dam1 complex for microtubules but does not abolish binding completely (Ramey et al., 2011; Westermann et al., 2005). We previously found that phosphorylation of Dam1p S20 decreases the apparent affinity of the Dam1 complex to microtubules, whereas phosphorylation of the C-terminal Aurora B sites together has little effect (Gestaut et al., 2008). Thus, both N- and C-terminal regions of Dam1p have been implicated in microtubule binding, although their specific roles remain elusive. Despite much work, key questions remain regarding how the Aurora B kinase phosphorylation of Dam1p regulates the kinetochore-microtubule interface during mitosis.

Cyclin dependent kinase 1 (Cdk1) also phosphorylates the Dam1 complex. The transition into and exit from mitosis is regulated by Cyclin dependent kinase 1 (Cdk1) activation by cyclin B (King et al., 1994). Cdk1 phosphorylates the Dam1 complex Ask1p S216 and S250. Although mutations blocking phosphorylation (S216A and S250A) do not confer a visible phenotype, addition of the *ASK1 ts* allele, *ask1-3*, compromises growth at the restrictive temperature (Li & Elledge, 2003). This suggests that the Cdk1 phosphorylation at Ask1p S216 and S250 act in a positive fashion to promote the Dam1 complex function; however, the mechanism by which this occurs is unknown.

Here, we show that the Aurora B kinase phosphorylation of Dam1p inhibits both the Dam1 binding to and oligomerization on microtubules. Our data suggest that inhibiting

oligomerization, and thereby cooperativity, has a more significant effect on disrupting the Dam1 complex binding to microtubules. In contrast, phosphomimetic mutations of the Ask1p Cdk1 phosphorylation sites enhances the Dam1 complex cooperative binding to microtubules; these mutations do not affect the interaction between the Dam1 and Ndc80 complexes nor the Dam1 complex's ability to bind microtubules as a single complex. Altogether, we demonstrate that regulation of the Dam1 complex oligomerization is extensive with both negative and positive regulation mechanisms.

Results

Aurora B kinase inhibits the Dam1 complex oligomerization

In a concurrent study, models of the Dam1 complex in the presence and absence of microtubules were built using protein cross-linking, mass spectrometry, and integrative structural modeling (Zelter et al., 2015; Figure 3.1). The models made testable predictions about the microtubule-binding and oligomerization interfaces of the Dam1 complex. Of the ten proteins that make up the Dam1 complex, only the C-terminal domains of Dam1p and Duo1p form cross-links to the microtubule. (Figure 3.2). Thus, Aurora B kinase phosphorylation of the Dam1p three C-terminal sites should regulate the interface with the microtubule. In contrast, the Dam1 complex model restricts the Dam1p S20 Aurora B phosphorylation site to the interface between the two Dam1 complex monomers (Figure 3.3). We hypothesized that phosphorylation of Dam1p at S20 should specifically affect oligomerization of the complex rather than the interface between the Dam1p and microtubule.

We tested these predictions by measuring the binding of Dam1 complexes to microtubules, both at the single-molecule level and when assembled into oligomers. First, we constructed two phospho-blocking mutants of the recombinant Dam1 complex (Figure 3.4A): (1) 'Dam1 CP', in which only the Dam1p C-terminal sites can be phosphorylated (Dam1p S20A, Ask1p S200A and Spc34p T199A) and (2) 'Dam1 NP', in which only the Dam1p N-terminal S20

site can be phosphorylated (Dam1p S257A, S265A, S292A, Ask1p S200A, and Spc34p T199A). These two mutants were then incubated with recombinant Aurora B kinase in the presence of ATP (phos) or buffer alone (mock). We used total internal reflection fluorescence (TIRF) microscopy to test the effects of phosphorylation on microtubule binding at the single-molecule level (40pM Dam1-GFP) (Umbreit et al., 2014). Consistent with our model, phosphorylation of the three C-terminal sites of Dam1p (Dam1 CP) decreased the residence time of single Dam1 complexes on microtubules by 20%, whereas phosphorylation of the N-terminal S20 site (Dam1 NP) had no effect (Figure 3.4B).

To measure the effects of phosphorylation on oligomerization, we performed the TIRF assay at concentrations that support the formation of oligomeric rings (2 nM total Dam1 complex) wherein small ‘tracer’ quantities of green fluorescent protein (GFP)-tagged Dam1 complex (1:100) were included to measure the behavior of individual oligomeric particles, as previously described (Umbreit et al., 2014). Relative to single molecules, the average residence time of non-phosphorylated Dam1-GFP complexes increased 45-fold, indicating their incorporation into oligomers of untagged Dam1 complexes (Figure 3.4 C). Tracer Dam1 CP complexes similarly exhibited increased residence time in the presence of excess untagged Dam1 CP complexes, indicating that phosphorylation of the C-terminal sites of Dam1p does not block oligomerization. However, Dam1 NP was defective in oligomerization, as indicated by a residence time increased by only two-fold. These results confirm the predictions based on our model that phosphorylation of the Dam1p N terminus inhibits the ability of the Dam1 complex to form oligomers, whereas phosphorylation of the C-terminal sites regulates the interface with the microtubule.

Cdk1 enhances the Dam1 complex oligomerization

Kinetochore particles purified from yeast harboring the *dad1-1 ts* allele have a decreased ability to make load-bearing attachments to microtubule tips, when compared those

purified from *DAD1* yeast (Akiyoshi et al., 2010). A collaborator incubated wt Dam1 complex (Dam1c-wt) or Dam1 complex harboring phosphomimetic mutations Ask1p S216D and S250D (Dam1c-2D) with kinetochores purified from *dad1-1* yeast. Compared to the Dam1c-wt, the Dam1c-2D had an increased ability to make load-bearing attachments to microtubule plus tips. We hypothesized that this result might be explained by the Ask1p phosphomimetic mutations: 1) increasing the Dam1 complex's affinity for microtubules as a single molecule; 2) promoting the Dam1 complex's oligomerization state, thereby enhancing the cooperative binding onto microtubules; 3) enhancing the affinity between the Dam1 and Ndc80 complexes.

We tested these hypotheses under the TIRF microscopy assay using the Dam1c-wt or Dam1c-2D. First, the Dam1c-wt and Dam1c-2D had similar microtubule residence times at a single-molecule level (50pM Dam1-GFP complex; Figure 3.5), suggesting that the phosphomimetic mutations do not affect single Dam1 complex binding to microtubules. Second, we tested the phosphorylation effects on oligomerization by incubating a higher concentration of the untagged Dam1 complex (1nM) in the presence of 'tracer' quantities of the Dam1-GFP complex (50pM). Under these oligomeric conditions, the Dam1c-2D mutant resided 50% longer on microtubules compared to the Dam1c-wt (Figure 3.5). These results suggest that Cdk1 phosphorylation of the Ask1p does not affect the single molecule Dam1 complex affinity for microtubules, but promotes oligomerization and cooperative binding onto microtubules.

Next, we tested the effects of Dam1c-2D mutations on the interaction between the Dam1 and Ndc80 complexes. The Ndc80-GFP complex alone had a short residence time on microtubules. The addition of either untagged 5nM Dam1c-wt or Dam1c-2D similarly increased the Ndc80-GFP complex microtubule residence time (Figure 3.6A), suggesting that the Ask1p phosphomimetic mutations do not enhance the interaction between the Dam1 and Ndc80 complexes. In addition, the 'tracer' experiment was repeated with 50pM GFP-tagged Dam1 complex in the presence of 5nM untagged Dam1 complex. The microtubule residence times between the Dam1c-wt and Dam1c-2D were similar under these conditions (Figure 3.6B),

suggesting that the Dam1c-2D mutations do not significantly affect the Dam1 complex oligomerization at a higher concentration of 5nM. Altogether, these results suggest that the Cdk1 phosphorylation of the Ask1p does not enhance the interaction between the Dam1 and Ndc80 complexes.

Discussion

Ever since the discovery of the Dam1 complex's ability to form high-order oligomeric rings around microtubules, its structure, function, and regulation have been extensively studied. The Dam1 complex imparts microtubule tip-tracking ability to the Ndc80 complex (Lampert et al., 2010; Tien et al., 2010) and might mediate the transition of lateral to end-on kinetochore-microtubule attachment (Tanaka et al., 2007). Although ring formation is not required for tracking with depolymerizing microtubule tips *in vitro*, oligomerization is important for establishing bioriented kinetochore-microtubule attachments *in vivo* (Gestaut et al., 2008; Umbreit et al., 2014). We show that the Aurora B kinase phosphorylates Dam1p to inhibit both the single-molecule and oligomeric interaction with microtubules. In contrast, Cdk1 phosphorylation of the Ask1p enhances oligomerization.

The exclusive cross-links between microtubules and the Dam1p/Duo1p C-terminal domains allowed us to make an informed hypothesis about the function of Aurora B kinase phosphorylation of the Dam1p C-terminal sites. *In vivo*, mutation of these sites confers mild defects such as sensitivity to benomyl, a microtubule depolymerizing drug, and synthetic lethality with mutations in Aurora B kinase (Cheeseman et al., 2002). Here we find that phosphorylation of the C-terminal sites of Dam1p also has mild effects and decreases the affinity of the Dam1 complex for microtubules, but only under conditions where the complex is a monomer. The same phosphorylation does not detectably affect the microtubule-binding affinity of the complex as an oligomer, probably because the loss of affinity for the microtubule is largely overcome by the cooperativity of forming rings. Consistent with our results, the Dam1p C

terminus is not required for Dam1 complex assembly nor for its accumulation at the microtubule tip (Kalantzaki et al., 2015; Ramey et al., 2011).

We were previously unable to pinpoint the exact site responsible for regulating the Dam1 complex oligomerization. In the molecular model of the Dam1 complex dimer, localization of the Dam1p S20 to the interface between the two monomers (far from the microtubule) allowed us to hypothesize that the Aurora B kinase phosphorylation of this site inhibits Dam1 complex oligomerization. We show here that S20 phosphorylation has no effect on the direct interaction with the microtubule but a pronounced effect on oligomerization. Considering the importance of oligomerization for maintaining attachments to dynamic microtubules, this presents a mechanism by which Aurora kinase B could potentially detach kinetochores by phosphorylation at a single site. Similarly, several different phospho-site mutations with mild phenotypic consequences confer severe phenotypes when combined with Dam1p S20A (Cheeseman et al., 2002). This further underscores the importance of S20 as a regulatory site.

For the first time, our data demonstrates a positive mechanism of regulating the microtubule binding activity of a kinetochore component. Aurora B kinase is understood as the central negative regulator of kinetochore-microtubule attachments, participating in the error correction pathway. The Dam1 and Ndc80 complexes are both phosphorylated by Aurora B kinase, decreasing their microtubule affinity (Gestaut et al., 2008; Tien et al., 2010; Umbreit et al., 2012). Aurora B kinase phosphorylation of the Dsn1p, a component of the outer kinetochore, promotes the interaction between the MIND complex and inner kinetochore proteins Ame1p and Mif2p (Akiyoshi et al., 2013; Dimitrova et al., 2016). To date, this is the only previously described example of a positive regulation of the kinetochore function. In addition, our data suggests that Ask1p phosphorylation by Cdk1 enhances the Dam1 complex oligomerization and cooperative binding on microtubules while not affecting its single molecule affinity for microtubules and the interaction between Dam1 and Ndc80 complexes.

Promotion of the Dam1 complex oligomerization by Cdk1 is consistent with previously known effects of Cdk1 activity. Cdk1 is activated by cyclin B to promote entry into mitosis and a major downstream effect is the formation of the bipolar mitotic spindle (Murray & Kirschner, 1989). This requires the elongation and stabilization of spindle microtubules for sister kinetochore biorientation and equal sister chromatid segregation. Since the Dam1 complex localizes to the whole length of the spindle and stabilizes microtubules (Cheeseman et al., 2002; Franck et al., 2007; Janke et al., 2002), the enhancement of oligomerization by Cdk1 might contribute to bipolar spindle formation. In addition to our results suggesting that Cdk1 phosphorylation of the Ask1p enhances kinetochore-microtubule interaction, future experiments will test if the Cdk1 phosphorylated Dam1 complex stabilizes microtubules and the mitotic spindle.

Materials and methods

Protein expression and purification

All ten Dam1 complex subunits were expressed in *Escherichia coli* (BL21 Rosetta; Novagen, Madison, WI) from a single polycistronic vector (Franck et al., 2007). The complex was affinity purified using a C-terminal 6xHis-tag on Spc34p. The eluate was purified on a size-exclusion column (Superdex 200 10/300GL; Amersham Biosciences, Piscataway, NJ), equilibrated with 20 mM phosphate at pH 7.0, 500 mM NaCl.

Dam1 complex phosphorylation

Expression of GST-Ipl1 (budding yeast Aurora B kinase) was from plasmid pSB196 (Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA) at 23 °C for 2 hr. GST-Ipl1 was purified using GSTrap HP, according to the manufacturer's instructions (GE Healthcare Biosciences), except that the elution buffer was 50 mM, 250 mM KCl, 10 mM glutathione, pH 8.0. pSB503 (Sue Biggins) was used to express GST-Sli15 (residues 554–698) at 37 °C. GST-

Sli15 was purified using glutathione–Sepharose 4B resin, following the manufacturer's instructions, except that the elution buffer was 20mM Tris buffer 200 mM NaCl, 1 mM β -mercaptoethanol, 1 mM EDTA, 10 mM glutathione, pH 8.0.

Ipl1p phosphorylation of recombinant Dam1 complex was carried out in a 50 μ l reaction containing 4 mM Dam1 complex, 0.5 mM GST-Ipl1, 0.5 μ M GST-Sli15 (residues 554-698), 200 mM NaCl, 10 mM ATP, 25 mM $MgCl_2$ and 50 mM HEPES buffer, pH 7.4. Reactions were incubated for 90min at 30°C. Glycerol was added (5% final) before snap-freezing and storing at -80°C. The mock/non-phosphorylated controls were carried out by substituting distilled water for the ATP. Under these conditions, we achieve nearly stoichiometric phosphorylation of the complex (Gestaut et al., 2008).

TIRF microscopy

Flow chambers were constructed using glass slides and functionalized coverslips as reported before (Gestaut et al., 2008; Tien et al., 2010; Umbreit et al., 2014). Coverslip was adhered to a glass slide with double-sided tape, to form individual flow channels between two adjacent strips of tape. 'Rigor' kinesin was added to each channel to nonspecifically bind to the coverslip. This allowed for the addition and immobilization of taxol-stabilized microtubules. For testing the effects of Aurora B kinase phosphorylation of the Dam1 complex, single-molecule imaging experiments were carried out by incubating 40pM phosphorylated or mock-treated Dam1-GFP NP (Dam1p S257A, S265A, S292A, Ask1p S200A and Spc34p T199A) or Dam1-GFP CP (Dam1p S20A, Ask1p S200A and Spc34p T199A) with Alexa-647-labelled microtubules. For testing the effects of Ask1p phosphomimetic mutations, 50 pM GFP-tagged Dam1c-wt, Dam1c-2D, or Ndc80 complex was used; additional 5 nM unlabelled Dam1c-wt or Dam1c-2D complex was added when testing the effects of Cdk1 phosphomimetic mutations on the interaction between Dam1 and Ndc80 complexes. In 'tracer' assays, GFP-tagged and unlabelled versions of the Dam1 complex were mixed to 1:100 ratio to a total concentration of 2

nM, and 1 nM or 5 nM for testing Aurora B phosphorylation and Cdk1 phosphomimetic mutations, respectively. Next, this solution was incubated with microtubules. GFP and Alexa-647 fluorescence channels were simultaneously recorded using a custom TIRF imaging system (Gestaut et al., 2010).

All TIRF assays were carried out in BRB80 (80 mM K-PIPES, 1 mM MgCl₂, 1 mM EGTA, pH 6.9) in the presence of oxygen scavenger system (200 mg/ml glucose oxidase, 35 mg/ml catalase, 25 mM glucose and 5 mM dithiothreitol). Experiments using GFP-tagged Dam1 complex had an additional 0.8 mg/mL κ -casein and 50 mM KCl. Experiments using GFP-tagged Ndc80 complex had an additional 8 mg/ml BSA.

Single particle tracking and analysis was carried out with custom software (available on request) developed in LabView (National Instruments) and Igor Pro (Wavemetrics) (Tien et al., 2010; Umbreit et al., 2014). Mean residence times were carried out through bootstrapping analysis (Umbreit et al., 2014). Each residence time data set was randomly resampled with replacement 1,000 times. All data sets presented formed normal distributions. Gaussian fits to these distributions yielded estimates of mean residence time and s.d.

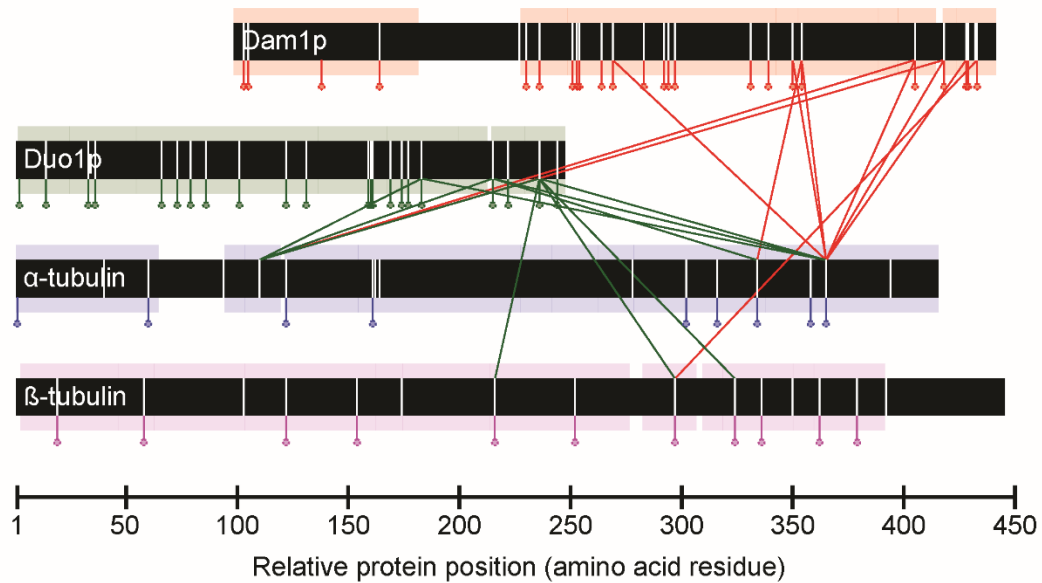


Figure 3.2

Dam1 complex to microtubule cross-links

Peptide sequence coverage (colored boxes), mono-links (colored vertical lines and circles) and lysines (vertical white lines) are also shown. Dam1p to Duo1p and tubulin to tubulin cross-links are hidden for clarity. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q -values ≤ 0.01 .

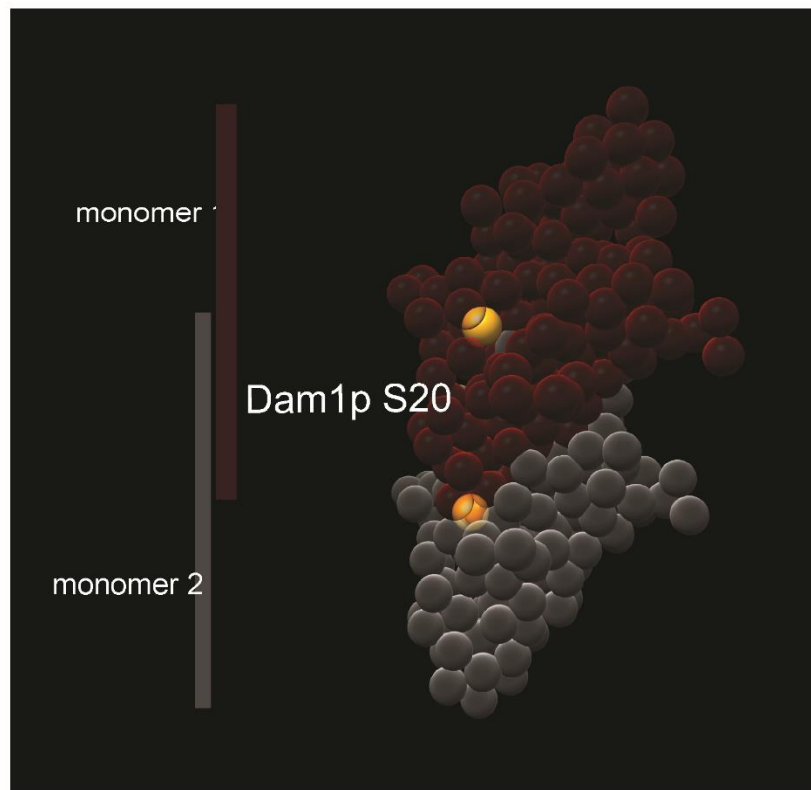


Figure 3.3

Dam1p S20 localizes at the Dam1 complex dimer interface

The Dam1 complex is shown as a dimer, with one monomer in red and the other in grey. The Aurora B kinase phosphorylation site Dam1p S20 lies at the interface between the two Dam1 complex monomers. Dam1p S20 beads are colored yellow.

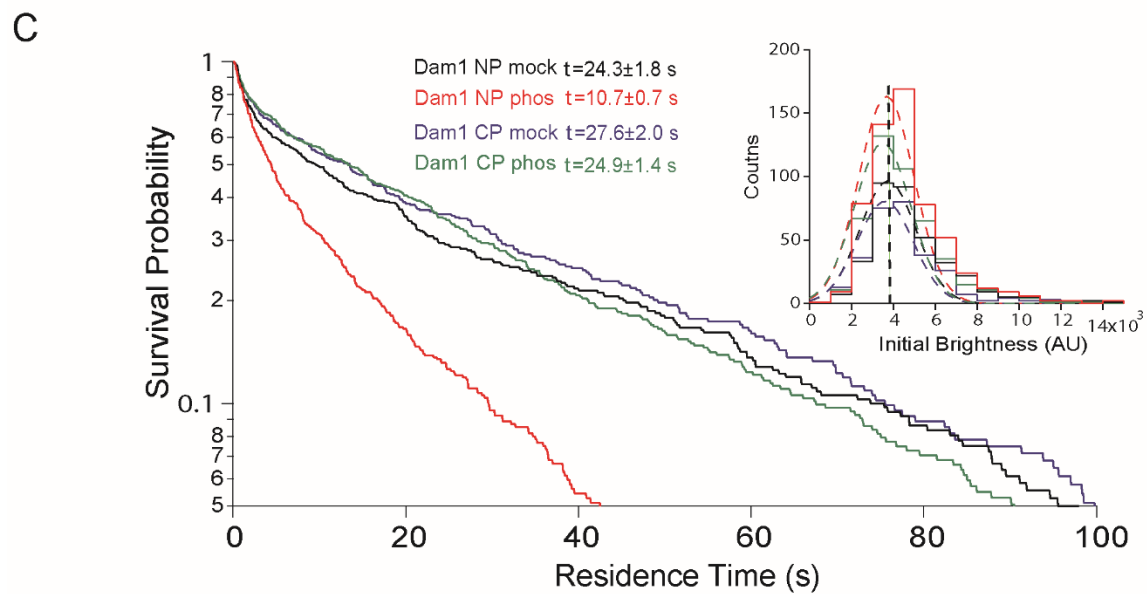
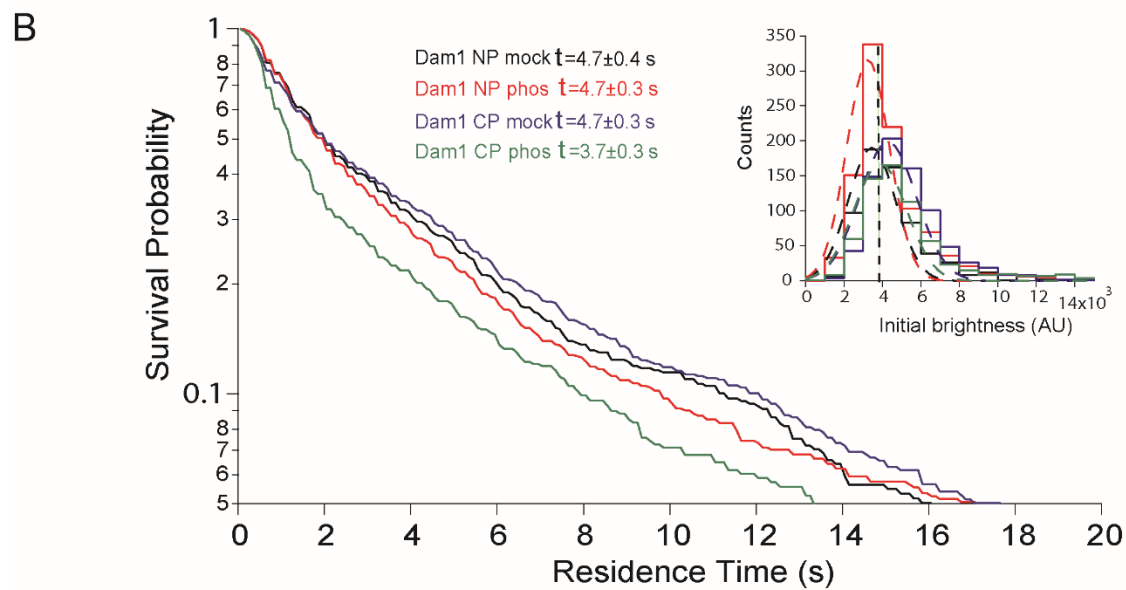
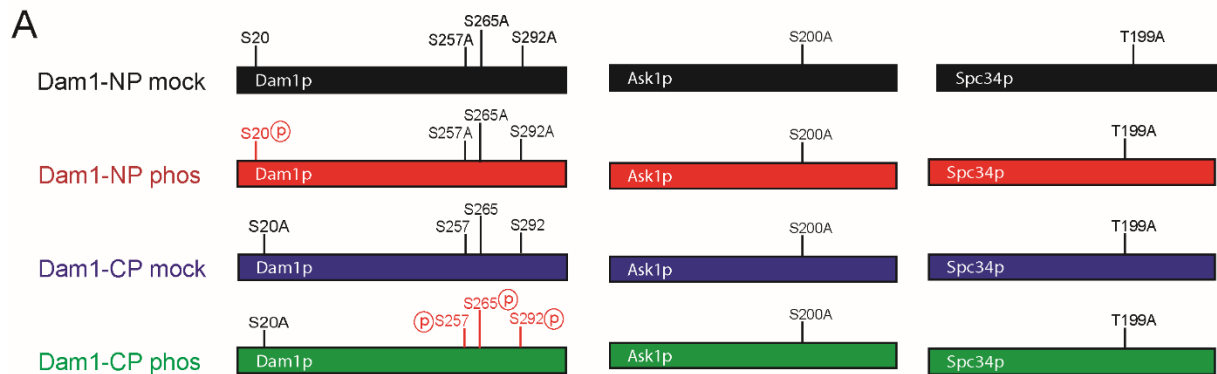


Figure 3.4

Aurora B kinase phosphorylation controls both microtubule binding and oligomerization of the Dam1 complex

(A) Schematic of phospho-blocking Dam1 complex constructs. Both constructs were phosphorylated by Aurora B kinase in the presence of ATP. Mock-treated Dam1 complex had ATP substituted with distilled water. (B) Survival curve summary of single-molecule Dam1-GFP TIRF experiments. Dam1 NP mock (n=638), Dam1 NP phos (n=1009), Dam1 CP mock (n=777) and Dam1 CP phos (n=646) were each incubated at 40pM for single-molecule Dam1-GFP complex imaging. Inset: GFP fluorescence (A.U.) distributions for Dam1 NP mock ($3,500 \pm 1,200$), Dam1 NP Phos ($3,300 \pm 1,100$), Dam1 CP mock ($4,300 \pm 1,500$) and Dam1 CP phos ($3,900 \pm 1,400$). (C) Survival curve summary of tracer Dam1-GFP TIRF experiments. GFP-tagged Dam1 NP mock (20 pM; n=360), Dam1 NP phos (n=587), Dam1 CP mock (n=294) and Dam1 CP phos (n=454) constructs were incubated with 2 nM corresponding non-tagged Dam1 constructs. Inset: GFP fluorescence (A.U.) distributions for Dam1 NP mock ($3,700 \pm 1,300$), Dam1 NP phos ($3,700 \pm 1,300$), Dam1 CP mock ($3,600 \pm 1,400$) and Dam1 CP phos ($3,500 \pm 1,400$) in tracer experiments. Inset panels show initial brightness of Dam1-GFP complex are similar across the whole TIRF data set, indicating that all data are from imaging single molecules of Dam1-GFP complexes either alone or incorporated into oligomers of unlabeled Dam1 complexes (in the tracer experiments). Vertical dashed line in inset panels represents the average height of single-step photobleach events under identical conditions ($3,900 \pm 1,900$ A.U., n=287). Average single-step photobleach duration under identical conditions is 130.9 ± 5.7 s (n=287). All errors are s.d. of the mean.

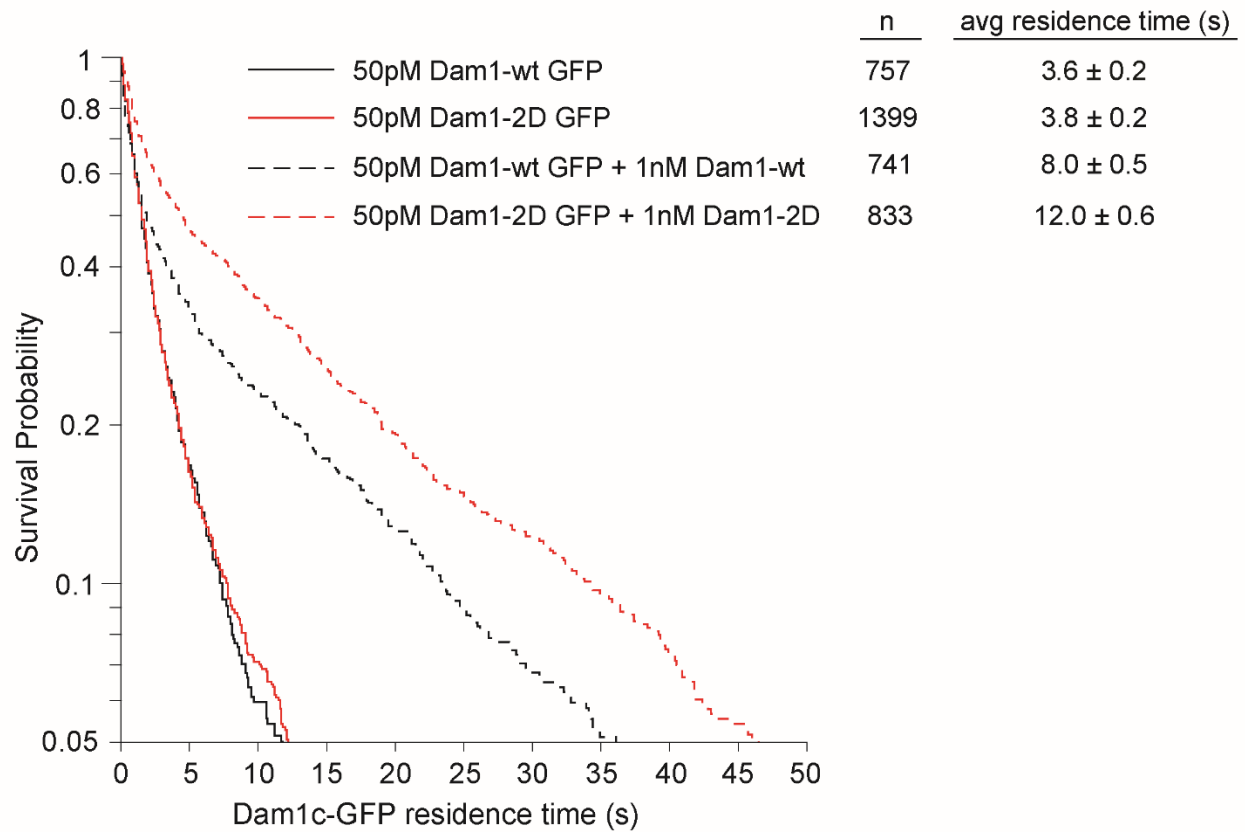


Figure 3.5
The Ask1p phosphomimetic mutations on Cdk1 phosphorylation sites promote Dam1 complex oligomerization
 Survival probability curves summarizing TIRF microscopy experiments using Dam1-GFP complex. Black and red curves represent Dam1c-wt and Dam1c-2D constructs, respectively. Solid and dashed lines show results from experiments carried out under single molecule and 'tracer' conditions, respectively.

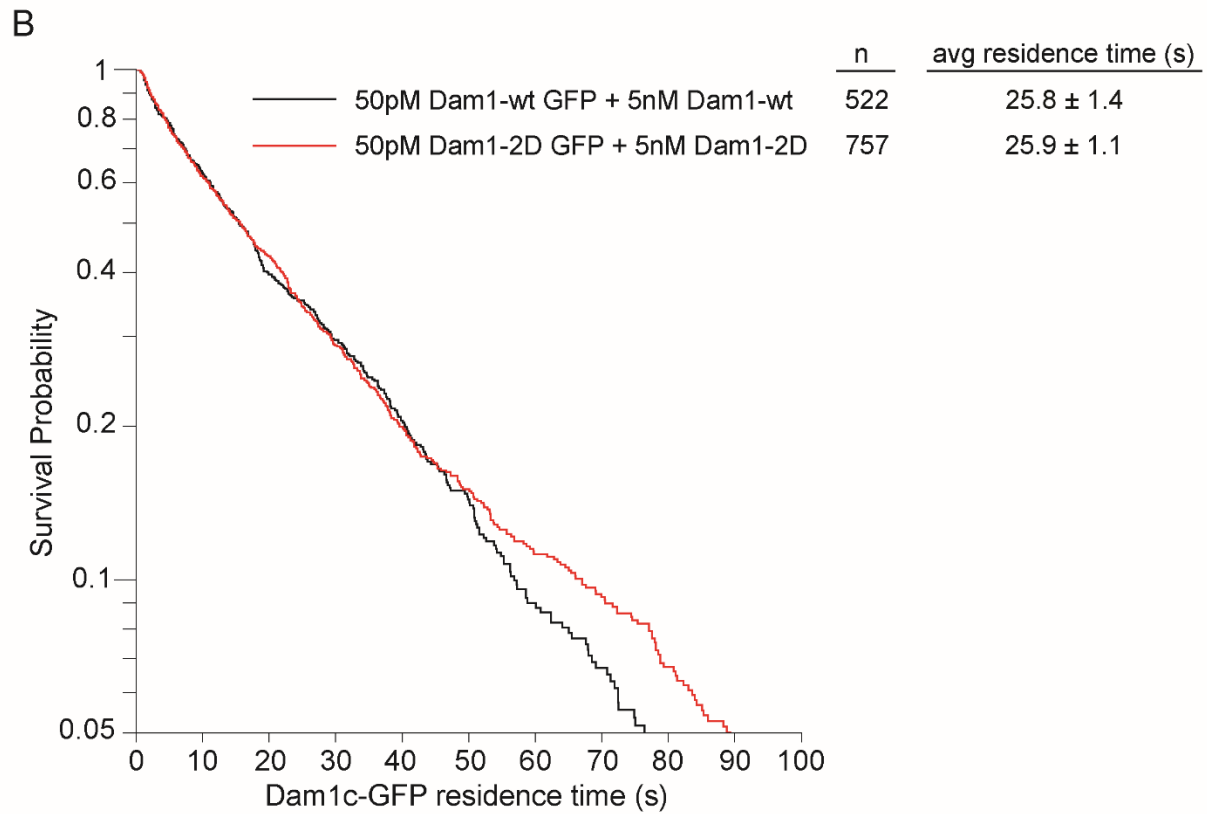
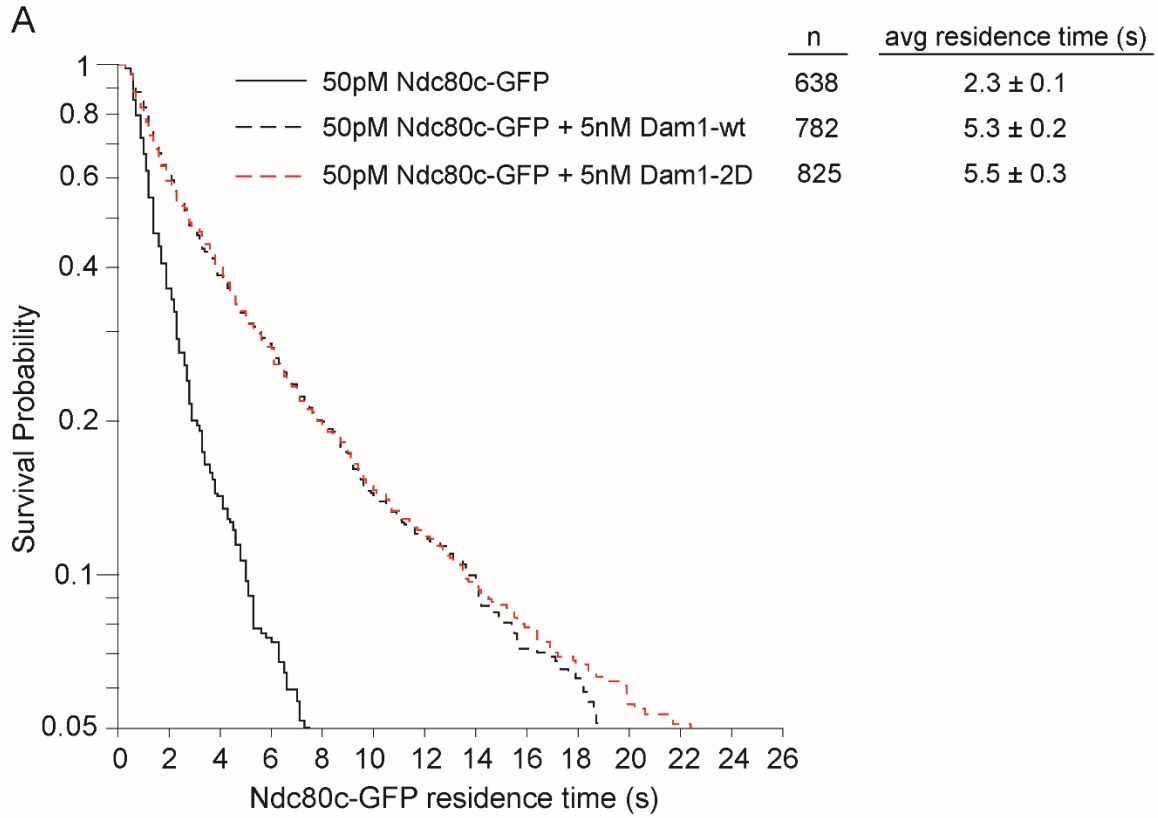


Figure 3.6

The Ask1p phosphomimetic mutations on Cdk1 phosphorylation sites do not affect interaction between Dam1 and Ndc80 complexes

Survival probability curves summarizing TIRF microscopy experiments. (A) TIRF experiments carried out with Ndc80-GFP complex alone, with the addition of Dam1c-wt or Dam1c-2D. (B) TIRF experiments carried out under 'tracer' conditions with 50pM GFP-tagged Dam1 complex plus 5nM non-tagged Dam1 complex.

Table 3.1 Plasmids used in this study

Plasmid	Relevant markers	Reference
pC4	<i>wt Dam1 complex (Spc34p-6xHis)</i>	Miranda et al., 2005
pC4-GFP	<i>pC4 with Dad1p-GFP</i>	
pJOK044	<i>pC4 with Dam1p S20A, Ask1p S200A, Spc34p T199A</i>	This study
pJOK056	<i>pC4-GFP with Dam1p S20A, Ask1p S200A, Spc34p T199A</i>	This study
pJOK057	<i>pC4 with Dam1p S257A, S265A, S292A; Ask1p S200A; Spc34p T199A</i>	This study
pJOK058	<i>pC4-GFP with Dam1p S257A, S265A, S292A; Ask1p S200A; Spc34p T199A</i>	This study
pJOK120	<i>pC4 with Ask1p S216D, S250D</i>	This study
pJOK126	<i>pC4-GFP with Ask1p S216D, S250D</i>	This study
pETDuet Ndc80/Nuf2	<i>wt Ndc80p, Nuf2p</i>	Wei et al., 2005
pEM33	<i>wt Spc24p-6xHis, Spc25p</i>	Wei et al., 2005

Chapter 4

Characterization of the budding yeast KMN network organization

Introduction

Kinetochores assemble onto centromeric DNA regions and make bioriented attachments on spindle microtubules during mitosis. The budding yeast kinetochore is composed of at least 40 different proteins, most of which are conserved from yeast to humans. Each budding yeast chromosome has a point centromere of ~125 base pairs, on which the inner kinetochore proteins assemble (Bloom & Carbon, 1982). Although many inner kinetochore proteins have been identified thus far, only CBF3 complex, Mif2p, Ame1p, and Okp1p are essential for viability; this suggests functional redundancy among inner kinetochore proteins. The outer kinetochore proteins further assemble onto the inner kinetochore scaffold and are all essential for viability. The Spc105 (Knl1 in human), MIND (Mis12 in human), and Ndc80 complexes make up the KMN network (Cheeseman & Desai, 2008). Finally, the Dam1 complex is recruited to the kinetochore by the Ndc80 complex (Janke et al., 2002).

The KMN network components make up the core outer kinetochore and are highly conserved from yeast to humans. The MIND and NDC80 complexes have both been obtained *in vitro* through recombinant expression and purification methods, allowing for their biochemical characterization. The heterotetrameric MIND complex (Dsn1p, Nsl1p, Mtw1p, Nnf1p) forms a “Y” structure and the N-terminal domains of Mtw1p and Nnf1p bind inner kinetochore proteins Mif2p and Ame1p (De Wulf et al., 2003; Dimitrova et al., 2016). The Ndc80 complex is a heterotetrameric complex with an elongated structure of ~55 nm (Wei et al., 2005). The Ndc80p and Nuf2p each have a globular calponin homology (CH) domain and an α -helical domain. These two proteins dimerize through a coiled coil domain; Spc24p and Spc25p also dimerize in a similar fashion, but have RWD domains instead of CH domains (Wei et al., 2007; Wei et al.,

2006). The two dimers interact through the ends of their coiled coil domains forming a tetramerization domain (Valverde et al., 2016). The C-terminal domains of MIND complex Mtw1p, Nsl1p, and Dsn1p interact with the RWD domains of Ndc80 complex Spc24p and Spc25p (Kudalkar et al., 2015). Thus, the MIND complex serves as the main connection between the microtubule binding components and the inner kinetochore.

Unlike the MIND and Ndc80 complexes, the Spc105 complex has been historically difficult to obtain *in vitro*. This has resulted in limited information about the Spc105 complex's biochemical behavior. The Spc105 complex is composed of Spc105p and Kre28p and the former has been implicated to have microtubule binding activity (Nekrasov et al., 2003; Pagliuca et al., 2009). In *C. elegans*, the KNL-1 binds microtubules and associates with the MIS-12 complex, but not the NDC-80 complex (Cheeseman et al., 2006); a Kre28p homolog in *C. elegans* has not been identified. In Human, Knl1 and Zwint proteins are the homologs of budding yeast Spc105p and Kre28p, respectively (Kiyomitsu et al., 2007; Pagliuca et al., 2009). The Knl1 C-terminal RWD domain interacts with the C-terminal tail of NSL1 of Mis12 complex, but does not interact with the Ndc80 complex (Petrovic et al., 2014; Petrovic et al., 2010). These studies were carried out using highly truncated forms of human Knl1 (amino acids 2106-2316), unable to show how the full-length Knl1 complex is organized within the KMN network.

Here, we show for the first time a recombinant expression and purification of the full length Spc105 complex. In addition, tandem affinity purification (TAP) tag purification of the native Spc105p-TAP co-purifies other components of the KMN network. Chemical cross-linking and mass spectrometry analysis reveals that the Spc105p and Kre28p interact through a narrow region of coiled-coil domain. We were not able to detect any cross-link amongst KMN network components in either recombinantly or natively purified components. Finally, we report four phosphorylation events on the Kre28p, all of which are not necessary for viability.

Results

Recombinant purification of the Spc105 complex

To gain insight into why the recombinantly purified Spc105 complex has been difficult to obtain *in vitro*, we initially carried out a secondary structure prediction of the Spc105p and Kre28p (McGuffin et al., 2000). While most of the Kre28p is predicted to form secondary structures, only the C-terminal half of Spc105p is predicted to fold into secondary structure (Figure 4.1A). In addition, further analysis showed that the N-terminal half of the Spc105p is predicted to be disordered (Figure 4.1B). This analysis suggests that the Spc105p N-terminal domain (~450 amino acids) is unstructured and may negatively contribute to the Spc105 complex's recombinant expression and purification *in vitro*.

As expected, our initial attempts at expressing and purifying the full length Spc105 complex were not successful (data not shown). In accordance with the secondary structure prediction data, we then added a N-terminal MBP and a C-terminal GFP tag to the Spc105p in attempt to stabilize and solubilize Spc105p. A FLAG tag was also added to the C terminus of GFP for purification purposes (Figure 4.1C). The dicistronic construct of MBP-SPC105-GFP-FLAG and KRE28 was bacterially expressed and purified in two steps using anti-FLAG antibody and amylose resin (see materials and methods). This purified sample separated on SDS-PAGE shows high purity and only minimal degradation (Figure 4.1D). With the addition of solubility tags, we demonstrate for the first time a full length expression and purification of the recombinant Spc105 complex.

Analysis of the recombinantly purified KMN network interaction

We asked how the Spc105 and Kre28p proteins interact to form a complex. This was done by applying a cross-linking and mass spectrometry analysis following protocols developed previously (Hoopmann et al., 2015; Kudalkar et al., 2015; Tien et al., 2014; Zelter et al., 2015). Application of this method to the newly purified Spc105 complex yielded good sequence

coverage and reactivity of the linkable residues (Table 4.1). The Spc105p and Kre28p formed robust cross-links between each other. Most of these cross-links occurred in a narrow region of about 200 amino acids for both Spc105p and Kre28p suggesting that these two proteins interact through a specific region (Figure 4.2). Analysis of the Spc105p and Kre28p with a coiled-coil prediction algorithm revealed that these narrow regions are also predicted to form coiled-coil domains (Figure 4.2). These results suggest that the Spc105 complex is formed through a coiled-coil domain.

Then we attempted to identify the interaction regions among the highly conserved KMN network components. We incubated the recombinant Spc105, MIND, and Ndc80 complexes together before adding the chemical cross-linker. In this reaction, cross-links between the Spc105p and Kre28p were consistent with those when only the Spc105 complex was cross-linked (Figure 4.3A, Figure 4.2). The cross-links of Ndc80 and MIND complexes within each complex and between the two complexes are consistent with previously published results (Figure 4.3; Kudalkar et al., 2015; Tien et al., 2014). In addition, sequence coverage and reactivity of the linkable residues were good (Table 4.2). These results demonstrate a robust cross-linking reaction and mass spectrometry detection of the digested peptides. However, the Spc105 complex did not form extensive cross-links to the Ndc80 or MIND complexes. Only one cross-link was observed between the Spc105 and Ndc80 complexes, and none between the Spc105 and MIND complexes (Figure 4.3 A, B).

Analysis of the natively purified KMN network interaction

Next, we purified the native Spc105 complex from budding yeast with the use of a Spc105p C-terminal tandem affinity purification tag on (Spc105p-TAP). We reasoned that there may be unknown post translational modifications on the KMN network component(s) that promote the assembly of the network. Adopting methods previously described (Fong et al., 2017; Fong et al., 2016), we carried out a one-step purification of the Spc105p-TAP with

magnetic beads conjugated to rabbit IgG, stringent washes, and finally TEV protease cleavage elution (see Methods and Materials). Analysis of this purified sample with SDS-PAGE and silver staining showed additional bands other than the two components of the Spc105 complex, even after the stringent washes (Figure 4.4A). Mass spectrometry analysis of this sample identified other kinetochore proteins with the most abundant ones being components of the KMN network (Figure 4.4B). These results suggest that purification of the native Spc105p co-purifies other KMN network components, and that the native Spc105 complex interacts strongly with the Ndc80 and MIND complexes.

The cross-linking and mass spectrometry analysis was then applied to this natively purified KMN network. The sequence coverage and reactivity of the linkable residues were robust (Table 4.3). However, no cross-link was detected between the Spc105 and Ndc80 complexes, and Spc105 and MIND complexes (Figure 4.5 A, B). In addition, there were no cross-links detected between the Ndc80 and MIND complexes, unlike what was observed with cross-linking of recombinant components (Figure 4.5C). Even though the native Spc105 complex co-purifies the Ndc80 and MIND complexes, we were not able to detect any cross-links among these KMN network components.

Kre28p phosphorylation is not essential for viability

Even though phosphorylation of the Spc105p and its involvement in the SAC has been extensively studied, information about the Kre28p phospho-regulation is still lacking. To address this, we analyzed the natively purified Spc105p-TAP sample with mass spectrometry searching for phosphorylated peptides (see materials and methods). Four phosphorylation events were detected through this method. Four Kre28p phospho-peptides were detected in the MS1 scan, each with a single phosphorylation event. Two sites (T210 and S214) were specifically identified based on the peptide fragments detected in the MS2 scan; however, the other two phospho-

peptides fragmented such that one of two close-by sites may be phosphorylated (T16/S21 and S215/S217). For the first time, we show that Kre28p is phosphorylated.

We next investigated the function(s) of these phosphorylation events. Since the N-terminal phosphorylation sites (T210, S214, S215/S217) are included in the predicted coiled-coil domain of the Kre28p, we hypothesized that these phosphorylation sites may be involved in the assembly of the Spc105 complex. The two certain Kre28p phosphorylation sites (S210, S214) and all four of the ambiguous sites (T16, S21, S215, S217) were mutated to Asp, mimicking phosphorylation (Kre28p-6D). Recombinantly expressing and purifying this mutant Spc105 complex did not have detectable differences during the purification process, when compared to the wild-type Spc105 complex (Figure 4.6B). This suggests that phosphorylation of the Kre28p does not affect the interaction between Spc105p and Kre28p *in vitro*.

Finally, the Kre28p phosphorylation events were tested for their function *in vivo*. We constructed yeast expression plasmids for *KRE28*, *kre28-6D*, and *kre28-6A* encoding the wild-type, phosphomimetic, and phospho-blocking versions of Kre28p. Using a plasmid shuffle assay, we found that both the Kre28p-6D and Kre28p-6A supported growth. The wild-type Kre28p also supported growth and the empty vector did not support growth, as expected (Figure 4.6C). These results suggest that Kre28p phosphorylation at these sites is not required for viability.

Discussion

Outer kinetochore components allow sister chromatids to attach to the spindle microtubules and ultimately make bioriented attachments. The KMN network is considered as the core outer kinetochore and microtubule binding unit (Cheeseman & Desai, 2008). In budding yeast, the KMN network alone is insufficient for strong kinetochore-microtubule attachments. The Ndc80 complex recruits the Dam1 complex, which imparts microtubule tip-tracking ability to the Ndc80 complex and is required for biorientation (Lampert et al., 2010; Tien et al., 2010;

Umbreit et al., 2014). Additionally, the Spc105 complex is the scaffold for recruiting spindle assembly checkpoint proteins; disruption of this checkpoint results in separation of sister kinetochores in the absence of correct attachments and aneuploidy (London & Biggins, 2014b). Understanding the structural organization of the KMN network protein complexes is crucial for uncovering how the outer kinetochore recruits other components for these functionalities.

We demonstrate for the first time a recombinant expression and purification method for obtaining the full length Spc105 complex *in vitro*. The association of Spc105p and Kre28p through coiled coil domains is similar to the organization of other kinetochore proteins and the human Knl1 complex. Human Knl1 and Zwint also have sequences predicted to form coiled-coil domains similar to the Spc105 complex proteins. Full length Zwint associates with Knl1 that contains this coiled-coil domain, but not with Knl1 not containing the coiled-coil domain (Petrovic et al., 2010). This suggests that the formation of coiled-coil domains within the Spc105 complex is conserved between budding yeast and human. In addition, previous biochemical experiments suggest the Spc105p:Kre28p stoichiometry to be 1:2 forming a complex of ~195 kDa (Pagliuca et al., 2009). The *C. elegans* KNL-1 recombinantly purified *in vitro* forms decameric oligomers, mediated by a conserved hydrophobic patch (Kern et al., 2015). In budding yeast, the reported copy number of Spc105p per kinetochore is variable from 2.4 to 14.5 (Dhatchinamoorthy et al., 2017; Joglekar et al., 2006; Lawrimore et al., 2011). Currently, the oligomeric state of the Spc105 complex and the stoichiometry between the Spc105p and Kre28p are unclear. The newly purified full length Spc105 complex will be appropriate for studying these characteristics in future experiments.

The Mis12 complex was shown to be the main organizer of the KMN network. In both humans and *C. elegans*, the Ndc80 complex and KNL-1 do not interact with each other, but independently interact with the Mis12 complex (Cheeseman et al., 2006; Petrovic et al., 2014; Petrovic et al., 2010). Organization of the budding yeast KMN network is less clear. The interaction between the MIND and Ndc80 complexes is important for recruiting the Ndc80

complex to the kinetochore and enhancement of the Ndc80 complex microtubule binding activity (Kudalkar et al., 2015). However, how the Spc105 complex is recruited to the outer kinetochore is not known. Similar to other organisms, the MIND complex may be the main recruiter of the Spc105 complex, or the Ndc80 complex may also be involved in this process. We were unable to detect any significant cross-links between the Spc105 complex and the other KMN network components. Co-elution of the MIND and Ndc80 complexes in the Spc105p-TAP purification suggests strong interaction among the KMN network components. Future experiments will focus on optimizing the detection of these interactions through cross-linking the native Spc105p-TAP sample in the crude lysate prior to purification, digestion, and mass spectrometry detection. Cross-linking the sample in crude lysate has the advantage of capturing interactions in a more biologically-relevant environment and a possible higher abundance of the protein of interest.

The KMN network is a hub of regulation important not only for signaling the spindle assembly checkpoint, but also for the error correction pathway. The Mps1 kinase is recruited to kinetochores lacking microtubule attachment and phosphorylates the Spc105p, starting the signaling cascade that ultimately results in activation of the spindle assembly checkpoint and inhibition of anaphase (Musacchio, 2015). The Ndc80p unstructured N-terminal tail is phosphorylated by Aurora B kinase, which decreases the Ndc80 complex's affinity for microtubules (Akiyoshi et al., 2009; Ciferri et al., 2008; Umbreit et al., 2012). This regulation is important for correcting aberrant kinetochore-microtubule attachments, and disrupting it results in chromosome missegregation (Alushin et al., 2012; DeLuca et al., 2006; Foley & Kapoor, 2013). Even though the four phosphorylation sites on Kre28p are not essential for viability, the phosphorylation sites taking place in the KMN network may regulate function. For example, the Cdk1 kinase phosphorylation of the Dam1 complex Ask1p is not necessary for cell viability, but significantly enhances the Dam1 complex oligomerization on microtubules (Li & Elledge, 2003; see Chapter 3). Future work will involve identifying what kinase(s) is responsible for phosphorylating the Kre28p and possible functions of these phosphorylation events.

Materials and methods

Recombinant protein expression and purification

The *MBP-SPC105-GFP-FLAG* and *KRE28* open reading frames were cloned into the pCDF-1b vector such that a single dicistronic transcript is transcribed (pJOK116). This construct was transformed into BL21 Rosetta 2 *E. coli* cells (Millipore Sigma, Burlington, MA) and induced for 16 hours, 18°C. These cells were then resuspended in cold FLAG buffer (50mM Hepes pH 7.6, 500mM NaCl) + cOmplete Protease Inhibitor Cocktail Tablet (Roche, Indianapolis, IN) + Benzonase nuclease (Sigma-Aldrich, St. Louis, MO). The purification was carried out at 4°C from here. These cells were then lysed with a French press and subjected to centrifugation at 30,000 x g, for 20 min. The cleared lysate was then incubated with anti-FLAG resin (Sigma-Aldrich; St Louis, MO) for 30 min before washing with FB + 10 mM MgATP (pH 7). Protein sample was eluted with FB + 100 µg/mL 3x FLAG peptide (Sigma-Aldrich, St. Louis, MO). This eluate was then incubated with amylose resin (NEB, Ipswich, MA) for 30 min before washing with FB + 10 mM MgATP (pH 7), and then MBP buffer (50 mM Hepes pH7.6, 150 mM NaCl; FB). Protein was eluted with MB + 10 mM maltose.

The *S. cerevisiae* Ndc80 and MIND complexes were independently expressed in BL21 Rosetta 2 *E. coli* cells (Millipore Sigma, Burlington, MA) using polycistronic vectors, as previously described (Kudalkar et al., 2015; Powers et al., 2009; Wei et al., 2005). Spc24-6xHis and Dsn1p-FLAG were used for purifying the Ndc80 and MIND complexes, respectively. The Ndc80 complex was affinity purified with Ni²⁺ charged MAC resin column (Bio-Rad, Hercules, CA). The MIND complex was affinity purified with anti-FLAG antibody M2 resin (Sigma-Aldrich, St. Louis, MO). Each of these eluates were further purified using size exclusion chromatography on a Superdex 200 column.

Native budding yeast TAP purification

S. cerevisiae Spc105 complex was purified with a TAP tag on the C terminus of Spc105p as previously described (Fong et al., 2017). Lysed cells were resuspended in purification buffer (20 mM HEPES buffer, pH 7.4, 0.5% Triton X-100, 2 mM MgCl₂, 100 μM GTP, 1 mM ATP, 1 mM DTT, 1 mM PMSF, 4 μg/ml aprotinin, 4 μg/ml chymostatin, 4 μg/ml leupeptin, 4 μg/ml pep- statin, 10 mM sodium fluoride, 1 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 5% glycerol) plus 300 mM NaCl and homogenized. This sample was subjected to centrifugation at 2000 x g for 10 min at 4°C. The cleared lysate was incubated with Dynabeads, conjugated to rabbit IgG (following manufacturer's directions), for 30 min at 4°C. The Dynabeads were then washed three times with purification buffer containing 500 mM KCl, and once with purification buffer containing 150 mM NaCl. Finally, these beads were resuspended in TEV cleavage buffer (40 mM HEPES buffer, pH 7.4, 200 mM NaCl, 2 mM MgCl₂, 1 mM GTP, 1 mM ATP, 1 mM EDTA, pH 8, 1 mM DTT, 5% glycerol) and incubated with 1 μg of TEV for 2 h at 4°C. The eluate was then analyzed with SDS-PAGE and silver staining, and mass spectrometry.

Identification of phosphorylation sites in Spc105 by Mass Spectrometry Analysis

The Spc105p-TAP was purified from yeast (EMMY002-7c) as described above. 650 μL of the purified sample were concentrated down to 100 μL according to manufacturer's instructions in an Amicon 10K MWCO filter unit (Amicon Ultra-0.5 Centrifugal Filter Unit cat number UFC501024; Millipore Sigma, Burlington, MA). 400 μL 25 mM ammonium bicarbonate were mixed with the sample in the filter unit and the sample was spun down to 100 μL. This process was repeated a total of 3 times leaving the protein in 100 μL of 25 mM ammonium bicarbonate. Protein was reduced in the Amicon unit by adding 2.75 μL 200 mM TCEP and incubating for 1 hour at 37°C. Alkylation was performed by adding 1.35 μL of 0.5 M IAC and incubating at room temperature for 20 minutes in the dark. 1.5 μL of 0.2 μg/μL sequencing

grade modified trypsin (Promega Corp cat #V5111; Madison, WI) was added to the Amicon filter unit and samples were digested at 37°C for 4 hours in an Eppendorf Thermomixer with shaking (1000 rpm). Digested sample was transferred to a clean eppendorf tube by pipetting. Filter unit was washed by spinning through 100 μ L 25 mM ammonium bicarbonate. Flow through was added to the digested sample. This process was repeated a total of 3 times. Finally, the Amicon unit was inverted and eluted according to the manufacturer's instructions. The combined sample from the steps above was placed in a speed vac at room temperature and volume was reduced to around 15 μ L. Sample was acidified with HCl (pH ~2) and stored at -80°C until analysis. MS analysis was performed as described below for XL-MS. Analysis of MS data was performed Comet (Eng et al., 2013) and a statistically meaningful q-value was assigned to each peptide spectrum match (PSM) using Percolator version 2.08 (Käll et al., 2007). Data was visualized using MSDataPI (Sharma et al., 2012).

Chemical Cross-linking and Mass Spectrometry Analysis (XL-MS)

Chemical crosslinking and mass spectrometry analysis (XL-MS) was carried out as described by (Zelter et al., 2015). The Spc105p-TAP was purified from yeast (EMMY002-7c) following steps described above. 1 mL the sample in TEV cleavage buffer (40 mM Hepes pH 7.6, 150 mM NaCl, 1 mM DTT) was brought up to 0.25 mM BS3 or 0.5 mM DSS using 14.5 mM stock crosslinker solutions. Samples were crosslinked for 15 or 30 minutes at room temperature (BS3 and DSS samples, respectively) before quenching the reaction by bringing it to 100 mM ammonium bicarbonate. Samples were concentrated and buffer exchanged into 25 mM ammonium bicarbonate using Amicon concentration filter units and digested as described for samples analyzed for phosphorylation sites above. For recombinant *E. coli* expressed Spc105 complex and Spc105/Ndc80/MIND complex crosslinking, reactions were 35 μ L total volume in 50 mM Hepes, 150 mM NaCl, 10 mM maltose, pH 7.6, 5% glycerol plus 0.5 mM DSS or 9.6 mM EDC plus 9.6 mM sulfo-NHS. Spc105/Ndc80c/MIND complexes where present were at 0.4 μ M

final concentration of each protein complex. Crosslinking was carried out at room temperature. For EDC/sulfo-NHS reactions crosslinking was done for 40 mins prior to quenching by addition of 4.2 μ L 0.33 mM BME. For DSS reactions crosslinking was performed for 10 mins before quenching by addition of 4.2 μ L 0.83 mM ammonium bicarbonate. After quenching, reactions were brought up to 0.1% PPS silent surfactant (Expedition Inc.; San Diego, CA) and 5 mM TCEP (tris(2 carboxyethyl)phosphine) and reduced for 60 mins at 60°C. Alkylation was performed at room temperature with 6 mM iodoacetamide, followed by trypsin digestion at 37°C for 6 hours in an Eppendorf Thermomixer with shaking (1000 rpm) at a substrate to enzyme ratio of 15:1 prior to acidification with 250 mM HCl (final concentration). Digested samples were stored at 80°C until analysis. Mass spectrometry and data analyses were performed as previously described (Zelter et al., 2015). In brief, 3 μ L of digested protein was loaded by autosampler onto a 150 μ m Kasil fritted trap packed with 2 cm of ReprosilPur C18AQ (3 μ m bead diameter, Dr. Maisch) at a flow rate of 2 μ L per min. After desalting with 8 μ L of 0.1% formic acid plus 2% acetonitrile, the trap was brought online with a fused silica capillary tip column (75 μ m i.d.) packed with 30 cm of ReprosilPur C18AQ (3 μ m bead diameter, Dr. Maisch). Peptides were eluted from the column at 0.25 μ L/min using an acetonitrile gradient. A QExactive HF (Thermo Fisher Scientific) was used to perform mass spectrometry in data dependent mode. Acquired spectra were converted into mzML using msconvert from ProteoWizard (Chambers et al., 2012). All proteins in the sample were identified using Comet (Eng et al., 2013). Crosslinked peptides were identified within proteins identified by comet, using Kojak version 1.4.3 (Hoopmann et al., 2015) available at (<http://www.kojak-ms.org>). A statistically meaningful q value was assigned to each peptide spectrum match (PSM) through analysis of the target and decoy PSM distributions using Percolator version 2.08 (Käll et al., 2007). Target databases consisted of all proteins identified in the sample analyzed. Decoy databases consisted of the corresponding set of reversed protein sequences. Data were filtered to show hits to the target proteins that had a Percolator assigned peptide level q value ≤ 0.01 and were identified by 2 or more PSMs.

Testing the function of the Kre28p phosphorylation in vivo

The function of Kre28p phosphorylation sites *in vivo* was assayed using the red/white plasmid shuffle (Davis, 1992; E. G. Muller, 1996) and strain JOKY065-7D (*ade2-1oc ade3Δ-100 can1-100 his3-11,15 leu2-3,112 lys2Δ::HIS3 trp1-1 ura3-1 kre28Δ::KANMX*) containing plasmid pJOK124 (*ADE3 LYS2 KRE28* in a 2μm vector). JOKY065-6D was transformed with pJOK127, pJOK129, pJOK130, and pRS316 (see Table 4.1) and plated on SD-ura plates. Colonies were grown at 25°C and observed for sectoring.

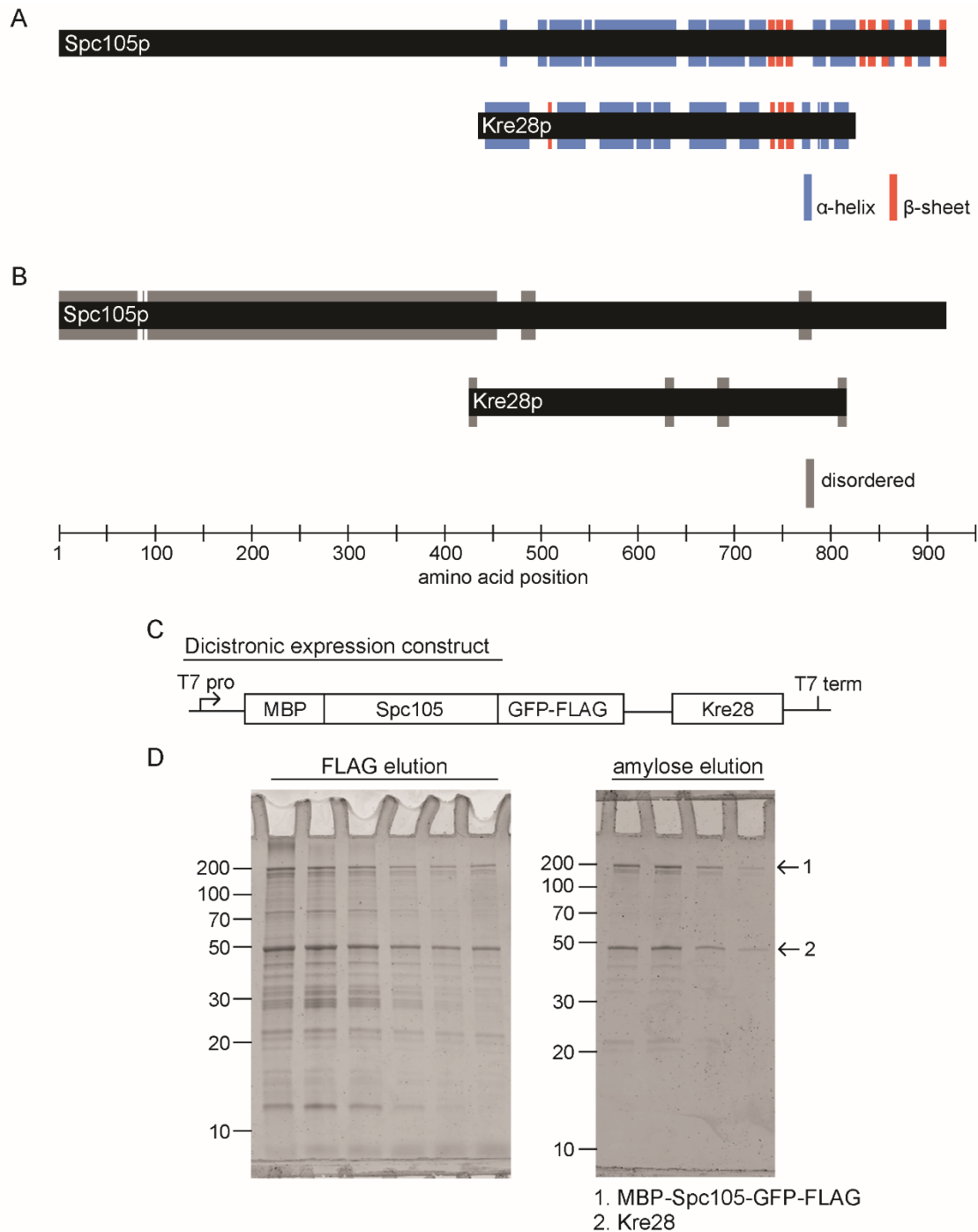


Figure 4.1 Purification of the Spc105 complex

(A) Secondary structure prediction of Spc105p and Kre28p using PSIPRED3 (McGuffin et al., 2000). (B) Disordered structure prediction of Spc105p and Kre28p using DISOPRED3 (D. T. Jones & Cozzetto, 2015). (C) Bacterial expression construct of the full length Spc105 complex under control by the T7 promoter and terminator. (D) Coomassie blue stained SDS-PAGE gels of elution fractions during Spc105 complex purification. Left gel shows elution fractions from the first FLAG-affinity step and right gel shows elution fractions from the second MBP-affinity step.

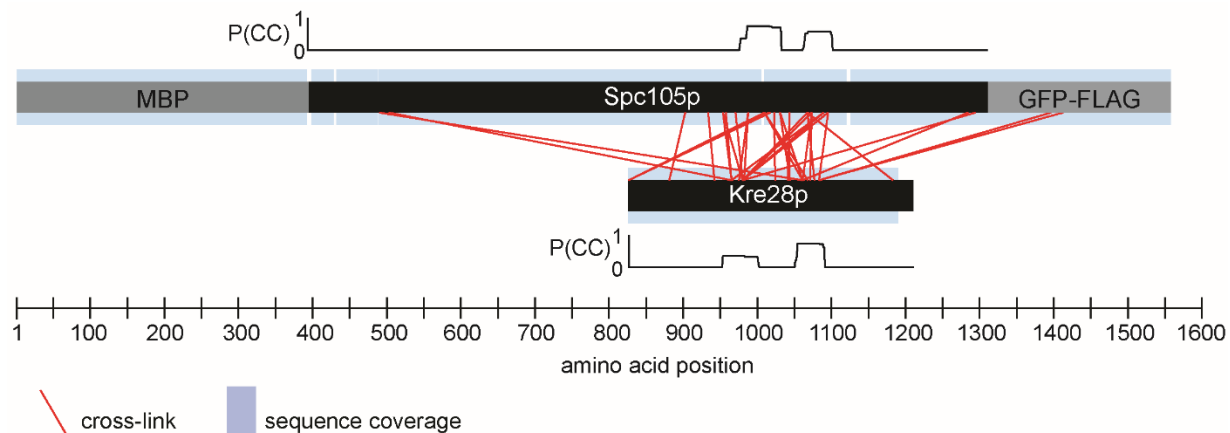


Figure 4.2 The Spc105p and Kre28p interact through a coiled-coil domain

Cross-links between the MBP-Spc105p-GFP-FLAG and Kre28p. Horizontal black and gray bars represent proteins and the blue bars represent mass spectrometry sequence coverage. Red lines represent cross-links between the two proteins from Percolator (Käll et al., 2007) peptides assigned q -values ≤ 0.05 . Data shown is the combination of two experiments using DSS and EDC cross-linkers. Graphs above and below the protein bars present the probability of each protein sequence forming coiled-coil domain based on PairCoil2 analysis (McDonnell, Jiang, Keating, & Berger, 2006).

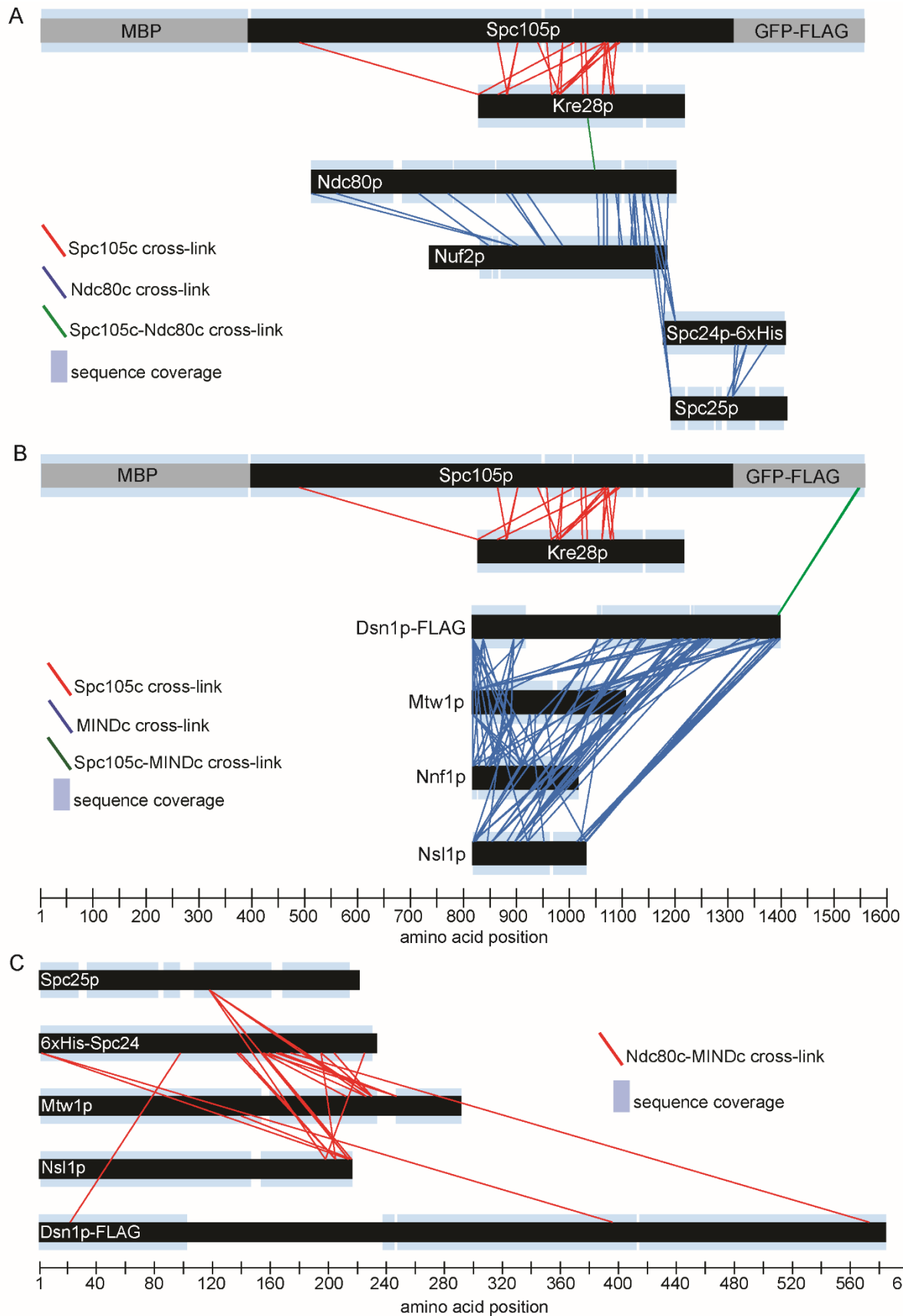


Figure 4.3 Cross-linking of recombinantly reconstituted KMN network

Recombinantly purified Spc105, Ndc80, and MIND complexes were combined before the addition of DSS or EDC cross-linker. Data shown here show cross-links combined from both experiments using Percolator (Käll et al., 2007) peptide q-value ≤ 0.01 . Horizontal black bars and blue bars represent protein and mass spectrometry sequence coverage, respectively. (A) Cross-links within Spc105 and Ndc80 complexes, and between the two complexes. (B) Cross-links within Spc105 and MIND complex, and between the two complexes. (C) Cross-links between the Ndc80 and MIND complexes. Proteins involved in the interaction of these two complexes are shown (Kudalkar et al., 2015). Cross-links within Ndc80 and MIND complexes are omitted for clarity.

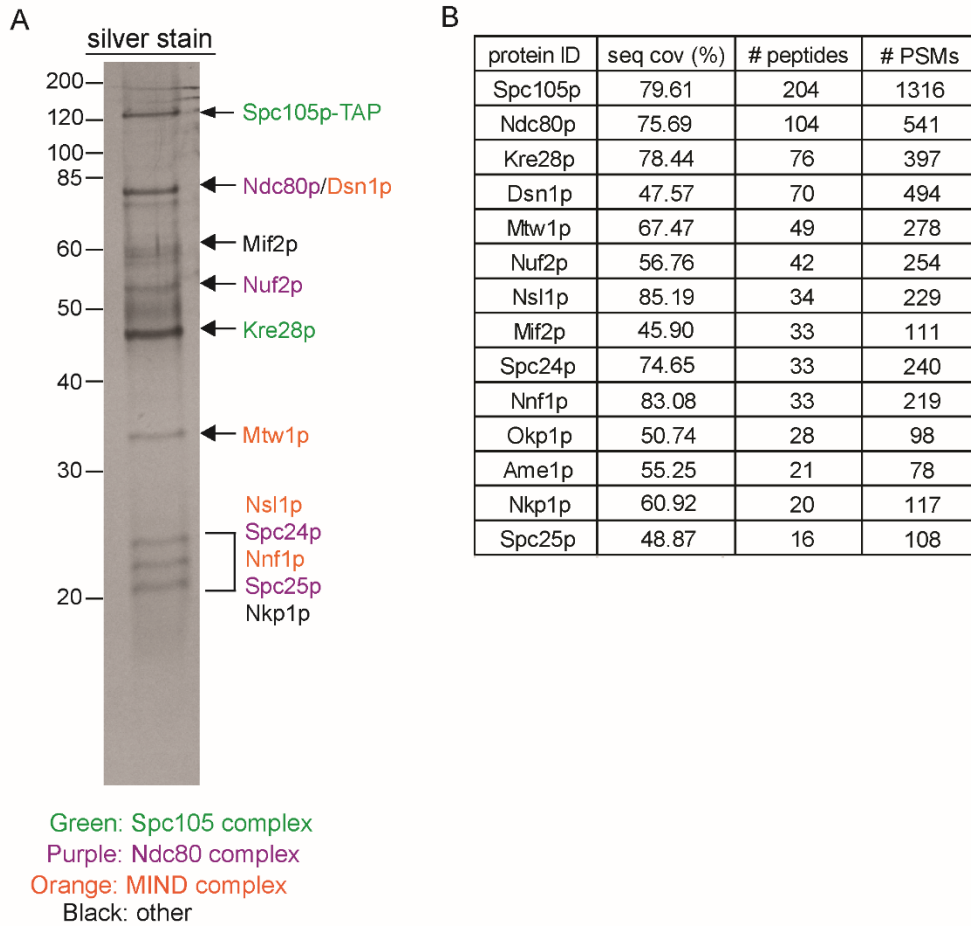


Figure 4.4 Native Spc105-TAP purification co-purifies other KMN network components

(A) Silver stained SDS-PAGE gel of the Spc105-TAP purification eluate. Protein bands are labeled based on predicted size and the abundance from mass spectrometry. (B) Data showing the most abundant kinetochore proteins from the Spc105-TAP purification. Proteins are arranged based on the number of peptides.

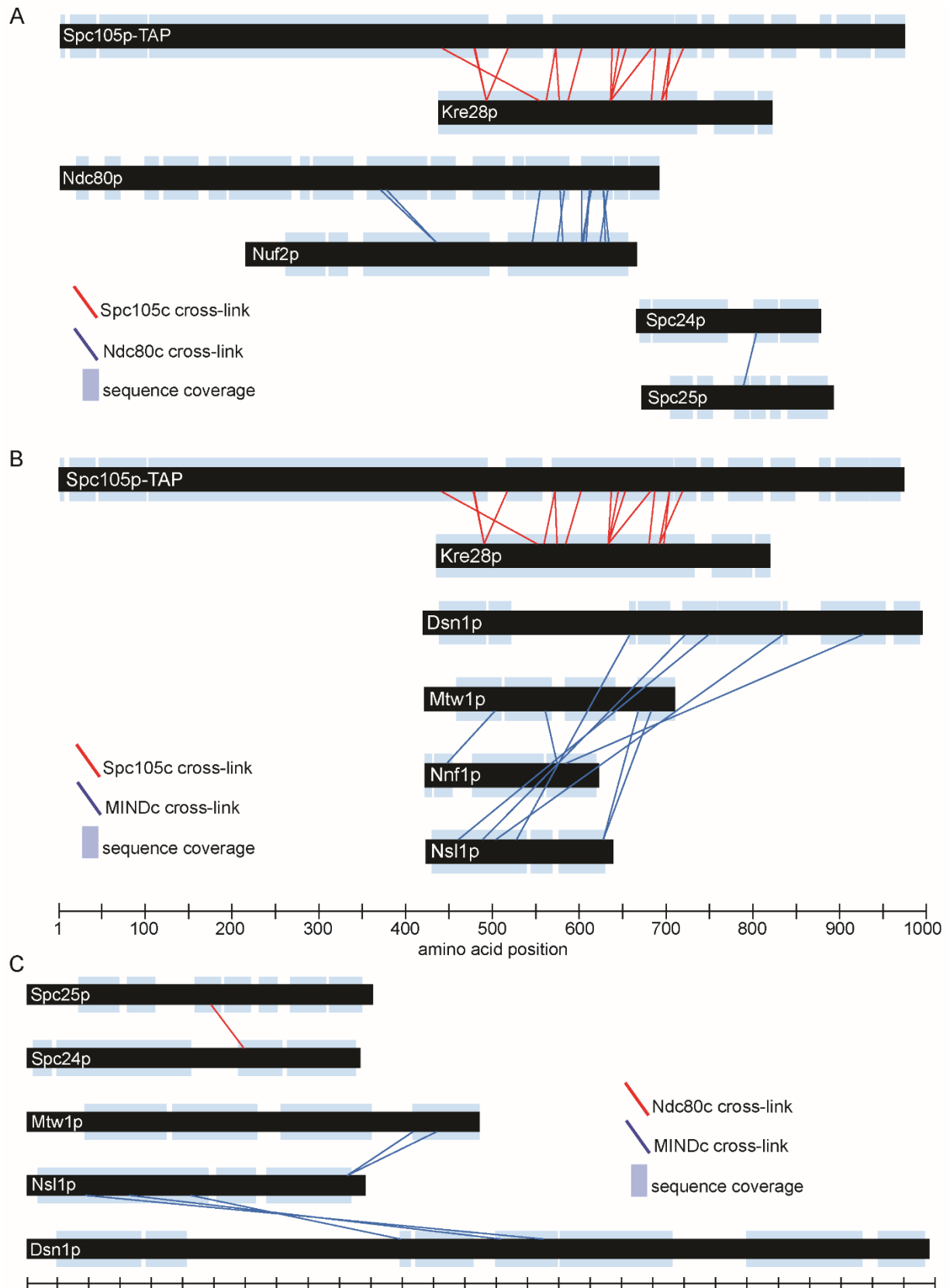


Figure 4.5 Cross-linking of the natively purified KMN network

The natively purified KMN network using Spc105-TAP sample was cross-linked using DSS or BS3. Data shown here show cross-links combined from both experiments using Percolator (Käll et al., 2007) peptide q-value ≤ 0.01 . Horizontal black bars and blue bars represent protein and mass spectrometry sequence coverage, respectively. (A) Cross-links within Spc105 and Ndc80 complexes, and between the two complexes. (B) Cross-links within Spc105 and MIND complex, and between the two complexes. (C) Cross-links between the Ndc80 and MIND complexes. Proteins involved in the interaction of these two complexes are shown (Kudalkar et al., 2015).

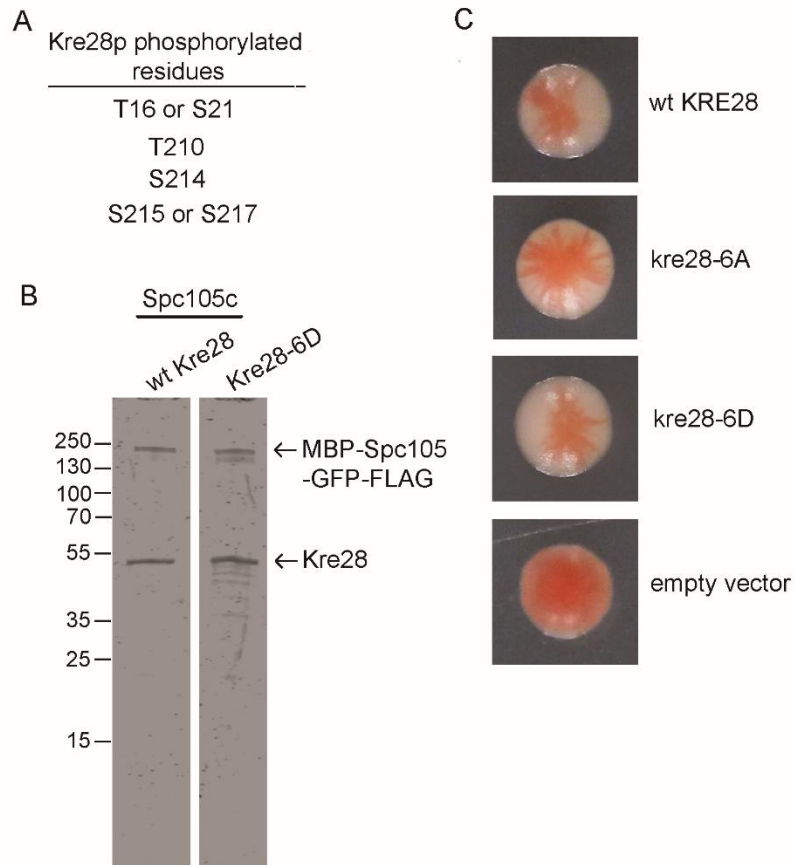


Figure 4.6 Kre28 phosphorylation sites are not essential for viability

(A) Four detected phosphorylation sites on the Kre28p. Natively purified Spc105-TAP sample was analyzed for phosphor-peptides. Two of four sites are ambiguous based on the detected fragments in the MS2 scan. (B) Coomassie Blue stained SDS-PAGE gels of the recombinantly purified wt Spc105 or Spc105-6D (Kre28 T16D, S21D, T210D, S214D, S215D, S217D) complex. (C) Red/white plasmid shuffle assay to test the viability of the Kre28 phospho-site mutations. Solid red colony indicate the inability of the empty vector to support growth. Sectoring white colonies show the ability of wt KRE28, kre28-6A, and kre28-6D to support growth.

Table 4.1 Statistics from recombinant Spc105 complex cross-linking and MS^a

protein	sequence coverage	proportion of reacted linkable residues ^b
MBP-Spc105-GFP-FLAG	0.99	0.69
Kre28-6xHis	1	0.62

Table 4.2 Statistics from recombinant KMN network cross-linking and MS^a

protein	sequence coverage	proportion of reacted linkable residues ^b
MBP-Spc105-GFP-FLAG	0.98	0.58
Kre28-6xHis	0.98	0.67
Ndc80	0.96	0.57
Nuf2	0.75	0.52
6xHis-Spc24	0.97	0.67
Spc25	0.84	0.21
Dsn1-FLAG	0.76	0.66
Mtw1	0.92	0.59
Nnf1	0.99	0.63
Nsl1	0.96	0.53

Table 4.3 Statistics from native KMN network cross-linking and MS^a

protein	sequence coverage	proportion of reacted linkable residues ^b
Spc105-TAP	0.84	0.86
Kre28	0.94	0.82
Ndc80	0.71	0.67
Nuf2	0.77	0.61
Spc24	0.8	0.78
Spc25	0.61	0.50
Dsn1	0.59	0.70
Mtw1	0.72	0.72
Nnf1	0.84	0.77
Nsl1	0.87	0.56

^aStatistics shown are for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.01

^bStatistic showing the proportion of reacted linkable residues in the total number of linkable residues sequenced

Table 4.4 plasmids used in this study

Plasmid	Relevant markers	Reference
pRS316	<i>CEN6 ARSH4 URA3 bla</i>	Sikorski and Hieter 1989
pJOK127	<i>KRE28</i> in pRS316	This study
pJOK129	<i>kre28 T16A S21A T210A S214A S215A S217A</i> in pRS316	This study
pJOK130	<i>kre28 T16D S21D T210D S214D S215D S217D</i> in pRS316	This study
pJOK116	<i>MBP-SPC105-GFP-FLAG, KRE28-6xHis</i> in <i>pCDF-1b</i>	This study
pJOK123	<i>MBP-SPC105-GFP-FLAG, KRE28-6D (T16D S21D T210D S214D S215D S217D)-6xHis</i> in <i>pCDF-1b</i>	This study
wt Ndc80, Nuf2	<i>NDC80 NUF2</i> in pETDuet	This study
wt Spc24, Spc25	<i>6xHis-SPC24 SPC25</i> in pRSFDuet	This study
wt MINDc-FLAG	<i>DSN1-FLAG MTW1 NNF1 NSL1</i> in pST39	This study

Table 4.5 yeast strains used in this study

Strain	Genotype ^a	Reference
W303	<i>ade2-1oc can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1</i>	
EMMY002-7C	<i>ade3Δ-100 SPC105-6xHis-TAP</i>	This study
JOKY065-7D	<i>ade3Δ-100 lys2Δ::HIS3 kre28Δ::KANMX</i>	This study

^aAll strains have the same markers as W303 except as noted

Chapter 5

Conclusions and future directions

The conserved outer kinetochore mediates essential functions during mitosis. These include making strong and processive attachments to microtubule tips, activating the error correction pathway, and signaling the spindle assembly checkpoint. The arrangement of outer kinetochore proteins impacts how the kinetochore carries out these functions. My thesis research detailed the interaction mechanism between the Ndc80 and Dam1 complexes, and how they are organized at the kinetochore-microtubule interface. I also determined how the Aurora B kinase regulates the interaction between these two complexes. In addition, I found that the Aurora B kinase and Cdk1 regulate the Dam1 complex microtubule-binding activity through several mechanisms. Finally, I report methods for the purification of recombinant full length Spc105 complex and native KMN network purification from budding yeast. The MIND and Ndc80 complexes are co-purified with the Spc105 complex from yeast; these advancements might provide a future platform for studying the architectural organization of the outer kinetochore. Overall, I have contributed to our understanding of the molecular architecture and regulation of the budding yeast kinetochore-microtubule interface.

The Ndc80 complex bridging two Dam1 complex rings: implications at the kinetochore-microtubule interface

The Ndc80 complex's ability to bridge two Dam1 complex rings *in vitro* has implications on how these proteins are organized *in vivo*. Unlike other kinetochore proteins, the Dam1 complex localizes along the length of the spindle microtubules (Cheeseman et al., 2001; Jones et al., 2001). After the Ndc80 complex attaches kinetochores laterally to microtubules, molecular motors transport the kinetochores and chromosomes toward the spindle pole (Rieder & Alexander, 1990; Tanaka et al., 2005; Yang et al., 2007). When the microtubule plus-ends depolymerize to catch up to the kinetochores, the lateral attachments are converted to end-on

attachments (Tanaka, 2010). During this process, the Ndc80 complex is exposed to many Dam1 complexes already localized to the microtubule. We show that the Ndc80 complex coiled-coil domain contains three Dam1 complex binding sites. Two sites together bind one Dam1 complex ring and the third site binds the second ring, resulting in the Ndc80 complex bridging two rings *in vitro*. Mutating any one of the three Ndc80 complex interaction sites results in chromosome segregation error. We postulate that the Dam1 complex might occupy all three binding sites on the Ndc80 complex *in vivo*.

Even though the Dam1 complex oligomerization and ring formation has been extensively studied *in vitro*, it is still unclear if rings are present at the kinetochore-microtubule interface in cells. Fluorescence brightness studies in budding yeast that counted the copy number of each kinetochore protein give conflicting results. The measurements suggest enough Dam1 complex molecules for one or two rings per kinetochore (Dhatchinamoorthy et al., 2017; Joglekar et al., 2006; Lawrimore et al., 2011). Regardless of the Dam1 complex copy number at each kinetochore, these studies still do not show direct evidence of ring formation in a cell. Ring formation is not required for the Dam1 complex to track with microtubule tips, but oligomerization is required for kinetochores to track with microtubule tips under load-bearing conditions (Gestaut et al., 2008; Umbreit et al., 2014). Hence, it is also possible that the Ndc80 complex recruits partial Dam1 complex rings to the kinetochore.

The Dam1 complex is specific to fungal organisms and the Ska complex is believed to be its functional homolog in human. A recent study showed that the Dam1 complex and Ska complex (Ska1, Ska2, Ska3 proteins) are widespread in eukaryotic species in an inverse manner, suggesting their functional redundancy and homology (Van Hooff et al., 2017). *In vivo*, kinetochore localization of the Ska complex is dependent on the Ndc80 complex (Janczyk et al., 2017; Zhang et al., 2017), and loss of the Ska complex is associated with chromosome segregation defects and mitotic delay (Hanisch et al., 2006; Welburn et al., 2009). *In vitro*, the Ska complex binds microtubules and makes load-bearing attachments to microtubule tips

(Helgeson et al., 2018; Welburn et al., 2009). The Ska complex enhances the microtubule tip tracking ability of the human Ndc80 complex (Schmidt et al., 2012). These characteristics of the Ska complex resemble the kinetochore functions of the Dam1 complex.

The Dam1 and Ska complexes also have differences. The heterodecameric Dam1 and heterotrimeric Ska complexes do not have sequence or structural homology (Abad et al., 2014; Jenni & Harrison, 2018; Jeyapragash et al., 2012). In Ndc80 complex-based microtubule tip attachments, the Ska complex does not affect the attachment lifetime under constant force and selectively strengthens load-bearing capacity when weakened by low Ndc80 complex concentration (Helgeson et al., 2018). Under similar conditions, the Dam1 complex enhances attachment lifetime and strengthens load-bearing attachments even at high Ndc80 complex concentrations (Tien et al., 2010). Additionally, cross-linking and mass spectrometry analysis show only the Ska3 protein extensively cross-linking with the coiled-coil domain of all four human Ndc80 complex proteins (Helgeson et al., 2018). In budding yeast, we detected cross-links between multiple Dam1 complex proteins and mainly the Ndc80p and Nuf2p coiled-coil domains. Despite the differences, these results suggest that the budding yeast and human Ndc80 complexes contain multiple sites on the coiled-coiled coil domain for binding the Dam1 and Ska complexes, respectively.

Rigid control of the Dam1 complex microtubule binding mechanisms

The Aurora B kinase phosphorylates the Dam1 complex at six different sites (Cheeseman et al., 2002). I show that these phosphorylation events disrupt the Dam1 complex's affinity and avidity for microtubules, and its interaction with the Ndc80 complex. In contrast, I also revealed that Cdk1 phosphorylates Ask1p to enhance the Dam1 complex's avidity for microtubules. The presence of multiple mechanisms controlling the Dam1 complex oligomerization on microtubules highlight the importance of oligomerization for the Dam1 complex's function at the kinetochore. This is also consistent with previous work showing the

requirement of oligomerization for establishing sister kinetochore biorientation (Umbreit et al., 2014).

Mechanisms of the Ska complex regulation at the outer kinetochore contain both similarities and differences compared to the Dam1 complex regulation. The Aurora B kinase also phosphorylates the Ska complex to inhibit its microtubule binding and kinetochore localization (Abad et al., 2014; Chan et al., 2012). Instead of enhancing microtubule binding, Cdk1 phosphorylation of the Ska complex promotes its kinetochore localization (Zhang et al., 2017). In budding yeast, Mps1 kinase phosphorylation of the Dam1 complex promotes efficient microtubule plus-end coupling of kinetochores (Shimogawa et al., 2006); however, the Mps1 kinase phosphorylation of the Ska complex inhibits kinetochore-microtubule attachments in human (Maciejowski et al., 2017). The effects of phosphorylation by different mitotic kinases do not exactly align between the budding yeast Dam1 complex and human Ska complex; however, these results suggest that stringent regulation of microtubule-binding kinetochore components is conserved throughout evolution.

Future work will focus on better understanding the architecture and regulation of the kinetochore-microtubule interface. In addition to my work on the organization of the Ndc80 and Dam1 complexes, we still do not clearly understand how the KMN network is organized. I showed that purifying the Spc105p-TAP from budding yeast co-purifies other KMN network proteins. The application of more advanced cross-linking and mass spectrometry methods may yield information about the architectural organization of the budding yeast outer kinetochore. Compared to the budding yeast, the interaction mechanisms between the human Ska and Ndc80 complexes still remain unclear. Further work will show how these two organisms are similar and different in their outer kinetochore organization.

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Appendix A

The Ndc80 complex bridges two Dam1 complex rings

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The Ndc80 complex bridges two Dam1 complex rings

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Abstract Strong kinetochore-microtubule attachments are essential for faithful segregation of sister chromatids during mitosis. The Dam1 and Ndc80 complexes are the main microtubule binding components of the *Saccharomyces cerevisiae* kinetochore. Cooperation between these two complexes enhances kinetochore-microtubule coupling and is regulated by Aurora B kinase. We show that the Ndc80 complex can simultaneously bind and bridge across two Dam1 complex rings through a tripartite interaction, each component of which is regulated by Aurora B kinase.

Mutations in any one of the Ndc80p interaction regions abrogates the Ndc80 complex's ability to bind two Dam1 rings in vitro, and results in kinetochore biorientation and microtubule attachment defects in vivo. We also show that an extra-long Ndc80 complex, engineered to space the two Dam1 rings further apart, does not support growth. Taken together, our work suggests that each kinetochore in vivo contains two Dam1 rings and that proper spacing between the rings is vital.

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Introduction

Kinetochores link replicated chromosomes to spindle microtubules. They form attachments flexible and strong enough to stay attached to microtubules during assembly and disassembly. Estimates of the strength of the attachments suggest that they are much stronger than required just to pull a chromosome through the cellular milieu (Fisher et al., 2009; Nicklas, 1965). Yet, incorrect attachments are weak enough to be detached and corrected.

The Dam1 and Ndc80 complexes are the main microtubule-binding components in the budding yeast kinetochore. The Ndc80 complex connects the Dam1 complex to the rest of the kinetochore. The two complexes interact on microtubules, significantly increasing the strength of Ndc80 complex attachment to microtubules (Lampert et al., 2010, 2013; Tien et al., 2010). How these two complexes interact on microtubules remains uncertain with conflicting results reported in the literature (Lampert et al., 2013; Maure et al., 2011).

In addition to providing strong attachments between chromosomes and spindle microtubules, kinetochores also serve as regulatory hubs. The Ndc80 complex and the Dam1 complex are at the center of this regulation. The Ndc80 complex is a scaffold for the spindle assembly checkpoint (Dou et al., 2015; Hiruma et al., 2015; Ji et al., 2015). Both the Dam1 and Ndc80 complexes are targets of Aurora B kinase, which corrects aberrant kinetochore-microtubule attachments to achieve bioriented attachments (Reviewed in Sarangapani and Asbury, 2014). Aurora B kinase phosphorylation of the Dam1 complex components Dam1p, Ask1p, and Spc34p together disrupts the interaction between the Dam1 and Ndc80 complexes (Lampert et al., 2013; Tien et al., 2010); however, which phospho-protein is responsible for disrupting the interaction has yet to be deciphered.

eLife digest The genetic material inside yeast, human and other eukaryotic cells is stored within structures called chromosomes. Every time a cell divides to make two “daughter” cells, all the chromosomes in the cell must be copied and then separated into two equal sets, with each set delivered to a different daughter cell. If the copied chromosomes divide into unequal sets the daughter cells may die or not work properly.

Chromosomes are attached to tube-like structures called microtubules, which pull the two sets of chromosomes to opposite ends of the cell just before it divides. Microtubules are constantly shrinking and growing, and chromosomes must stay attached the whole time, or they will not be correctly separated. Many proteins are involved in attaching chromosomes to microtubules, including two groups known as the Dam1 complex and the Ndc80 complex. The Dam1 complex forms a ring around microtubules, while the Ndc80 complex forms a long flexible rod that can bend in the middle. However, it was not clear how these shapes allow these complexes to perform their roles in cells.

Kim et al. studied these two complexes from yeast cells. The experiments show that the Ndc80 rod bridges two Dam1 rings, not one as previously assumed. Both of these rings and the rod are required for chromosomes to attach to microtubules. Cells with defective Ndc80 rods that can only bind to one ring do not distribute their chromosomes correctly when they divide. Kim et al. also show that the Ndc80 rod holds the Dam1 rings a specific distance apart, which also appears to be important for the chromosomes to be correctly divided between daughter cells.

The next step following on from this work is to find out exactly why both rings are needed and why they seem to need to be a set distance apart. Human and other eukaryotic cells divide in a similar way to yeast cells, so these findings may help us to understand what goes wrong in Down’s syndrome and other diseases caused by cells having the wrong number of chromosomes.

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The spontaneous assembly in vitro of the Dam1 complex into microtubule-encircling rings suggests a compelling mechanism for kinetochore-microtubule coupling. The ring might be pushed by the curled depolymerizing microtubule protofilaments and prevented from falling off of flared assembling ends (Asbury et al., 2011; McIntosh et al., 2008). We don’t know the organization of the Dam1 complex in the cell, although oligomerization is essential for formation of strong kinetochore-microtubule attachments (McIntosh et al., 2013; Umbreit et al., 2014). Estimates of the number of Dam1 complexes at the kinetochore in vivo have suggested numbers as low as 10, which is not enough to form a single 16-membered ring, and as high as 32, which is enough for two rings (Aravamudhan et al., 2013; Joglekar et al., 2006; Lawrimore et al., 2011). Despite this uncertainty, previous budding yeast kinetochore models have assumed one Dam1 complex ring (Aravamudhan et al., 2015; Cheeseman and Desai, 2008; Joglekar et al., 2009; Tanaka et al., 2007).

In this study, we provide evidence that the kinetochore requires the Ndc80 complex to bind and bridge two Dam1 complex rings in vivo and that Aurora B kinase regulates the interactions at both rings. Our work suggests that the specific ring-ring distance, defined by the Ndc80 complex bridge, is important for supporting growth. The presence of two Dam1 rings at the kinetochore, in a specific orientation bound to the Ndc80 complex, would have implications for how attachment strength is established and modulated by tension.

Results

Three Ndc80p regions interact with Dam1p, Ask1p, and Spc34p of the Dam1 complex

We identified where the Dam1 and Ndc80 complexes interact using protein cross-linking and mass spectrometry analysis (Figure 1A and Figure 1—figure supplement 1) following protocols developed previously (Hoopmann et al., 2015; Kudalkar et al., 2015; Tien et al., 2014; Zelter et al., 2015). The full decameric Dam1 complex and tetrameric Ndc80 complex were cross-linked in the

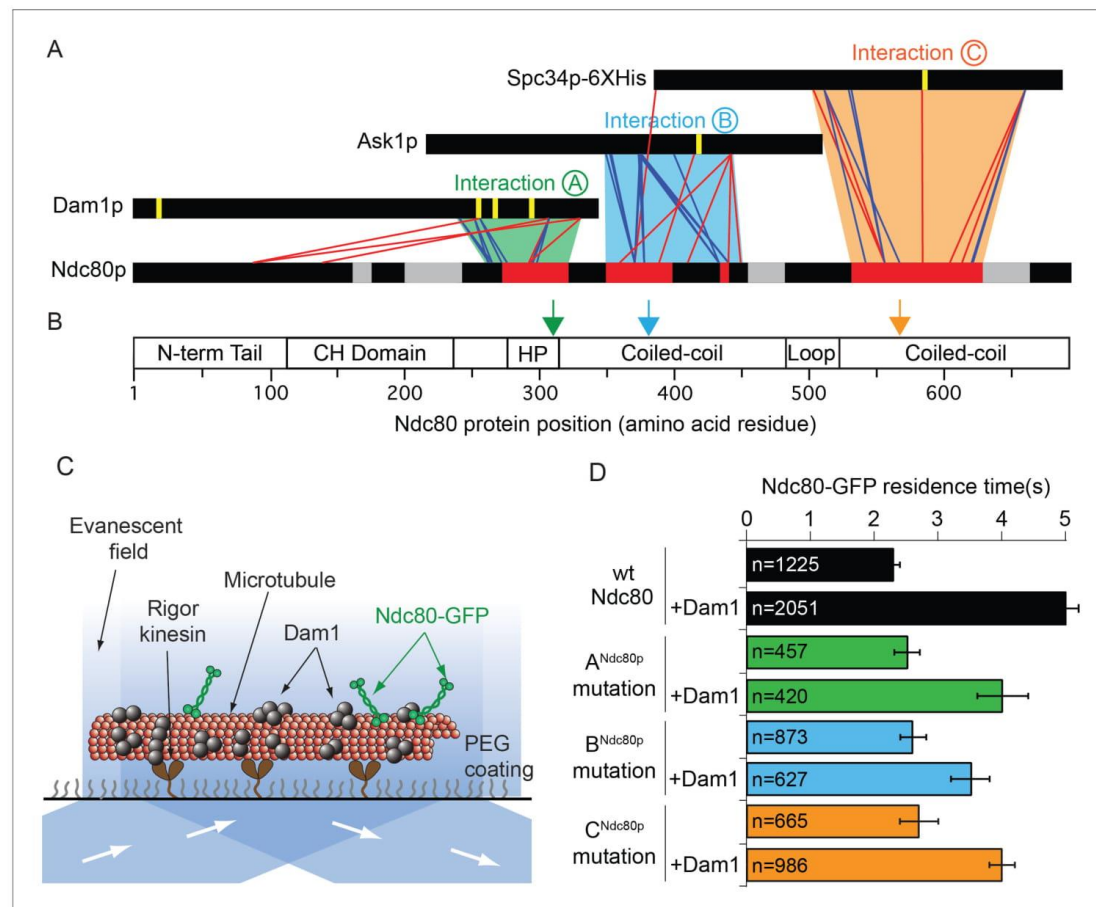


Figure 1. Dam1p, Ask1p, and Spc34p form cross-links to three distinct Ndc80p regions. (A) Cross-links between the Dam1p, Ask1p, and Spc34p of the Dam1 complex and Ndc80p of the Ndc80 complex. Dam1 and Ndc80 complexes were cross-linked in the presence of microtubules. Horizontal black bars represent proteins and the six vertical yellow lines indicate Aurora B kinase phosphorylation sites on the Dam1 complex. Red and blue lines show cross-links formed with DSS and EDC cross-linkers, respectively. For clarity, only the cross-links between the Dam1 complex proteins and Ndc80p are displayed. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.05 . Red bars on Ndc80p indicate regions where clusters of lethal mutations mapped (from Tien et al., 2013) to cross-linked regions. Grey bars on Ndc80p indicate clusters of lethal insertions outside of cross-linked regions. Green, blue, and orange trapezoids represent putative interactions (A, B, and C) between the Dam1 and Ndc80 complexes. (B) Bar diagram of Ndc80p with structural features. Green, blue and orange arrows indicate the positions of lethal mutations in interaction regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} used in this study. (CH: calponin homology; HP: hairpin) (C) Diagram showing the setup of TIRF microscopy experiments. Single molecule Ndc80-GFP complex binding on microtubules was visualized in the presence or absence of the Dam1 complex. (D) Lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} partially disrupts the Ndc80 complex's interaction with Dam1 complex. Average residence time of Ndc80-GFP mutant and wild-type complexes on microtubules in the presence or absence of Dam1 complex. Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Materials and methods for additional details). Ndc80-GFP complex microtubule residence time raw data are included in **Figure 1—source data 1**. Refer to **Supplementary file 1A** for statistical analysis of data in part (D).

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The following source data and figure supplements are available for figure 1:

Source data 1. Table of Ndc80-GFP microtubule residence times for **Figure 1D**.

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Figure 1 continued on next page

Figure 1 continued

Figure supplement 1. Dam1 and Ndc80 complexes robustly react with DSS and EDC cross-linking agents.

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Figure supplement 2. Dam1p, Ask1p, and Spc34p cross-link to Nuf2p, in agreement to the coiled-coil structure of Ndc80p and Nuf2p.

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Figure supplement 3. Ndc80 mutations outside of regions A, B, and C do not disrupt the interaction between Dam1 and Ndc80 complexes.

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Figure supplement 3—source data 1. Table of Ndc80-GFP microtubule residence times for Figure 1—figure supplement 3C.

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presence of taxol-stabilized microtubules. The C-terminal regions of Dam1p, Ask1p, and Spc34p formed cross-links to three distinct regions of Ndc80p (Figure 1A; Figure 1—figure supplement 1). The corresponding regions of Nuf2p also formed cross-links to these same regions of the Dam1 complex (Figure 1—figure supplement 2). These three C-terminal regions of the Dam1 complex include five sites phosphorylated by Aurora B kinase. Phosphorylation of all five sites fully disrupts the interaction between the Dam1 and Ndc80 complexes (Lampert et al., 2010; Tien et al., 2010). Thus, these sites likely represent primary interaction sites through which the Dam1 complex binds the Ndc80 complex.

To identify the cognate binding site(s) on the Ndc80 complex, we compared the cross-linking data to a map of functional regions on Ndc80p identified in a previous linker-scanning mutagenesis screen (Tien et al., 2013). Three of these regions map near or within the regions that cross-link to Dam1p, Ask1p, and Spc34p (Figure 1A). We define these interactions as A, B, and C, and we will refer to the specific regions on each protein that are involved in these interactions as A^{Dam1p} and A^{Ndc80p}, B^{Ask1p} and B^{Ndc80p}, and C^{Spc34p} and C^{Ndc80p}, respectively (Figure 1A and Table 1).

We tested if the lethal mutations identified in our screen in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} (Figure 1B) interfere with interactions between the Ndc80 and Dam1 complexes on microtubules using single-molecule total internal fluorescence (TIRF) microscopy (Figure 1C). Importantly, Ndc80 complexes carrying a mutation in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} demonstrated homogeneous behavior in size exclusion chromatography, similar to the wild type Ndc80 complex (Figure 1—figure supplement 3E). These mutations also did not alter the residence time of the Ndc80 complex alone on microtubules, consistent with previous observations (Tien et al., 2013). As reported previously, the presence of the Dam1 complex significantly increases the residence time of wild-type Ndc80 complex on microtubules (Tien et al., 2010). However, addition of the Dam1 complex only partially increased the residence time of the mutant complexes as compared to the wild-type Ndc80 complex (Figure 1D and Figure 1—figure supplement 3A,B). Ndc80-GFP complex containing Ndc80p insertional mutations outside the regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} had similar microtubule and Dam1 complex binding characteristics as the wild-type Ndc80 complex (Figure 1—figure supplement 3C,D). These observations suggest regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} each contribute to the interaction of Ndc80 complex with Dam1 complex; and the insertional mutants in these

Table 1. The interacting regions in the Dam1 and Ndc80 complexes.

Interaction	Protein region	Amino acids	Phosphorylated residues	Five amino acid mutation (insertion) position
A	A ^{Dam1p}	241–330	S257, S265, S292	n/a
	A ^{Ndc80p}	262–322	n/a	314
B	B ^{Ask1p}	133–225	S200	n/a
	B ^{Ndc80p}	350–448	n/a	383
C	C ^{Spc34p}	118–274	T199	n/a
	C ^{Ndc80p}	532–630	n/a	563

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three regions were previously identified as lethal because they disrupted the interaction between the Dam1 and Ndc80 complexes.

Dam1p, Ask1p, and Spc34p participate in interactions with Ndc80 complex that can be separately regulated by Aurora B kinase

We then asked if the corresponding regions in the Dam1 complex also contribute to the interaction between Dam1 and Ndc80 complexes. Aurora B kinase phosphorylates the Dam1 complex at six different sites: Dam1p S20, S257, S265, S292; Ask1p S200; Spc34p T199 (Cheeseman *et al.*, 2002). Phosphorylation of Dam1p S20 significantly decreases Dam1 complex oligomerization, while phosphorylation of the three C-terminal sites in Dam1p slightly inhibits direct binding of the Dam1 complex to microtubules (Gestaut *et al.*, 2008; Zelter *et al.*, 2015). In our prior work, we showed that phosphorylating all five sites besides S20 fully disrupts interaction between the Dam1 and Ndc80 complexes (Tien *et al.*, 2010). As shown in Figure 1A, these five sites fall into the three interaction regions defined above, A^{Dam1p}, B^{Ask1p}, and C^{Spc34p} (Table 1). We therefore systematically phosphorylated combinations of these five sites to dissect their contributions to the interaction between the Dam1 and Ndc80 complexes.

We first purified recombinant mutant Dam1 complexes containing different combinations of Ser/Thr to Ala mutations, together with the S20A mutation in all cases. These mutants were treated with Aurora B kinase to produce a series of Dam1 complexes, each with a unique subset of phosphorylated sites. Each phosphorylated Dam1 complex was then tested for its ability to bind the wild-type Ndc80 complex in the single-molecule TIRF assay.

Simultaneously phosphorylating all three regions, A^{Dam1p}, B^{Ask1p}, and C^{Spc34p} completely disrupted the interaction between the Dam1 and Ndc80 complexes, as reported previously (Figure 2A and Figure 2—figure supplement 1A; Tien *et al.*, 2010). Individually phosphorylating regions A^{Dam1p}, B^{Ask1p}, or C^{Spc34p} caused only partial disruption. Phosphorylating region B^{Ask1p} together with C^{Spc34p} also only partially disrupted the interaction, but phosphorylating A^{Dam1p} in combination with either B^{Ask1p} or C^{Spc34p} was sufficient to fully disrupt the interaction (Figure 2B and Figure 2—figure supplement 1B,C). Neither the alanine mutations themselves, nor phosphorylation of a mutant Dam1 complex with all six sites mutated to alanine had any effect, confirming that the observed disruptions were due to phosphorylation at the known sites (Figure 2A and Figure 2—figure supplements 1A and 2). Together these data demonstrate that each of these regions contributes to the interaction between the Dam1 and Ndc80 complexes and is regulated by phosphorylation.

The Dam1 and Ndc80 complexes interact at three different sites

We next mixed and matched phosphorylation of the Dam1 complex with the mutations in Ndc80p. Full disruption by phosphorylation of the Dam1 complex was recapitulated by substituting a Dam1 complex phosphorylation with its corresponding mutation in Ndc80p. For example, phosphorylation of regions A^{Dam1p} and C^{Spc34p} fully disrupted the interaction between the two complexes (Figure 2B). Similarly, phosphorylation at region A^{Dam1p} plus mutation in region C^{Ndc80p} fully disrupted the interaction between the two complexes (Figure 2C and Figure 2—figure supplement 1E,F).

Phosphorylation combinations that partially disrupted the interaction between the Dam1 and Ndc80 complexes were also recapitulated by substituting a phosphorylation event with mutation in the corresponding binding region of Ndc80p. Phosphorylation at regions B^{Ask1p} and C^{Spc34p} partially disrupted the interaction between the two complexes (Figure 2B). Similarly, mutation in region B^{Ndc80p} plus phosphorylation at region C^{Spc34p}, or phosphorylation at region B^{Ask1p} plus mutation in C^{Ndc80p} partially disrupted the interaction between the two complexes (Figure 2C and Figure 2—figure supplement 1D). Together, our results demonstrate that the Dam1 and Ndc80 complexes interact at three different sites, each of which is phospho-regulated by Aurora B kinase.

The Ndc80 complex binds two Dam1 complex rings

The Ndc80 complex is predicted to be a long fibril. The coiled-coiled length between regions A^{Ndc80p} and C^{Ndc80p} is predicted to be 29 nm (Lupas and Gruber, 2005) (Figure 3D). The width of a Dam1 complex ring is only ~7 nm (Ramey *et al.*, 2011). Assuming that the Ndc80 complex adopts

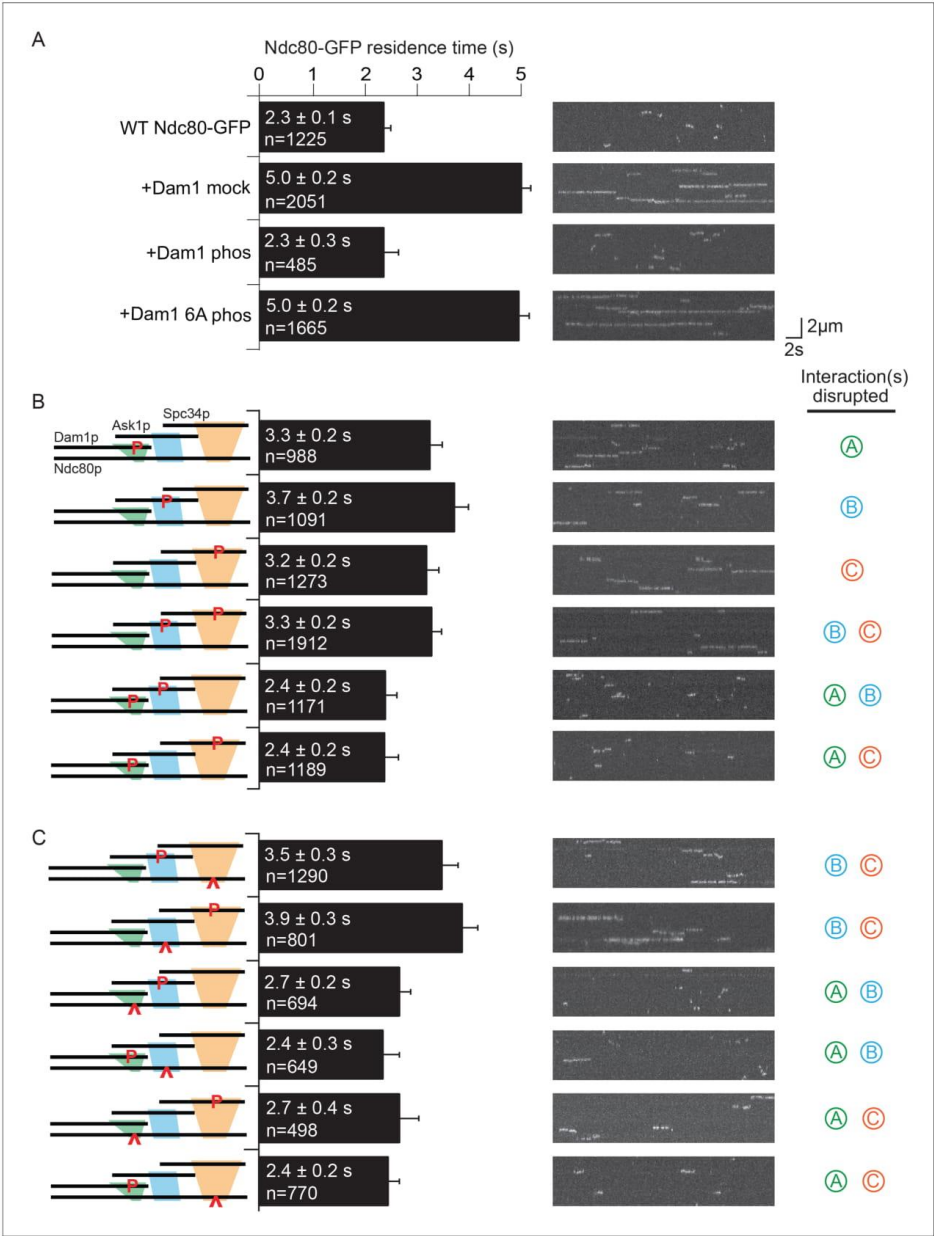


Figure 2. Dam1 and Ndc80 complexes interact through three distinct sites. (A) Average microtubule residence time of the wild-type Ndc80-GFP complex alone, in the presence of mock treated, phosphorylated Dam1 complex, or phosphorylated Dam1 6A mutant complex. Dam1 6A: all six Dam1 complex phosphorylation sites mutated to Ala. (B) Average microtubule residence time of wild-type Ndc80-GFP complex in the presence of selectively phosphorylated Dam1 complex. Diagram on the left indicates phosphorylated proteins. (C) Average microtubule residence time of Ndc80-GFP

Figure 2 continued on next page

Figure 2 continued

complex with lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the presence of selectively phosphorylated Dam1 complex. Diagrams on the left indicate phosphorylated protein (P) and Ndc80p region with a lethal mutation (^l). On the right are representative kymographs for each experiment. Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Materials and methods or additional details). Ndc80-GFP complex microtubule residence time raw data are included in **Figure 2—source data 1**. Refer to **Supplementary file 1C** for statistical analysis.

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The following source data and figure supplements are available for figure 2:

Source data 1. Table of Ndc80-GFP microtubule residence times for **Figure 2**.

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Figure supplement 1. Microtubule residence time survival probability plots of experiments in **Figure 2**.

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Figure supplement 2. Mutating Dam1 complex Aurora B kinase phosphorylation sites to Ala does not affect the interaction between Dam1 and Ndc80 complexes.

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Figure supplement 2—source data 1. Table of Ndc80-GFP microtubule residence times for **Figure 2—figure supplement 2**.

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a conformation roughly parallel to the microtubule axis (Alushin et al., 2010; Joglekar et al., 2009), a single ring could not span this distance. Thus we considered that multiple Dam1 complex rings might bind to the Ndc80 complex. Using negative stain electron microscopy, we imaged the Dam1 complex on microtubules in the absence and presence of the wild-type Ndc80 complex.

The Dam1 complex formed rings on microtubules as previously reported (Figure 3A,B; Miranda et al., 2005; Westermann et al., 2005). In the absence of the Ndc80 complex, the Dam1 complex rings tended to bind in doublets of rings, at an inter-ring distance of 13.3 ± 2.4 nm (avg $\pm$ s.d.) (Figure 3A–C and Figure 3—figure supplement 1A). This suggests that Dam1 complex rings have an affinity for each other along the longitudinal axis of microtubules and have a tendency to stack together. The stacked double-ring organization may be analogous to the paired ring helices previously reported to form at higher concentrations of Dam1 complex (Miranda et al., 2005). The percent of Dam1 ring pairs found in closely spaced doublets was higher in the presence of the Ndc80 complex, 49.1% (estimated by Gaussian fitting to the peak, see Materials and methods), as compared to without the Ndc80 complex, 18.8%. In addition, the two rings in each doublet were consistently held apart by a distance of 33.1 ± 3.8 nm, similar to the predicted coiled-coil length between regions A^{Ndc80p} and C^{Ndc80p} (Figure 3 and Figure 3—figure supplement 1B). This suggests that one Ndc80 complex bridges two Dam1 rings by binding one ring at region A^{Ndc80p} and a second ring at region C^{Ndc80p}.

To further test this hypothesis, 10 coiled-coil heptad repeats were inserted into the Ndc80p and Nuf2p coiled-coil domains to increase the length of the coiled-coil domain between the interaction regions by a predicted 10.5 nm (Figure 3D). In the presence of the 10-heptad mutant Ndc80 (Ndc80^{10-hep}) complex, Dam1 ring doublets were consistently separated by 42.1 ± 2.1 nm, 9 nm farther apart than in the presence of wild-type Ndc80 complex (Figure 3 and Figure 3—figure supplement 1C). Finally, mutation in regions A, B or C of Ndc80p abolished its ability to bind two rings as the Dam1 inter-ring distance in these cases was the same in the absence of Ndc80 complex (Figure 3—figure supplements 1D–F and 2). These results strongly support the hypothesis that the Ndc80 complex bridges two Dam1 complex rings in vitro.

The Ndc80 10-heptad insertion complex is lethal in vivo

We next explored the possibility that Ndc80 complex might also bridge across two Dam1 complex rings in vivo. In particular, our observations raise the question of whether the specific 33 nm inter-ring distance, delimited by the Ndc80 complex, is important. To test this we constructed yeast expression plasmids for *ndc80*^{10-hep} and *nuf2*^{10-hep}, encoding Ndc80p and Nuf2p each with 10-heptad repeats inserted. Using a plasmid shuffle assay, we found that even when introduced together, *ndc80*^{10hep} and *nuf2*^{10hep} could not support growth, even though the encoded proteins were

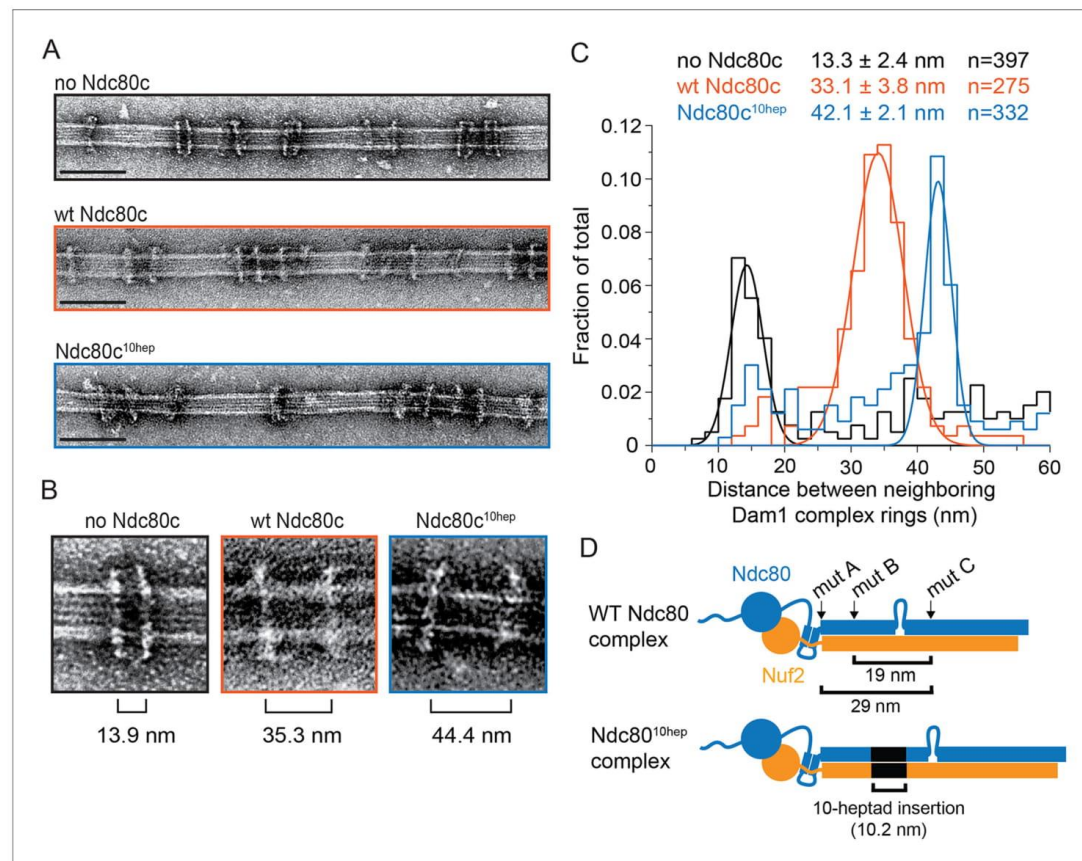


Figure 3. The Ndc80 complex binds two Dam1 complex rings. (A) Representative EM images of Dam1 complex rings on microtubules in the absence or presence of the Ndc80 complex. Three experiments include no Ndc80 complex (Ndc80c), wild-type Ndc80c, and Ndc80c^{10hep}. Protein concentrations were 20 nM tubulin and 25 nM Dam1 and Ndc80 complexes. Scale bars: 100 nm. (B) Zoomed in images of a Dam1 complex ring doublet for the three experiments. Distances from middle of one ring to that of next ring are indicated. (C) Distribution of closest neighboring inter-ring distances measured for the three different conditions. Measurements made between 0–60 nm are shown. The cluster of distance measurements was fitted with a Gaussian distribution and the vertices ± standard deviations for each fit are listed. The raw data of Dam1 complex inter-ring distance measurements are included in **Figure 3—source data 1**. (D) Diagram demonstrating the predicted coiled-coil distances between the Ndc80p lethal mutations. Diagram below shows the location of additional 10-heptad insertion between the hypothesized binding sites of two Dam1 complex rings. Refer to **Supplementary file 1E** for statistical analysis of data in part (C).

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The following source data and figure supplements are available for figure 3:

Source data 1. Table of Dam1 complex inter-ring distance measurements for **Figure 3C**.

DOI: [10.7554/eLife.21069.016](https://doi.org/10.7554/eLife.21069.016)

Figure supplement 1. Full distributions of Dam1 complex inter-ring measurements for various experiments shown in **Figure 3** and **Figure 3—figure supplement 2**.

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Figure supplement 2. Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} disrupts the Ndc80 complex's ability to bind two Dam1 complex rings.

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Figure supplement 2—source data 1. Table of Dam1 complex inter-ring distance measurements for **Figure 3—figure supplement 2C**.

DOI: [10.7554/eLife.21069.019](https://doi.org/10.7554/eLife.21069.019)

expressed (Figure 4A,B). The Ndc80^{10hep} complex is competent to assemble in vitro, to bind microtubules, to interact with the Dam1 complex on microtubules, and to bind two Dam1 complex rings (Figure 3; Figure 4C). The only known difference is that the inter-ring distance is increased by 9 nm. These results suggest that the Ndc80^{10hep} complex cannot support viability due to an altered Dam1 complex inter-ring distance.

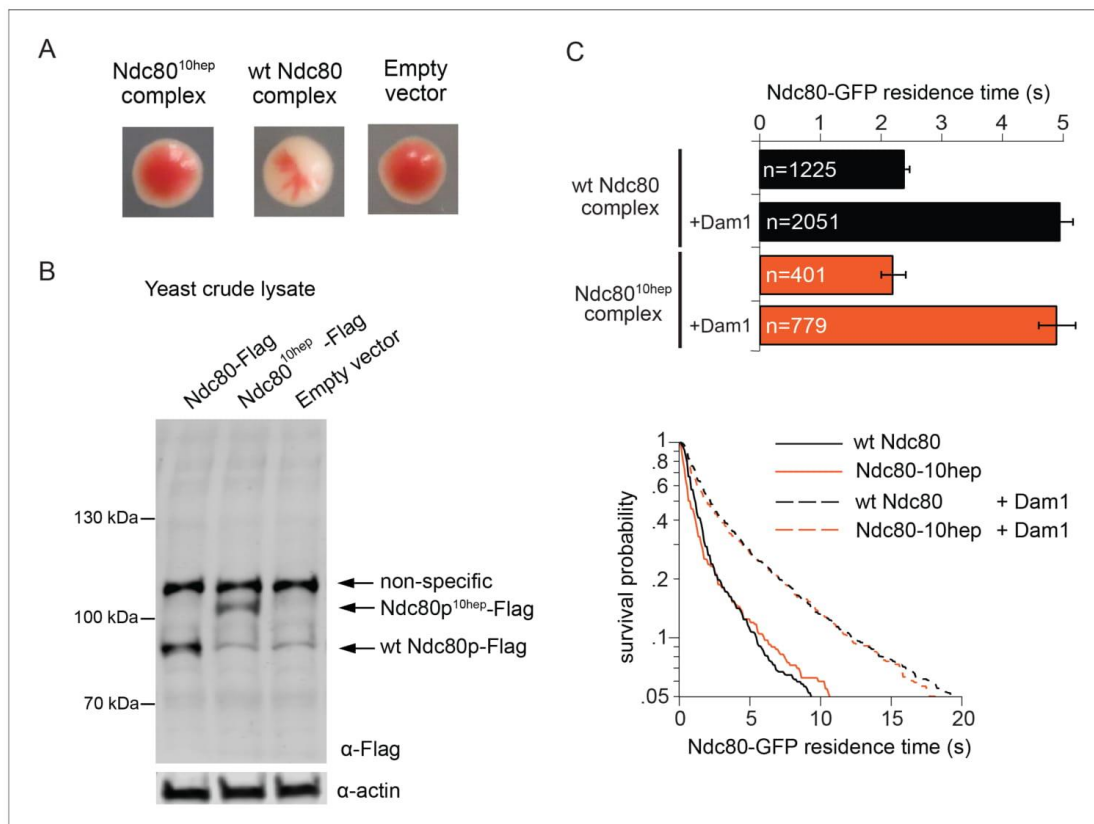


Figure 4. The Ndc80^{10hep} complex does not support growth. (A) Red/white plasmid shuffle assay to test the viability of the Ndc80^{10hep} complex. Solid red colonies indicate the inability of the Ndc80^{10hep} complex and empty vector to support growth. Sectoring white colony indicates the ability of the wild-type Ndc80 complex to support growth. (B) α -FLAG immunoblot of crude lysate of cells expressing either wild-type Ndc80p, Ndc80p^{10hep}, or empty vector. α -actin immunoblot of the same lysates shows equal loading of the lysates. (C) Wild-type or Ndc80^{10hep} complex average residence time (top) on microtubules in the absence and presence of Dam1 complex as derived from the survival probability curves (bottom). Bars represent average residence time $\pm$ error of the mean (estimated by bootstrapping analysis; see Materials and methods or additional details). Ndc80-GFP complex microtubule residence times raw data are included in [Figure 4—source data 1](#). Refer to [Supplementary file 1F](#) for statistical analysis of data.

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The following source data is available for figure 4:

Source data 1. Table of Ndc80-GFP microtubule residence times for [Figure 4C](#).

DOI: [10.7554/eLife.21069.021](https://doi.org/10.7554/eLife.21069.021)

The Ndc80 mutants exhibit biorientation and attachment defects

The Ndc80p mutants in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} cannot bind two Dam1 complex rings, and consequently have decreased interaction with the Dam1 complex on microtubules. To determine if these Ndc80p mutants confer defects in chromosome biorientation and attachment, we used an auxin-inducible degron system for degrading the wild-type Ndc80-AID (Nishimura *et al.*, 2009). Non-degradable wild-type or mutant *NDC80* was integrated at the *URA3* locus. Ndc80-AID was efficiently degraded in the presence of auxin (Figure 5—figure supplement 1). Budding index analyses revealed that cells carrying wild-type *NDC80* progressed through mitosis as expected. Cells without *NDC80* also budded and divided on schedule, despite major defects in kinetochore attachment (see below), as expected since activation of the SAC requires the Ndc80 complex (Dou *et al.*, 2015; Hiruma *et al.*, 2015; Ji *et al.*, 2015). In contrast, cells carrying Ndc80p mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} arrested as large-budded cells (Figure 5—figure supplement 2) suggesting defects in kinetochore attachment and indicating that the checkpoint remained functional.

We performed a detailed analysis of the phenotype conferred by the *NDC80* mutant alleles by imaging wild-type, depleted, and mutant cells with *CEN3* and spindle pole bodies (SPB) tagged with GFP and mCherry, respectively. Cultures were synchronized at G1, released from synchrony and then imaged after 60 min. In the majority of wild-type *NDC80* cells, *CEN3* was bioriented with both *CEN3*-GFP puncta on the spindle axis; 78% were in metaphase and 22% were in anaphase as judged by the spindle length. In the majority of the Ndc80-depleted cells, *CEN3*-GFP was off of the spindle axis, consistent with complete detachment. A mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}, resulted in 61, 59, and 75% of the cells with monooriented *CEN3* and 34, 39, and 21% of cells with detached *CEN3*, respectively. (Figure 5A,B). In addition, in the majority of cells depleted of Ndc80 or with a mutation in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} the SPBs were separated by more than 2 μ m (Figure 5C). These long spindles and the high frequency of detached *CEN3* suggest that few if any of the kinetochores were bioriented. Thus, we conclude that Ndc80p carrying a mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} disrupts kinetochore biorientation and attachment in vivo.

The Ndc80 mutants have defects in recruiting the Dam1 complex to the kinetochore

The Ndc80 complex is responsible for recruiting the Dam1 complex to the kinetochore (Janke *et al.*, 2002). Since Ndc80 complexes carrying a mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} are defective in binding the Dam1 complex in vitro, we asked if Dam1 complex kinetochore localization is also compromised in vivo. We again used the auxin-inducible degron system for degrading the wild-type Ndc80-AID, while wild-type or mutant *NDC80* was integrated at the *URA3* locus; Mtw1-YFP, Dad4-CFP, and Spc110-mCherry marked the kinetochore, Dam1 complex, and SPB, respectively.

Cells expressing wild-type *NDC80* had two distinct clusters of Mtw1-YFP between two SPBs, as expected for bioriented sister kinetochores. Consistent with previous studies (Cheeseman *et al.*, 2001; Hofmann *et al.*, 1998; Li *et al.*, 2002), the Dam1 complex localized along the spindle but also showed two distinct clusters that colocalized with the Mtw1-YFP. (Figure 6A,B). None of the mutants showed the organized kinetochores seen in wild-type cells. Instead, in the milder mutants (mutation in region B^{Ndc80p}, C^{Ndc80p}, or B^{Ndc80p} and C^{Ndc80p}) kinetochores were spread along the spindle and a few were detached. In the more severe mutants (mutation in region A^{Ndc80p}, A^{Ndc80p} and B^{Ndc80p}, A^{Ndc80p} and C^{Ndc80p}, A^{Ndc80p}, B^{Ndc80p} and C^{Ndc80p}, or Ndc80-depleted) few kinetochores were associated with the spindle. Instead, most were detached from the microtubules between the two SPBs (Figure 6—figure supplement 1).

Unlike wild-type *NDC80*, Dad4-CFP in mutant Ndc80 cells decorated the spindle but concentrated poorly at the Mtw1-YFP clusters that did occur (Figure 6B and Figure 6—figure supplement 1). In wild-type *NDC80* cells, an increase or decrease in Mtw1-YFP fluorescence correlated with an increase or decrease in Dad4-CFP fluorescence, respectively; however, Ndc80-depleted cells showed poor correlation. One or multiple mutations in region A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} resulted in significantly decreased correlation between Mtw1-YFP and Dad4-CFP (Figure 6C). Single mutations in region B^{Ndc80p} or C^{Ndc80p} resulted in less severe defects. These observations suggest that mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p} had little effect on binding of Dam1 complex to the microtubules, but disrupted the ability of Ndc80 to recruit Dam1 complex to the kinetochore in vivo, thereby causing severe defects in kinetochore attachment and biorientation.

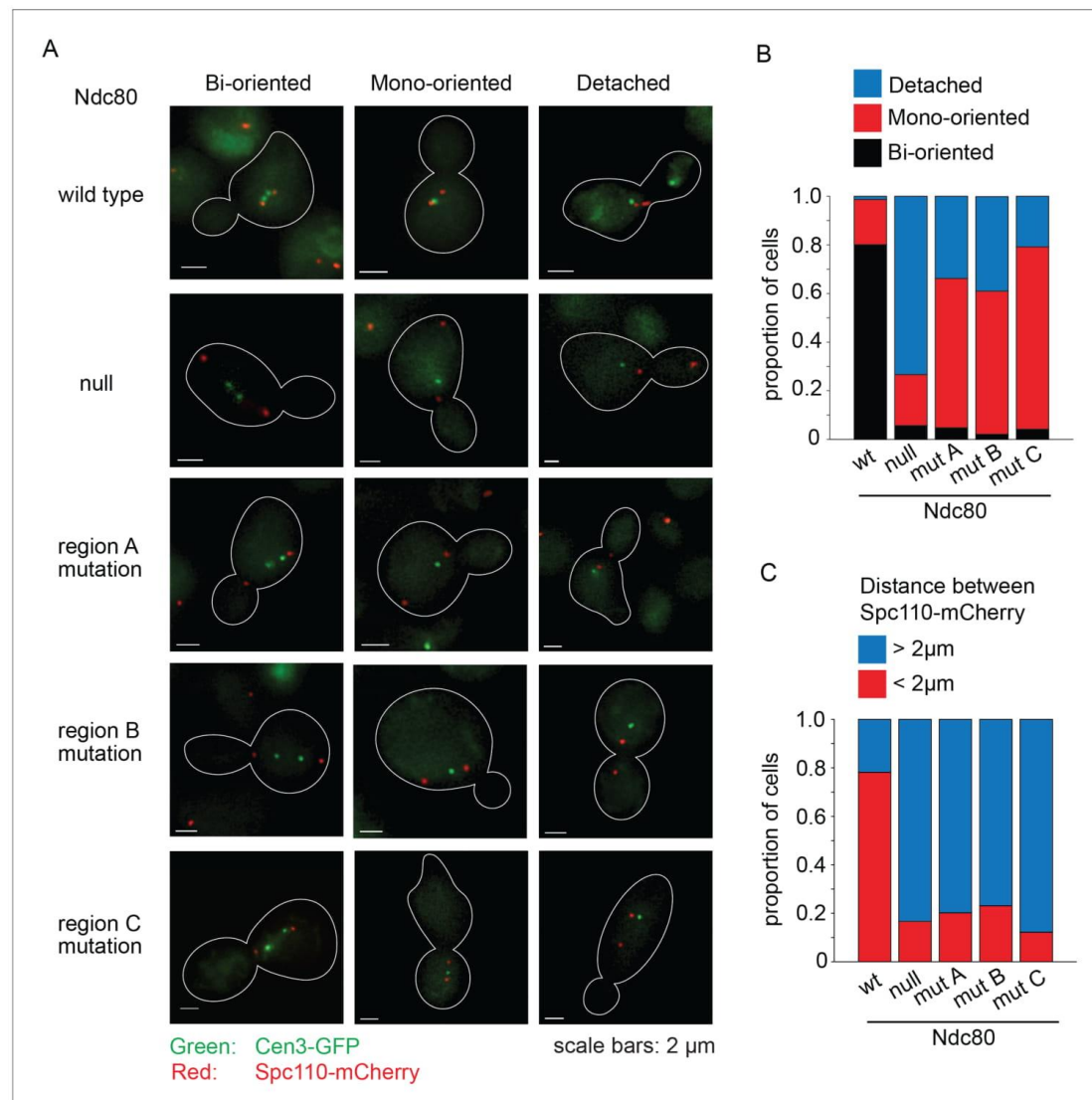


Figure 5. Lethal mutations in A^{Ndc80p} , B^{Ndc80p} , and C^{Ndc80p} have biorientation and microtubule attachment defects in vivo. (A) Representative images from Ndc80-AID degon experiments with cells carrying an extra copy of *NDC80* wild-type, no *NDC80* (null), or a mutations in A^{Ndc80p} , B^{Ndc80p} , or C^{Ndc80p} . Cells were treated with 6 μ M α -factor for two hours prior to treatment with 6 μ M α -factor and 0.5 mM auxin for one additional hour. At time 0, cells were released from α -factor and incubated in YPD medium containing 0.5 mM auxin. Images were taken at 60 min after release from α -factor arrest. Representative images are shown. (B) Stacked bar graphs showing proportion of the cells containing bioriented, monooriented or detached CEN3-GFP. Spindles with two CEN3-GFP puncta positioned between the two SPBs were counted as bioriented. Spindles with one CEN3-GFP puncta positioned between the two SPBs were counted as monooriented and spindles with one (or rarely two) CEN3-GFP puncta positioned off the spindle axis were counted as detached. Only cells with two Spc110-mCherry puncta and at least one CEN3-GFP punctum were included in the analysis. (C)

Figure 5 continued on next page

Figure 5 continued

Stacked bar graph showing the distance between SPBs. Wild-type *NDC80* *n* = 236 cells; no *NDC80* *n* = 210 cells; A^{Ndc80p} *n* = 210 cells; B^{Ndc80p} *n* = 190 cells; C^{Ndc80p} *n* = 260 cells.

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The following figure supplements are available for figure 5:

Figure supplement 1. Wild-type Ndc80-AID is degraded upon the addition of auxin.

DOI: [10.7554/eLife.21069.023](https://doi.org/10.7554/eLife.21069.023)

Figure supplement 2. Lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} causes mitotic arrest.

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Discussion

The discovery that the Dam1 complex forms oligomers and rings around microtubules galvanized the mitosis field by providing a molecular explanation for how kinetochores are able to maintain processive attachment to the flared ends of a depolymerizing microtubule. Rings are not required for the Dam1 complex to track depolymerizing microtubules in the absence of tension (Gestaut *et al.*, 2008; Grishchuk *et al.*, 2008). However, oligomerization of the Dam1 complex is required to maintain attachments under tension as would be experienced during metaphase (Umbreit *et al.*, 2014; Volkov *et al.*, 2013). Prior models of the kinetochore have all assumed that each kinetochore contains only one Dam1 complex ring. Here we show that the Ndc80 complex bridges two rings of the Dam1 complex in vitro. Lethal mutations in Ndc80p that block binding of either one of the two rings result in loss of the Dam1 complex from the kinetochore and failure in biorientation and attachment of kinetochores to the mitotic spindle. These results suggest that faithful microtubule attachments require two Dam1 complex rings per kinetochore in vivo.

Counting of kinetochore components by fluorescence microscopy has yielded conflicting results for the number of Dam1 complexes at the yeast kinetochore depending on the standard used. Assuming two Cse4 histone molecules per kinetochore, Joglekar and coworkers report enough Dam1 complexes to form one ring at a kinetochore during metaphase and a partial ring during anaphase (Aravamudhan *et al.*, 2013; Joglekar *et al.*, 2006). Lawrimore and coworkers measured 5.5 Cse4 histone molecules per kinetochore, giving enough Dam1 complex for two rings at a kinetochore during anaphase (Lawrimore *et al.*, 2011); whereas Coffman and coworkers measured 8 Cse4 histone molecules per kinetochore (Coffman *et al.*, 2011). Our results show that the Ndc80 complex can bind two Dam1 rings in vitro. Given the results of the latter two groups, we propose that there are two Dam1 complex rings at the kinetochore.

The region before the hairpin and the loop region of Ndc80p have both been identified as binding sites for the Dam1 complex (Lampert *et al.*, 2013; Maure *et al.*, 2011). Our results indicate that the interaction between the Ndc80 and Dam1 complexes is more extensive forming a tripartite network. The C-terminal domains of Dam1p, Ask1p, and Spc34p interact with three distinct regions of Ndc80p, one of which is the hairpin consistent with the previous report (Lampert *et al.*, 2013). Our results are also consistent with a previous study showing an interaction between Ndc80p and the C-terminal region of Dam1p (Kalantzaki *et al.*, 2015). However, we did not find any evidence for an interaction between the Ndc80p 'loop' region and Dam1p (Maure *et al.*, 2011). The inability of the loop deletion mutant to assemble the Dam1 complex into the kinetochore might be due to an indirect effect.

Comparing the predicted distances between regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p}, and the 33 nm Dam1 complex inter-ring distance from EM experiments suggests that regions A and B contribute to the Ndc80 complex interaction with the ring closer to the N-terminus of Ndc80p/Nuf2p, while region C contributes to the Ndc80 complex interaction with the second ring (Figure 7). Our TIRF microscopy and live cell imaging results suggest that disrupting interaction A is more severe than disrupting interaction B or C. A recent computational model of the structure of the Dam1 complex bound to microtubules positions region A^{Dam1p} and B^{Ask1p} near the microtubule surface, while region C^{Spc34p} is positioned away from the microtubule surface (Zelter *et al.*, 2015). Accordingly, our model of the interaction between Dam1 and Ndc80 complexes shows the Ndc80 complex positioned under the first Dam1 ring and above the second Dam1 complex ring further from Ndc80p N-terminus (Figure 7).

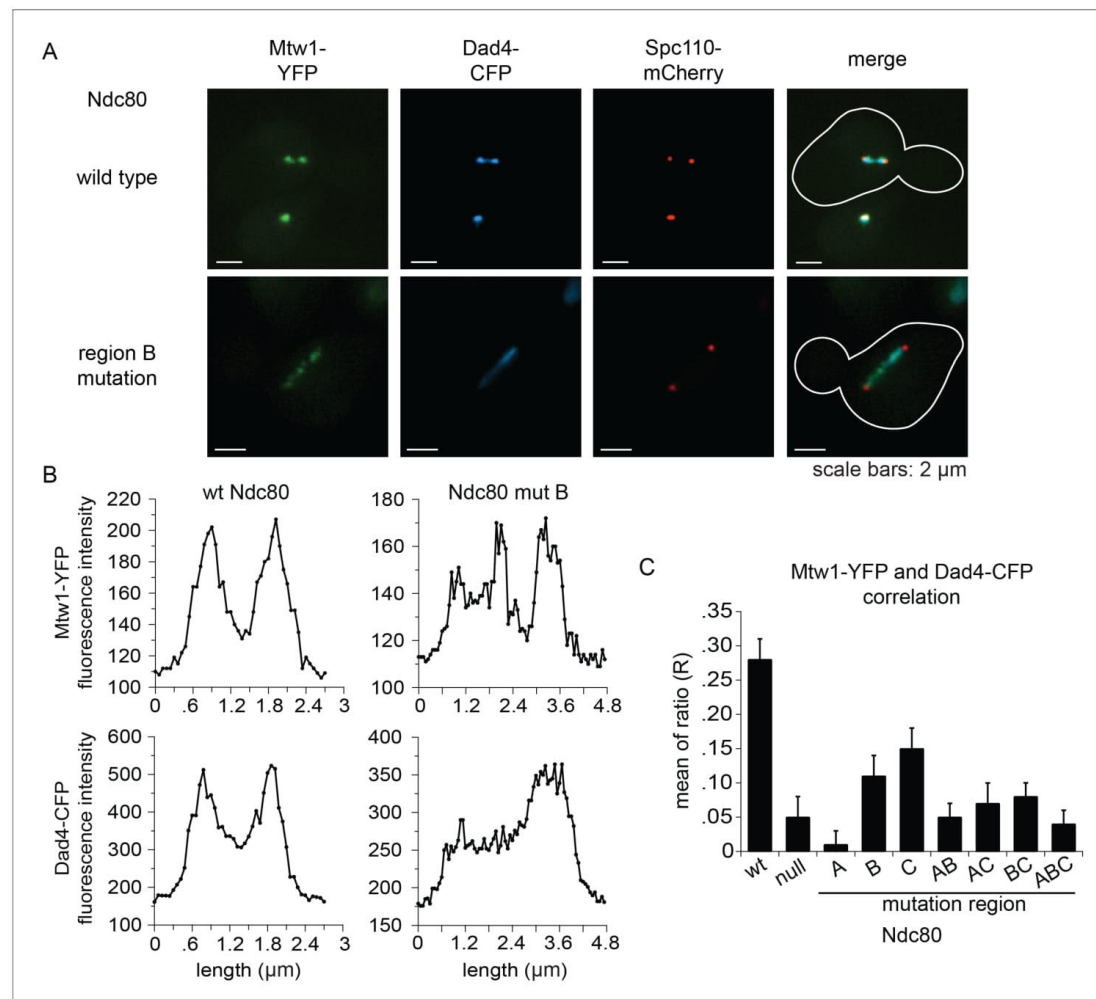


Figure 6. Lethal mutations in A^{Ndc80p} , B^{Ndc80p} , and C^{Ndc80p} confer defects in Dam1 complex recruitment to the kinetochore. (A) Representative images from *NDC80-AID* degenon experiments with cells also containing wild-type *NDC80* or mutation in region B^{Ndc80p} . The inner kinetochore was visualized with Mtw1-YFP, the Dam1 complex with Dad4-CFP, and the SPB with Spc110-mCherry. Cells were treated with 6 μ M α -factor for two hours prior to the addition of 0.5 mM auxin for one additional hour. At time 0, cells were released from α -factor arrest and incubated in YPD medium containing 0.5 mM auxin. Images were taken at 60 min after release from α -factor arrest. (B) Line scan traces of Mtw1-YFP and Dad4-CFP from the images in (A). Only cells with two SPBs in the same plane of focus were selected for analysis. Each line scan extended from one SPB to the other. Each point represents one pixel. (C) Summary of correlation analysis carried out between Mtw1-YFP and Dad4-CFP. For each line scan, a correlation of the positive or negative changes of pixel intensity along the line between the Mtw1-YFP and Dad4-CFP channels was calculated to examine co-localization along the spindle (see Materials and methods). Between 18 and 33 cells were analyzed for each mutant. Mtw1-YFP and Dad4-CFP fluorescence intensity raw data are included in **Figure 6—source data 1**. Refer to **Supplementary file 1G** for statistical analysis of data.

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The following source data and figure supplement are available for figure 6:

Source data 1. Tables of Mtw1-YFP and Dad4-CFP fluorescence intensities for cells analyzed in **Figure 6C**.

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Figure 6 continued on next page

Figure 6 continued

Figure supplement 1. Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} results in severe defects in kinetochore biorientation and attachment.
DOI: 10.7554/eLife.21069.027

Aberrant kinetochore-microtubule attachments are phosphorylated and destabilized by Aurora B kinase (Biggins et al., 1999; Cheeseman et al., 2002; Hauf et al., 2003; Pinsky et al., 2006; Tanaka et al., 2002). Aurora B kinase phosphorylation of the Dam1p, Ask1p, and Spc34p C-terminal sites together fully disrupts the interaction between the Dam1 and Ndc80 complexes (Tien et al., 2010). This regulation mechanism has been thought of as binary, either full or no disruption of the interaction.

We show that phosphorylation of certain combinations results in only partial disruption and full disruption does not require phosphorylation of all three proteins. Our results suggest how the Aurora B kinase might fine tune the interaction between Dam1 and Ndc80 complexes, resulting in a range of disruption.

The inability of *ndc80*^{10hep} and *nuf2*^{10hep} together to support growth in vivo might be explained in several ways. The addition of extra heptad repeats in the coiled-coil domains might destabilize formation of the heterotetrameric Ndc80 complex, for example. However, we do not currently favor this hypothesis because the extra-long mutant version of Ndc80p is expressed in vivo, and because the Ndc80^{10hep} complex is stable and binds to both microtubules and to the Dam1 complex in vitro, similar to wild-type Ndc80 complex. Unnatural orientation of the Ndc80p/Nuf2p globular head in the Ndc80^{10hep} complex, due to the addition of extra heptad repeats, might also disrupt the specific geometry that the Ndc80 complex takes in relation to other kinetochore proteins. However given

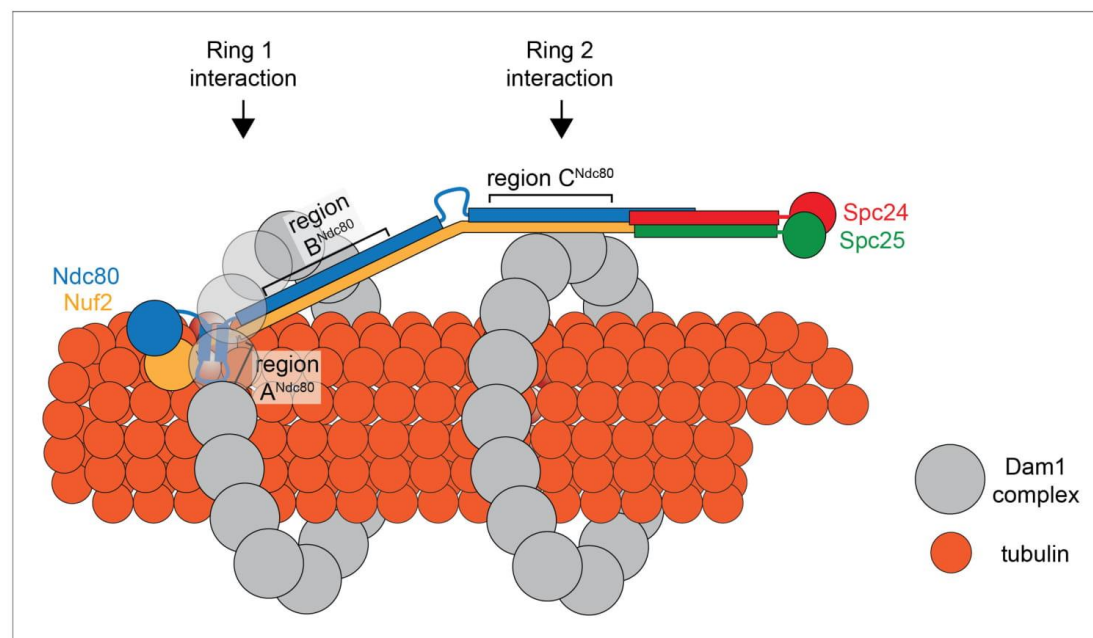


Figure 7. Model of the Ndc80 complex bridging two Dam1 complex rings, separated by 33 nm. Results in this study support ring 1 interaction being comprised of interaction A and B, and ring 2 interaction being comprised of interaction C (refer to Figure 1). This image depicts only one Ndc80 complex across two Dam1 complex rings, but multiple Ndc80 complexes would be present in vivo.

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the presence of the flexible loop in Ndc80p, it seems unlikely that the two ends of the complex are held in a rigid orientation relative to each other. Compared to the wild-type Ndc80 complex, the Ndc80^{10hep} complex further separates the Dam1 complex rings in vitro. We propose that the specific 33 nm Dam1 complex inter-ring distance is vital. The transition from 13 nm, in the absence of the Ndc80 complex, to 33 nm Dam1 complex inter-ring distance could signal the establishment of kinetochore-microtubule attachment or biorientation. Deviation from the specific 33 nm distance might disrupt a yet unknown mechanism for detecting kinetochore-microtubule attachments, or bioriented attachments, or both. Future work will focus on characterizing the specific effects of the extra-long Ndc80 complex and the role of the Dam1 complex inter-ring distance in vivo.

Materials and methods

Protein expression and purification

The *S. cerevisiae* Ndc80 and Dam1 complexes were independently expressed in *E. coli* using polycistronic vectors, as previously described (Gestaut et al., 2008; Miranda et al., 2005; Powers et al., 2009; Tien et al., 2010; Wei et al., 2005). The Dam1 complex component Spc34p C-terminus was tagged with His₆ and the Ndc80 complex component Spc24 N-terminus was tagged with FLAG or His₆. Each complex was affinity-purified before further purification through gel filtration. For TIRF microscopy, Nuf2p C-terminus of the Ndc80 complex was tagged with GFP.

Dam1 complex phosphorylation

GST-Ipl1 and GST-Sli15 (residues 554–698) were purified as previously described (Gestaut et al., 2008; Tien et al., 2010; Zelter et al., 2015). GST-Ipl1 (pSB196, Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA) and GST-Sli15 (residues 554–698) (pSB503, Sue Biggins) were expressed at 23°C and 37°C, respectively for 2 hr. GST-Ipl1 was purified using GSTrap HP (GE Healthcare Biosciences, Pittsburgh, PA) following manufacturer's instructions, except that the elution buffer was 50 mM Tris buffer (pH 8.0), 250 mM KCl, 10 mM glutathione. HiTrap 26/10 Desalting column (GE Healthcare) was used to exchange the buffer to 50 mM HEPES buffer (pH 7.4), 100 mM NaCl. GST-Sli15 was purified with glutathione-Sepharose 4B resin (GE Healthcare) following manufacturer's instructions, except that the elution buffer was 20 mM Tris buffer (pH 8.0), 200 mM NaCl, 1 mM β-mercaptoethanol, 1 mM EDTA, 10 mM glutathione.

4 μM recombinant Dam1 complex was incubated with 0.5 μM GST-Ipl1, 0.5 μM GST-Sli15, 200 mM NaCl, 10 mM ATP, 25 mM MgCl₂, and 50 mM HEPES buffer, pH 7.4. Reaction mixtures were incubated for 90 min at 30°C. Under these conditions, we achieve nearly stoichiometric phosphorylation of the complex (Gestaut et al., 2008). Mock treated (non-phosphorylated) controls of the Dam1 complex was carried out by substituting ATP with dH₂O.

TIRF microscopy

Glass slides and functionalized coverslips were used to construct flow chambers, as reported previously (Gestaut et al., 2008, 2010; Tien et al., 2010; Zelter et al., 2015). A coverslip was adhered to a glass slide with double-sided tape, forming a flow chamber between two adjacent strips of tape. 'Rigor' kinesin was flowed through the chamber to non-specifically bind to the coverslip. Taxol-stabilized, Alexa-647-labelled microtubules were then flowed in and incubated for 5 min for immobilization. For all TIRF microscopy experiments, Ndc80-GFP complex was incubated at 50 pM for single-molecule imaging. Each phosphorylated or mock-treated Dam1 complex mutant construct was incubated at 2.5 nM. GFP and Alexa-647 fluorescence channels were simultaneously recorded using a custom TIRF imaging system (Gestaut et al., 2010). All TIRF microscopy experiments were carried out in BRB80 (80 mM PIPES buffer (pH 6.8), 1 mM EGTA, 1 mM MgCl₂) in the presence of 8 mg/ml BSA, 0.04 mg/ml κ-casein, and an oxygen scavenger system (200 μg/ml glucose oxidase, 35 μg/ml catalase, 25 mM glucose, and 5 mM DTT). Each experimental condition was assayed between three and seven times yielding between 401 and 2051 measurements. This sample size adequately identified possible differences between different conditions.

Analysis of the single particle tracking was carried out as previously described (Tien et al., 2010; Umbreit et al., 2014; Zelter et al., 2015). Custom analysis software was developed in Labview (National Instruments) (RRID: SCR_014325) (Source code files 1–3) and Igor Pro (Wavemetrics)

(RRID: SCR_000325) (Source code files 4–6). Bootstrapping analysis was used to calculate the mean residence times (Umbreit et al., 2014). Randomly resampling each dataset (residence times from each TIRF microscopy experimental condition) with replacement was repeated 1000 times. Carrying out this method with each dataset yielded normal distributions. Gaussian fits to these distributions were used to estimate the mean residence times and the width of the fit was used as an estimate of error. Statistical analysis of the TIRF data was performed with pairwise z-tests. P-values were computed from the pairwise z-scores: $z = (\mu_1 - \mu_2) / (\delta_1^2 + \delta_2^2)^{0.5}$. μ_1 and μ_2 are bootstrap average residence times of Ndc80-GFP complex. δ_1^2 and δ_2^2 are the corresponding estimates of error. Tables of pairwise comparisons can be found in *Supplementary file 1A-D* and *F*.

Chemical Cross-linking and mass spectrometry analysis (XL-MS)

XL-MS experiment was performed twice, using EDC and DSS cross-linking agents (biological replicate). The two experiments identified similar interaction regions between the Dam1 and Ndc80 complexes. XL-MS was performed as described in Zelter et al. (2015). Briefly, 10 μ g of taxol stabilized microtubules were mixed with 10 μ g Dam1 complex and 10 μ g Ndc80 complex in 100 μ L BRB80 at 25°C and allowed to stand for 5 mins. For DSS cross-linking, 3 μ L 14.5 mM DSS in DMSO was added, and the mixture allowed to cross-link for 2 mins at 25°C before quenching by addition of 10 μ L 0.5 M NH_4HCO_3 . For EDC cross-linking 7.5 μ L 145 mM EDC plus 3.75 μ L 145 mM Sulfo-NHS were added to the reaction, and the mixture allowed to cross-link for 30 mins at 25°C before quenching by addition of 10 μ L 0.5 M NH_4HCO_3 . Quenched reactions were spun at 58,000 rpm in a TLA100 rotor for 10 min at 37°C. The pellet was resuspended in 100 μ L ice cold buffer reduced with 10 mM dithiothreitol (DTT) at 37°C for 30 min followed by 30 mins alkylation at RT with 15 mM iodoacetamide (IAA). Digestion with trypsin at a substrate to enzyme ratio of 60:1 was performed overnight at room temperature with shaking. Digested samples were acidified with 5 M HCL prior to being stored at –80°C until analysis. Mass spectrometry and data analysis was performed on either a Q-Exactive or Q-Exactive HF (Thermo Fisher Scientific, Waltham, MA) as previously described (Zelter et al., 2015). Each cross-linked sample was run four times and the data were combined before analysis (technical replicate). Mass spectra were converted into mzML using msconvert from ProteoWizard (Chambers et al., 2012). Standard linear peptide searches were performed using Comet to identify all proteins in the sample (Eng et al., 2013). Cross-linked peptides were identified using the Kojak version 1.4.2 cross-link identification software (Hoopmann et al., 2015) following the author's instructions (<http://www.kojak-ms.org>). Kojak results were exported to Percolator version 2.08 (Käll et al., 2007) to produce a statistically validated set of cross-linked peptide spectrum matches (PSMs) at the desired false discovery rate (FDR) threshold. Percolator (RRID: SCR_005040) is a semi-supervised algorithm that assigns a statistically meaningful q value to each PSM through analysis of the target and decoy PSM distributions. Decoy PSMs derive from peptide sequences known to be false. In the current work the target databases consisted of all proteins identified in the sample analyzed, while the decoy databases consisted of the corresponding set of reversed protein sequences. Cross-link PSMs were considered to be false if at least one of the peptides was from a decoy protein sequence. The data presented in this paper was filtered to show only hits to the target proteins that had a Percolator assigned peptide level q value ≤ 0.05 . The complete, unfiltered list of all PSMs and their Percolator assigned q values, are available on the ProXL web application (Riffle et al., 2016) at: http://proxl.yeastrc.org/proxl/viewProject.do?project_id=24 along with the raw MS spectra and search parameters used.

Electron microscopy

Cleared tubulin was polymerized in a total volume of 40 μ L BRB80 (80 mM PIPES buffer (pH 6.8), 1 mM EGTA, 1 mM MgCl_2) containing 1.75 mM GTP, 1 mM MgCl_2 , and 3.5% DMSO at 37°C for 30 min. Microtubules were pelleted and resuspended in BRB80 containing 10 μ M taxol. All samples were prepared in BRB80 +10 μ M taxol by mixing 20 nM microtubules, and 25 nM Dam1 in the absence or presence of 25 nM Ndc80 complex. Samples were incubated at room temperature for 15 min. Carbon-coated copper grids were negatively discharged in a glow discharge device. A 5 μ L volume of sample was applied on a discharged grid for 20 s before being blotted. 6 μ L 2% uranyl acetate was then applied on the grid and incubated for 1 min. The grid was blotted and air dried. EM samples were viewed on a transmission electron microscope (Morgagni; FEI, Hillsboro, OR)

operating at 100 kV. Images were recorded on a bottom-mounted Orius (Gatan, Pleasanton, CA) camera at 22,000x magnification. ImageJ was used to measure the Dam1 complex inter-ring distances. Distances from middle of a ring to the middle of the next closest ring, on both sides, were measured.

The EM microscopy experiments were performed with two independent copper grids. Similarity between the two replicates allowed us to combine the data. Statistical analysis of the EM data was performed with pairwise z-tests. P-values were computed from the pairwise z-scores: $z = (\mu_1 - \mu_2) \cdot (\delta_1^2 + \delta_2^2)^{-0.5}$. μ_1 and μ_2 are the center of the Gaussian fits around the single large cluster in each sample and δ_1^2 and δ_2^2 are the corresponding estimates of error. A table of pairwise comparisons can be found in *Supplementary file 1E*.

Yeast strain construction and validation

Strains used in this study are derivatives of W303 (*Supplementary file 2A*). We have previously verified the genotype of the W303 by whole-genome tiling as described (Gresham et al., 2006). For strains constructed for this study, *NDC80* alleles were integrated at the *URA3* locus as previously described (Widlund and Davis, 2005). Plasmids containing the *NDC80* alleles were sequenced before transformation. Successful integration was verified by PCR. Genes were tagged with genes encoding fluorescent proteins as described (Wach et al., 1997) and verified by PCR.

Test for function of Ndc80^{10hep} complex

To test if the Ndc80^{10hep} complex is functional in vivo, we used a red/white plasmid shuffle assay (Davis, 1992; Muller, 1996) using strain JTY5-8B (*ade2-1oc ade3Δ-100 leu2-3,112 ura3-1 ndc80Δ::natMX*), harboring pJT12 (*NDC80 ADE3* in a 2 μm vector). JTY5-8B was transformed with pJOK13 (*URA3 ndc80^{10hep}*) and selected for growth on SD-ura low adenine plates. Transformation with pJOK13 yielded non-sectored red colonies (strain JOKY3) demonstrating that the *ndc80^{10hep}* is not viable when paired with wild-type *NUF2* (data now shown). JOKY3 was then grown in SD-lys-ura media before being transformed with pJOK018 (*LEU2 nuf2^{10hep}*), pJOK019 (*LEU2 NDC80*), or pRS315 (*LEU2*) and plated on SD-ura-leu plates. Four colonies from each transformation were streaked onto SD-ura low-ade plates to screen for sectoring colonies (technical replicate). Two transformations were performed for each condition (biological replicate), both trials gave the same result.

Yeast live-cell imaging and analysis

Strains for live-cell imaging were constructed using previously described strains: *SPC110-mCherry*, *pCUP1-GFP-LacI2* and *CEN3::33LacO* (Wargacki et al., 2010); *NDC80-3V5-IAA7* and *pGPD1-TIR1* (Miller et al., 2016). Sue Biggins kindly provided the Ndc80-AID strain. Strains containing *NDC80*, *ndc80-314* (ins A^{Ndc80p}), *ndc80-383* (ins B^{Ndc80p}), or *ndc80-563* (ins C^{Ndc80p}) were constructed through an integration method previously described (Widlund and Davis, 2005). YFP and CFP were integrated into Mtw1 and Dad4, respectively, through homologous recombination with PCR products amplified with primers containing homologous sequence of its target locus.

Asynchronously growing cells (30 Klett units) were arrested with 6 μM α-factor for 2 hr. Auxin (IAA (0.5 mM) was then added and the incubation continued for an additional hour. The cells were released from α-factor into YPD +0.5 mM IAA medium and imaged at 15 min time-intervals from time 0 to 120 min. At each time point, cells were also fixed in formaldehyde for budding index analysis. Budding index was determined twice (from two different colonies) and yielded the same conclusion for each of the five yeast strains shown in Figure 5. Data is shown for one of the two experiments. Cells were placed on agar pads prior to imaging, as previously described (Muller et al., 2005). Cells were imaged using a DeltaVision system (Applied Precision, Issaquah, WA) equipped with IX70 inverted microscope (Olympus, Center Valley, PA), a Coolsnap HQ digital camera (Photometrics, Tucson, AZ), and a U Plan Apo 100X objective (1.35 NA).

For CEN3 tracking biorientation assay, *CEN3-LacI* was visualized with *GFP-LacO* and the spindle pole bodies were visualized with *SPC110-mCherry*. Both GFP and mCherry were imaged with 0.4 s exposures with 16, 0.2 μm z-sections. Images were binned 2 × 2 with a final resolution of 512 × 512. Biorientation analysis was done manually for those cells with 2 mCherry puncta using Imaris software (Bitplane, Switzerland) (RRID: SCR_007370). For all the strains tested, the 60 min time point was chosen for data analysis. The biorientation assay was performed twice for each strain, each time with a

different colony and on a different day. The combination of two experiments yielded sample sizes between 190 and 260 cells.

For Dam1 complex kinetochore localization assay, kinetochore, Dam1 complex, and spindle pole bodies were visualized with Mtw1-YFP, Dad4-CFP, and Spc110-mCherry respectively. All three proteins were imaged with single focal plane 0.4 s exposures. Images were not binned with a final resolution of 1024×1024 . Line scans analyses were obtained of Mtw1-YFP and Dad4-CFP fluorescence using Imaris software (Bitplane) (RRID: SCR_007370) and extended from one SPB to the other. For each line scan, a correlation of the sign (positive or negative) of pixel intensity changes across the line between the Mtw1 and Dad4 channels was calculated to examine co-localization along the spindle. This was calculated as: $R = N_{\text{agree}} - N_{\text{disagree}} / N - 1$, where N_{agree} is the number of pixels that changed with the same sign (both channels increased or decreased), N_{disagree} is the number of pixels that changed with opposite signs (one channel increased and one decreased) and N is the length of the line scan. For each strain, a mean R was calculated from all line scans. The localization assay was performed twice for each strain, each time with a different colony and on a different day. The combination of two experiments yielded sample sizes between 18–33 cells. Statistical analysis of the Mtw1-YFP and Dad4-CFP mean R -scores was performed with Student's t -test. A table of the pairwise comparisons can be found in *Supplementary file 1G*.

Western blot analysis

During the yeast live-cell imaging, samples were collected for western blot analysis. After the addition of auxin, cell samples were collected at various time points. 0.7 ml cells and 0.7 ml 20% trichloroacetic acid were incubated at 4°C for 1 hr. Samples were pelleted for at 20,000 $\times g$ for 10 min at 4°C. The pellet was washed by resuspending in 1 ml 4°C ethanol. The sample was then pelleted and resuspended once more before a final pelleting step. Supernatant was discarded and sample was air dried before resuspending in 50 μ l 0.1N NaOH, SDS-PAGE sample buffer. Western blot analysis was performed, probing for Ndc80p (gift from Arshad Desai, Ludwig Cancer Research Center, University of California San Diego) and β -Actin (Abcam, Cambridge, MA). Protein levels analysis was carried out using Image Studio Lite (Li-Cor, Lincoln, Nebraska) (RRID: SCR_014211). Each western blot was performed twice, yielding the same conclusion for each experiment.

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Additional files

Supplementary files

- Supplementary file 1. Tables of pairwise statistical comparisons.

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- Supplementary file 2. Table of yeast strains and plasmids used in this study.

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- Source code 1. LabVIEW TIRF analysis step 1.

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- Source code 2. LabVIEW TIRF analysis step 2.

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- Source code 3. LabVIEW TIRF analysis step 3.

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- Source code 4. Igor Pro compile TIRF data.

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- Source code 5 Igor Pro analyze TIRF data.

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- Source code 6. Igor Pro mean squared displacement TIRF analysis.

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Figures and figure supplements

The Ndc80 complex bridges two Dam1 complex rings

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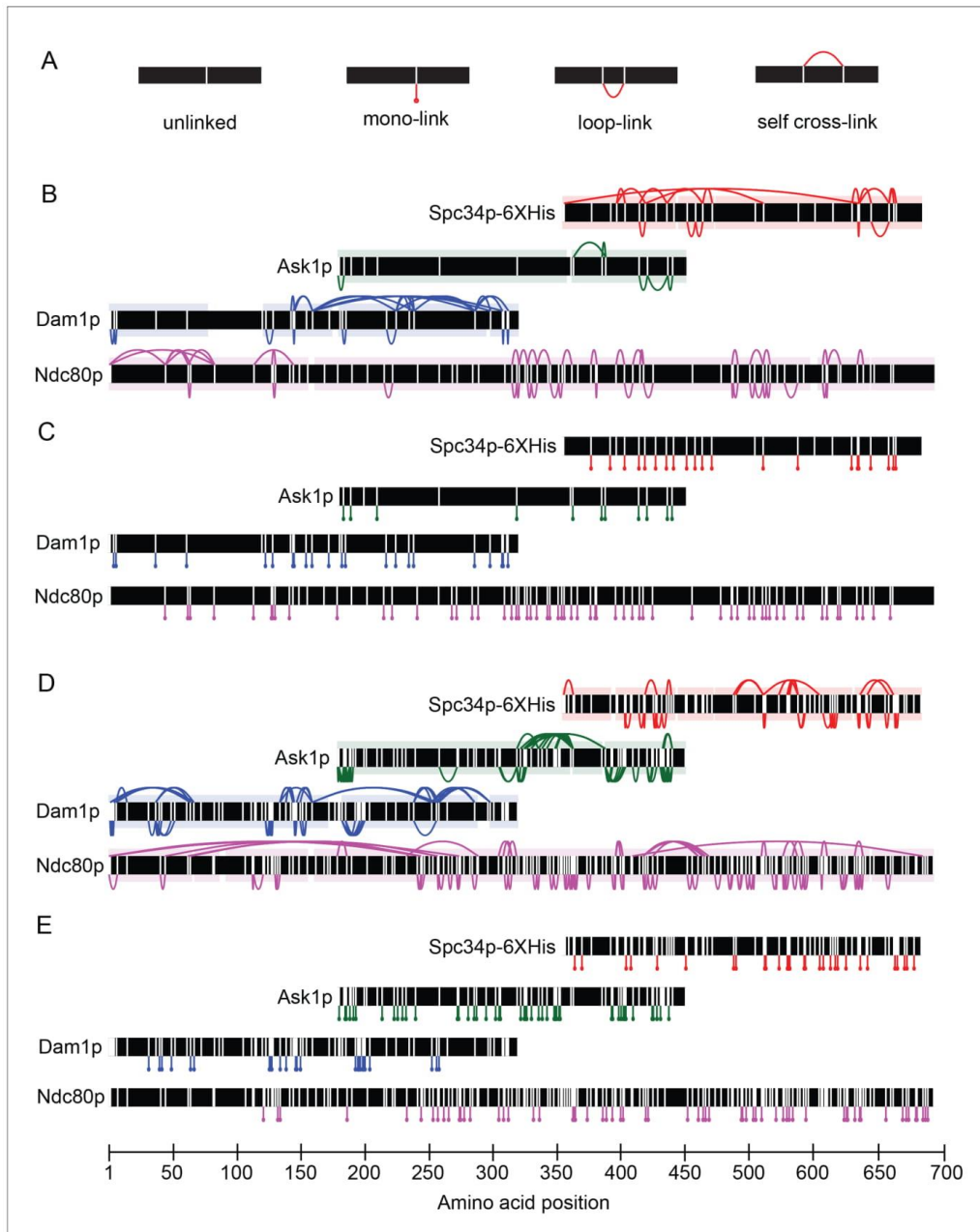


Figure 1—figure supplement 1. Dam1 and Ndc80 complexes robustly react with DSS and EDC cross-linking agents. (A) Four types of peptides identified by mass spectrometry after cross-linking and trypsin digestion. Horizontal black bars represent protein sequence and vertical white lines. Figure 1—figure supplement 1 continued on next page

Figure 1—figure supplement 1 continued

represent residues able to react with the cross-linking agent. Unlinked: peptide that did not react with cross-linking agent; mono-link: only one end of the cross-linking reagent reacted with a residue; loop-link: intra-protein cross-link without a tryptic site in between the two cross-linked residues; self cross-link: intra-protein cross-link with a tryptic site in between the two cross-linked residues. Dam1 and Ndc80 complexes were cross-linked in the presence of microtubules using DSS (panels B and C) or EDC (panels D and E). (B, D) Cross-links above and below the black bars represent self-crosslinks and loop-links, respectively. Data are shown for peptides with Percolator (Käll *et al.*, 2007) assigned q-values ≤ 0.05 . Highlighted regions above and below black bars represent sequence coverage from mass spectrometry. (C, E) Lines below the black bars represent mono-links.

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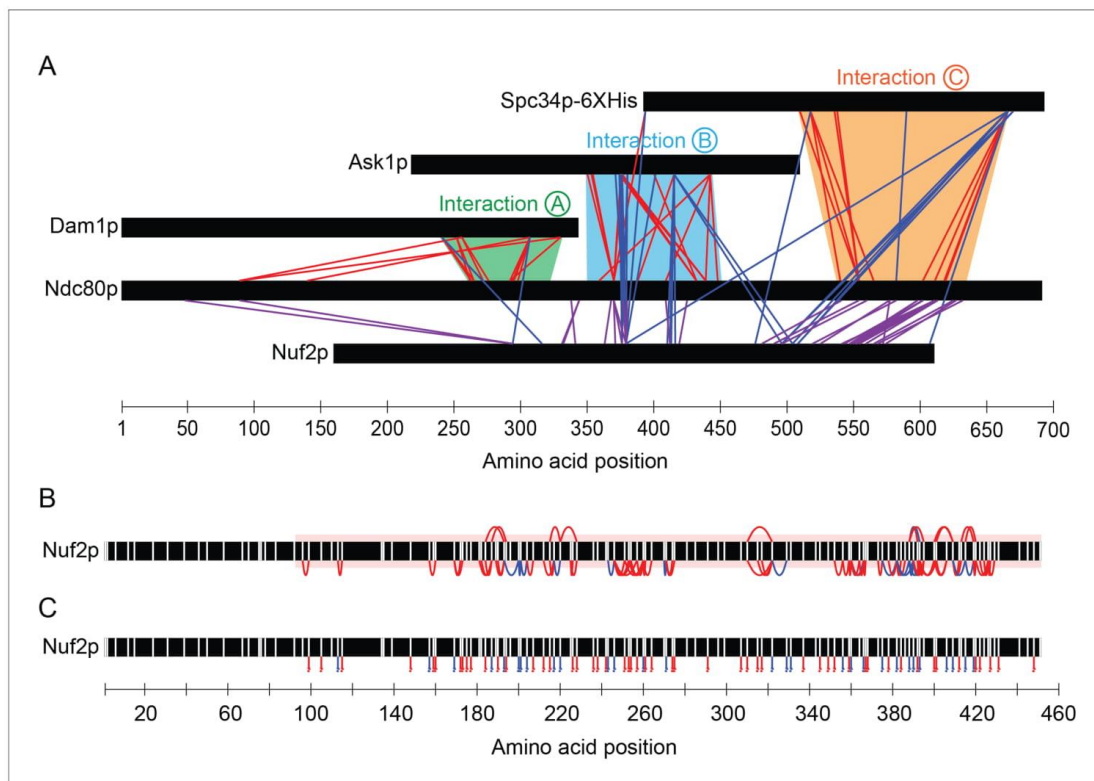


Figure 1—figure supplement 2. Dam1p, Ask1p, and Spc34p cross-link to Nuf2p, in agreement to the coiled-coil structure of Ndc80p and Nuf2p. (A) Cross-links between the Dam1p, Ask1p, and Spc34p of the Dam1 complex and Ndc80p and Nuf2p of the Ndc80 complex. For clarity, cross-links between the Dam1 complex proteins are not shown. Red lines represent cross-links between Ndc80p and Dam1p, Ask1p, or Spc34p. Blue lines represent cross-links between Nuf2p and Dam1p, Ask1p, or Spc34p. Purple lines represent cross-links between Ndc80p and Nuf2p. (B) Cross-links above and below the black bar represent self-crosslinks and loop-links, respectively, for Nuf2p. (C) Lines below the black bar represent mono-links for Nuf2p. Data from cross-linking experiments using DSS and EDC cross-linking agents are both shown. Data are shown for peptides with Percolator (Käll et al., 2007) assigned q-values ≤ 0.05 .

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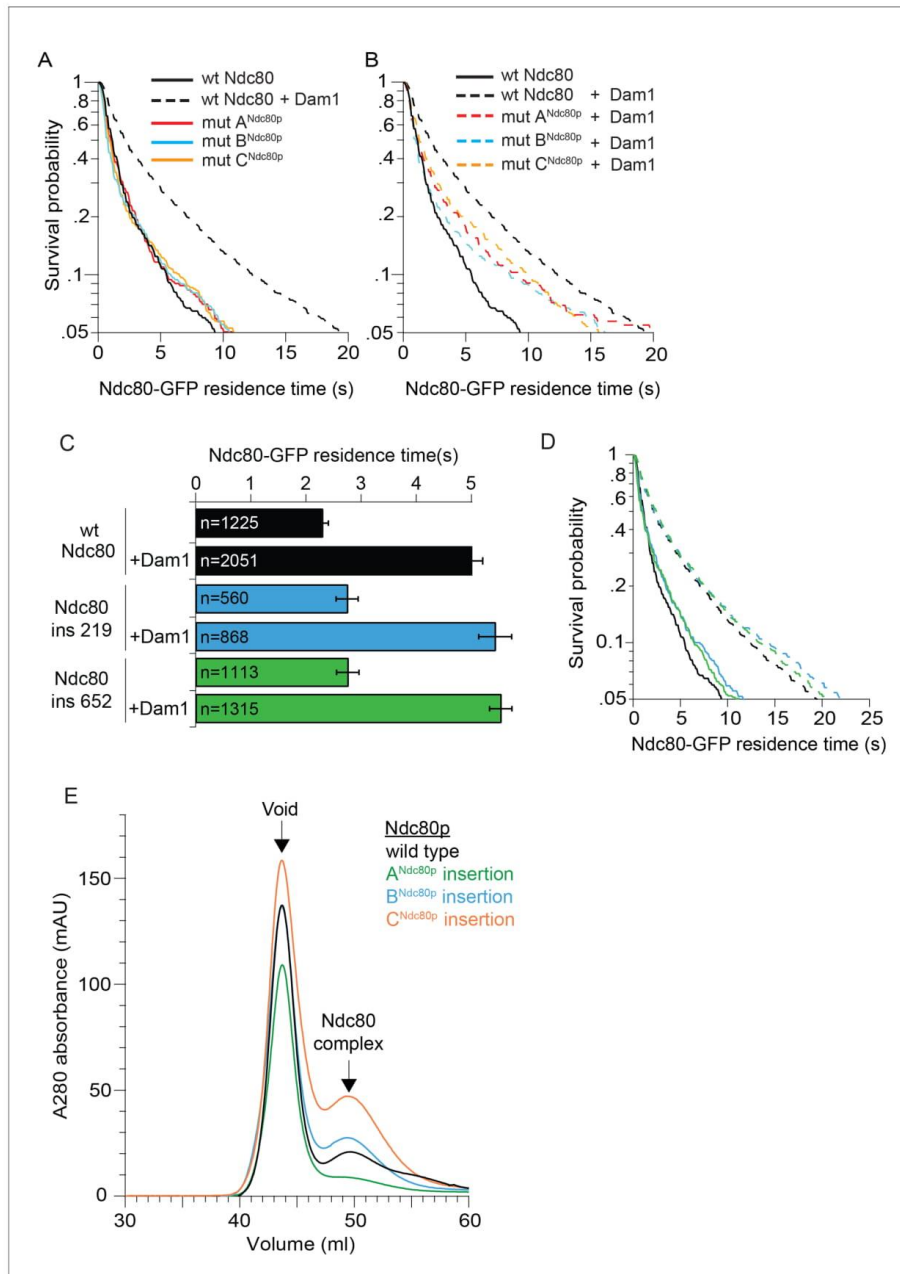


Figure 1—figure supplement 3. Ndc80 mutations outside of regions A, B, and C do not disrupt the interaction between Dam1 and Ndc80 complexes. (A), (B) Survival probability of the data shown in **Figure 1D**: microtubule residence times of Ndc80-GFP complex with a lethal mutation in region A. **Figure 1—figure supplement 3 continued on next page**

Figure 1—figure supplement 3 continued

A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the absence (A) and presence (B) of wild-type Dam1 complex. Data here have a range of n = 420 to 2051. (C) Average microtubule residence times of Ndc80-GFP complexes with wild-type Ndc80p, or lethal five amino acid insertional mutation at position 219 or 652. Bars represent average residence time ± error of the mean (estimated by bootstrapping analysis; see Materials and methods for additional details). Ndc80-GFP complex microtubule residence time raw data are included in **Figure 1—figure supplement 3—source data 1**. Refer to **Supplementary file 1B** for statistical analysis. (D) Survival probability curves of the data shown in (C). (E) Superdex 200 size exclusion chromatography of the Ndc80 complex with wild-type Ndc80p or insertional mutation in A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}.

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The following source data is available for figure 1:

Figure supplement 3—Source data 1. Table of Ndc80-GFP microtubule residence times for **Figure 1—figure supplement 3C**.

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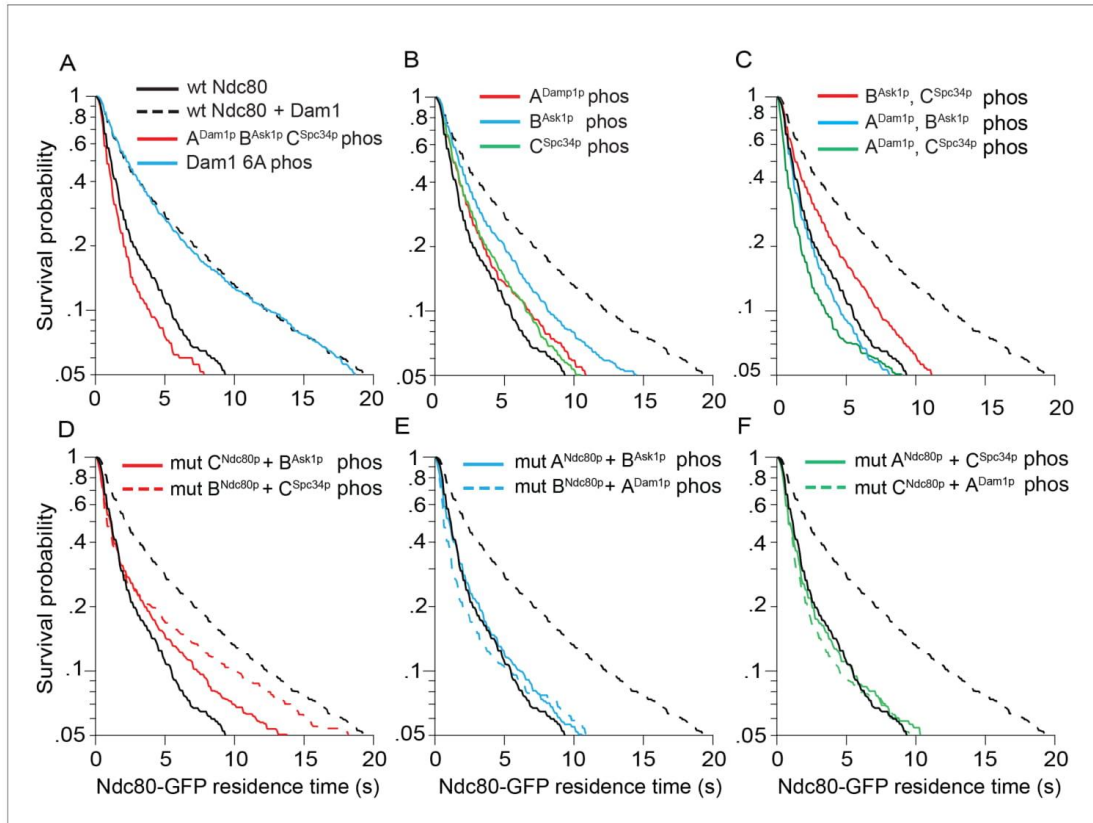


Figure 2—figure supplement 1. Microtubule residence time survival probability plots of experiments in Figure 2. For all graphs, solid and dashed black lines represent curves for wild-type Ndc80 complex in the absence and presence of wild-type Dam1 complex, respectively. (A–C) Wild-type Ndc80-GFP complex microtubule residence time in the presence of variably phosphorylated Dam1 complex. Dam1 6A phos: all Dam1 complex phosphorylation sites mutated to Ala and treated with Aurora B kinase. (D–F) Microtubule residence time of Ndc80-GFP complex with a lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} in the presence of variably phosphorylated Dam1 complex. Data here have a range of $n = 485$ to 2051.

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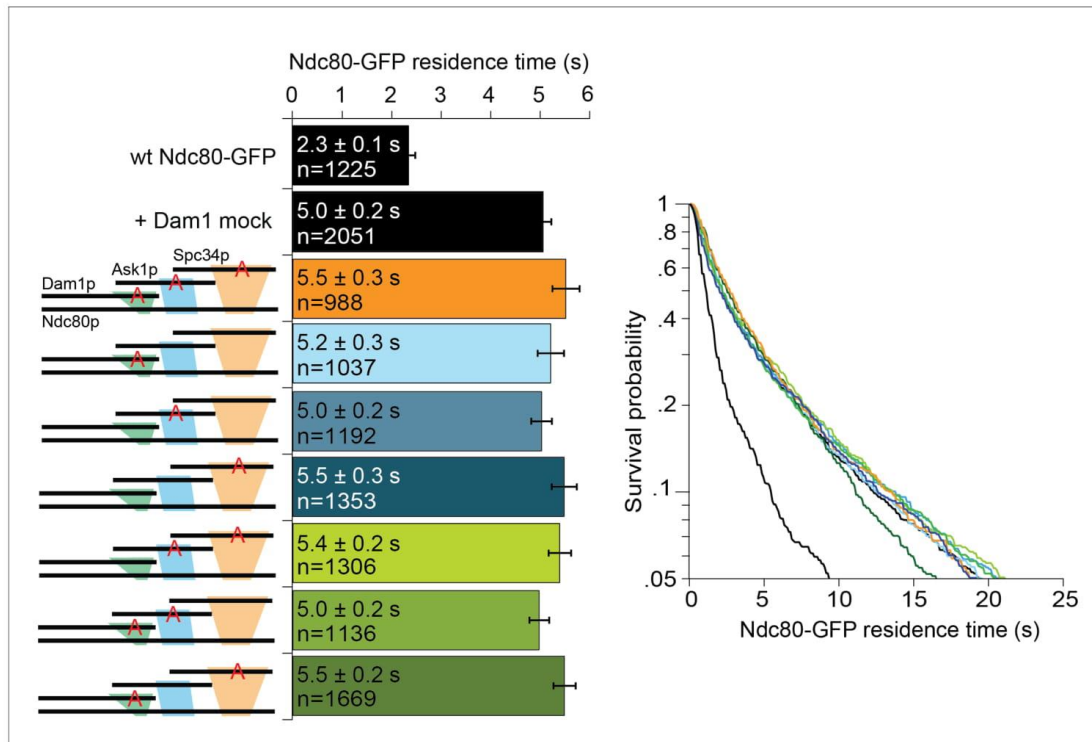


Figure 2—figure supplement 2. Mutating Dam1 complex Aurora B kinase phosphorylation sites to Ala does not affect the interaction between Dam1 and Ndc80 complexes. Average microtubule residence time of wild-type Ndc80-GFP complex in the presence of mock treated phosphorylation-blocking Dam1 complex mutant constructs. A red 'A' identifies the phosphorylation sites that are mutated to alanine. Mock treatment was carried out by substituting ATP with H₂O. Survival probability plots of the average microtubule residence time shown on the right. Ndc80-GFP complex microtubule residence time raw data are included in **Figure 2—figure supplement 2—source data 1**. Refer to **Supplementary file 1D** for statistical analysis.

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The following source data is available for figure 2:

Figure supplement 2—Source data 1. Table of Ndc80-GFP microtubule residence times for **Figure 2—figure supplement 2**.

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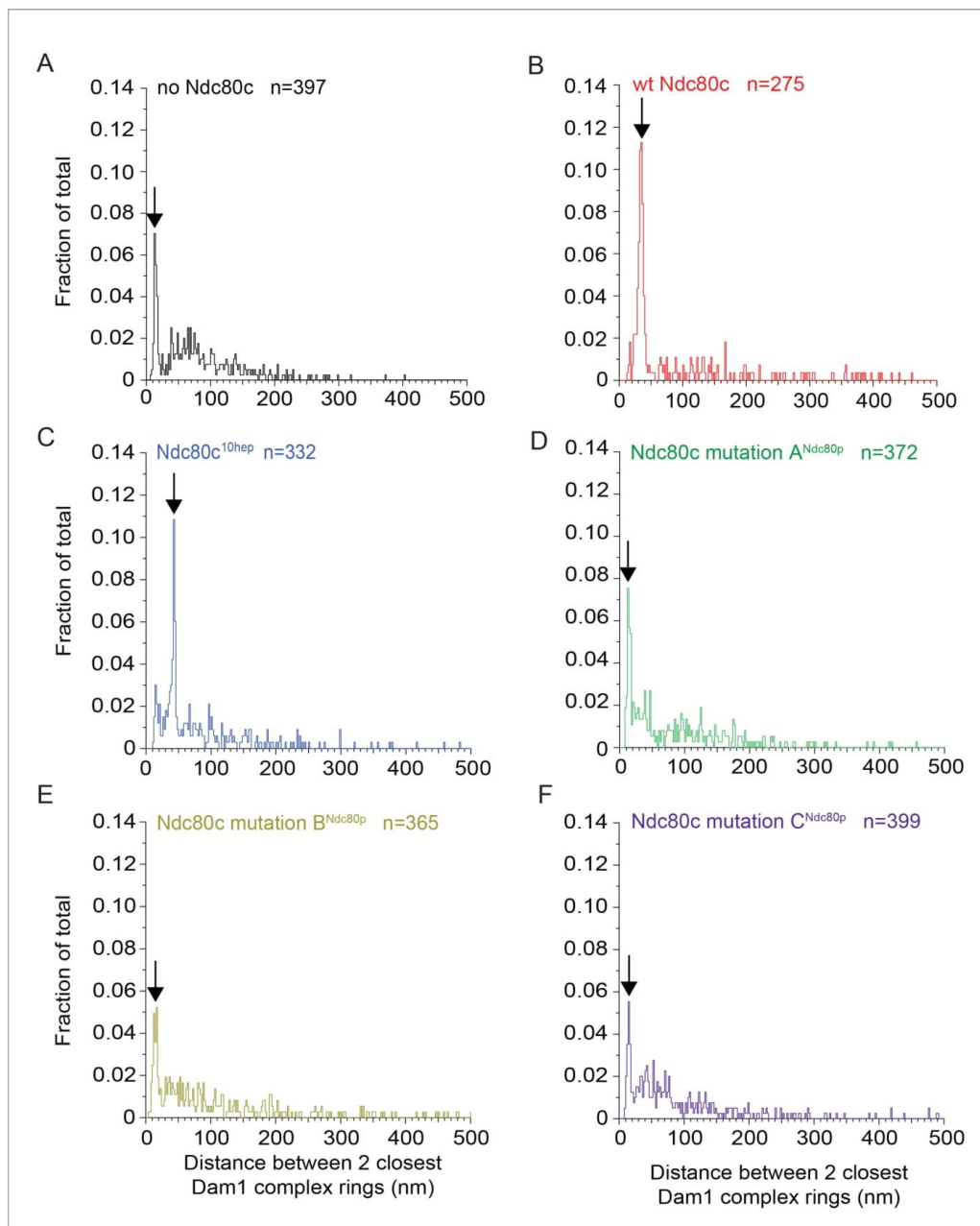


Figure 3—figure supplement 1. Full distributions of Dam1 complex inter-ring measurements for various experiments shown in *Figure 3* and *Figure 3—figure supplement 2*. Six conditions include Dam1 complex rings on microtubules in the absence of Ndc80 complex (Ndc80c) (A), presence of Ndc80c (B), presence of Ndc80c mutation A (C), presence of Ndc80c mutation B (D), presence of Ndc80c mutation C (E), and presence of Ndc80c mutation D (F). Figure 3—figure supplement 1 continued on next page

Figure 3—figure supplement 1 continued

of wild-type Ndc80c (B), Ndc80c^{10hep} (C), Ndc80c lethal mutation A^{Ndc80p} (D), B^{Ndc80p} (E), and C^{Ndc80p} (F). The arrows identify the sharp peaks that contain at least 20% of the events.

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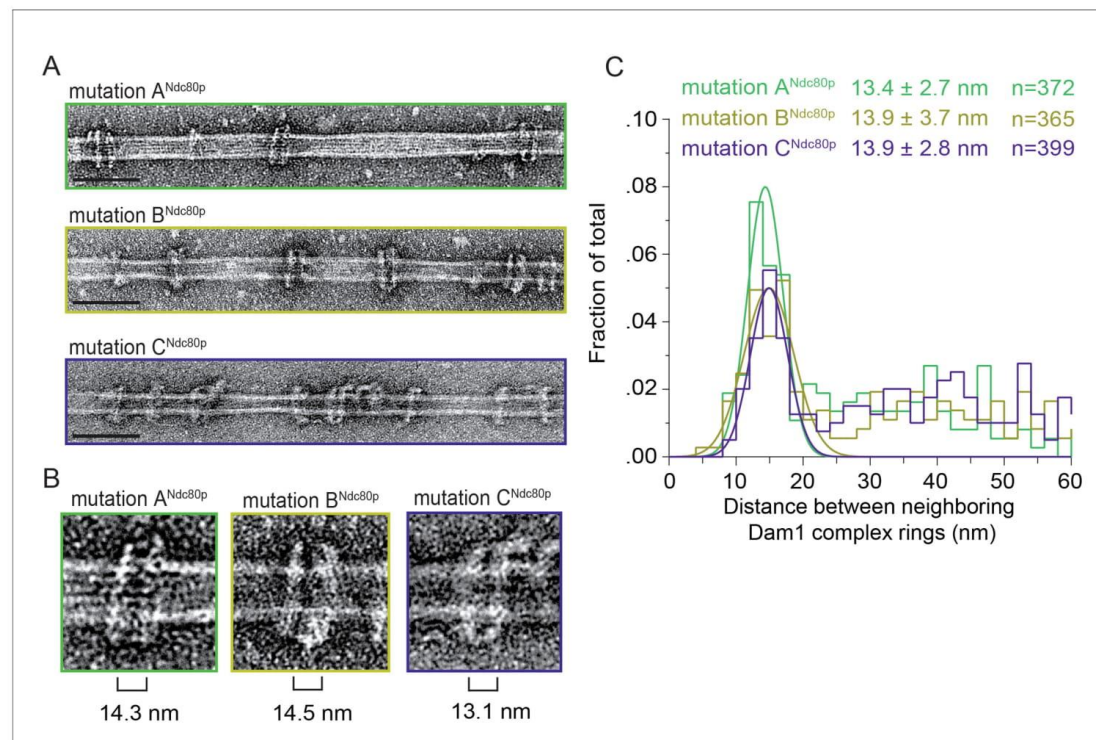


Figure 3—figure supplement 2. Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} disrupts the Ndc80 complex's ability to bind two Dam1 complex rings. (A) Representative EM images of Dam1 complex rings on microtubules in the presence of the Ndc80 complex with a lethal mutation in region A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}. Scale bars: 100 nm. Protein concentrations were 20 nM tubulin and 25 nM Dam1 and Ndc80 complexes. (B) Zoomed in images of a Dam1 complex ring doublet for the three experiments. Distances from middle of one ring to those of the closest two rings were measured. (C) Distribution of closest inter-ring distances measured for the three experiments. Measurements made between 0–60 nm are shown. The cluster of distance measurements was fitted with a Gaussian distribution and the vertex ± standard deviation for each fit is listed. The raw data of Dam1 complex inter-ring distance measurements are included in **Figure 3—figure supplement 2—source data 1**. Refer to **Supplementary file 1E** for statistical analysis of data in part (C).

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The following source data is available for figure 3:

Figure supplement 2—Source data 1. Table of Dam1 complex inter-ring distance measurements for **Figure 3—figure supplement 2C**.

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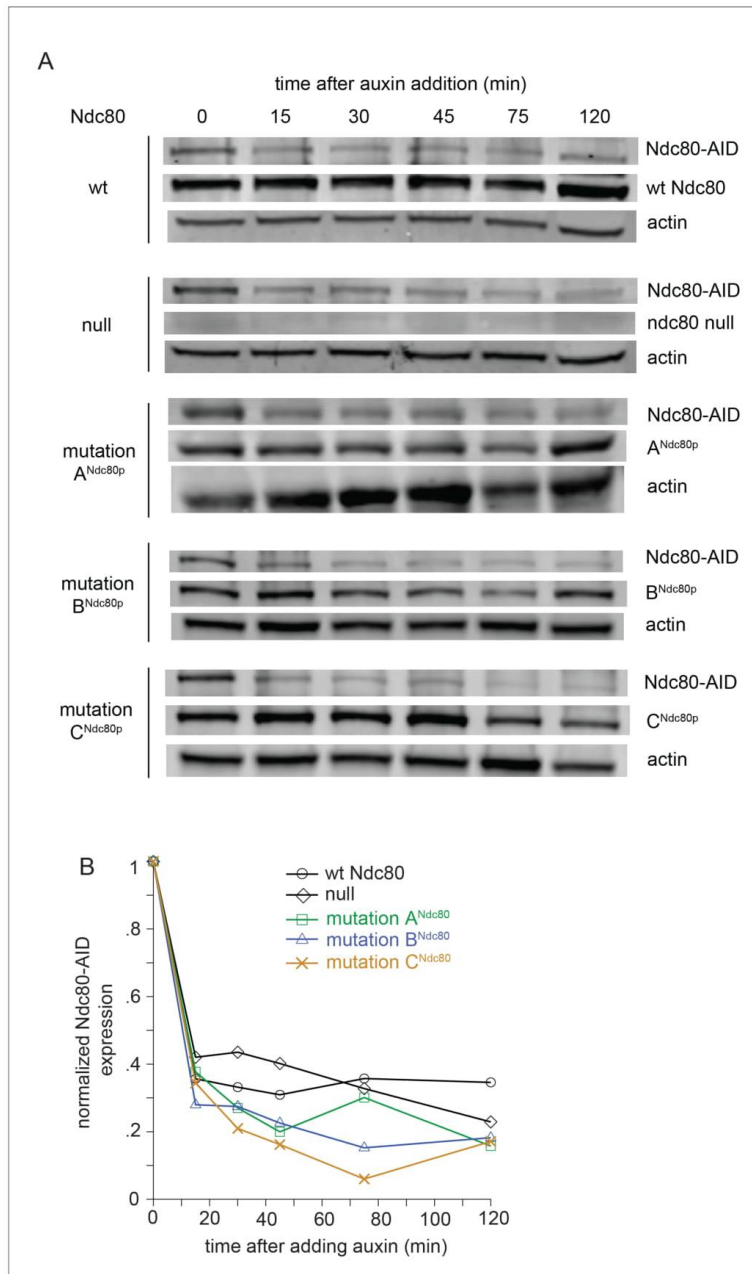


Figure 5—figure supplement 1. Wild-type Ndc80-AID is degraded upon the addition of auxin. (A) Western blot images from Ndc80-AID degron experiments, blotting for Ndc80-AID and β -actin. Whole cell protein precipitation was carried out for Ndc80-AID cells carrying an extra copy of *NDC80*. Figure 5—figure supplement 1 continued on next page

Figure 5—figure supplement 1 continued

wild-type, no *NDC80* (null), lethal mutation in A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p}. Samples were collected at various time points after adding auxin (Images were taken 120 min after auxin addition). (B) Normalized Ndc80-AID protein levels analyzed by western blot band intensities.

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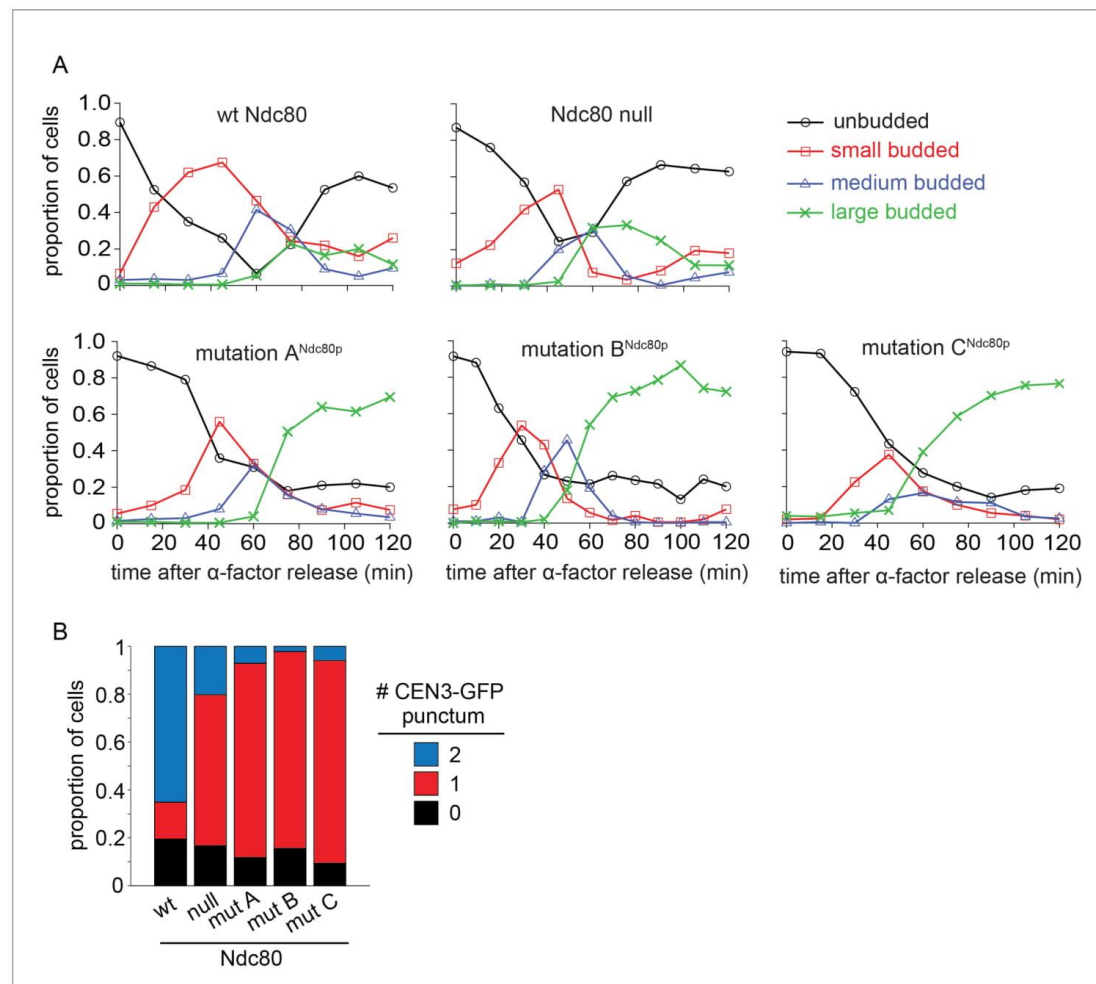


Figure 5—figure supplement 2. Lethal mutation in region A^{*Ndc80p*}, B^{*Ndc80p*}, or C^{*Ndc80p*} causes mitotic arrest. (A) Budding index analyses of *Ndc80*-AID degenon experiments. *Ndc80*-AID cells carrying an addition copy of *NDC80* wild-type, no *NDC80* (null), lethal mutation in region A^{*Ndc80p*}, B^{*Ndc80p*}, or C^{*Ndc80p*} were fixed at various time points after release from α -factor arrest (time 0). (B) Stacked bar graphs showing the percentage of cells with zero, one, or two CEN3-GFP spots for the various cell types.

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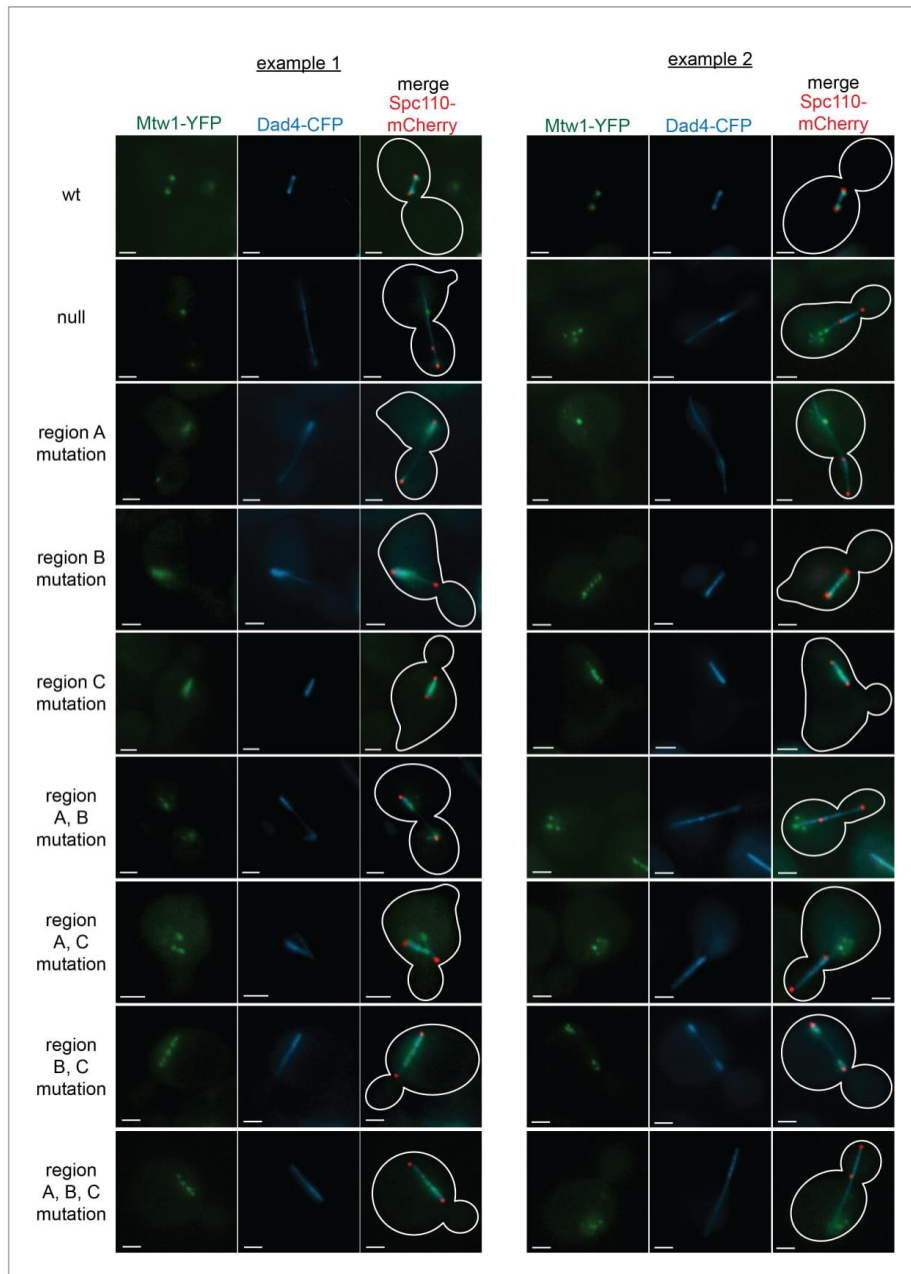


Figure 6—figure supplement 1. Mutations in regions A^{Ndc80p}, B^{Ndc80p}, or C^{Ndc80p} results in severe defects in kinetochore biorientation and attachment. Examples of commonly observed phenotypes in experiments with NDC80-AID degron cells carrying an additional copy of *ndc80*
 Figure 6—figure supplement 1 continued on next page

Figure 6—figure supplement 1 continued

containing single or multiple mutations in regions A^{Ndc80p}, B^{Ndc80p}, and C^{Ndc80p}, or not carrying a second copy of *NDC80* (null). Each example shows Mtw1-YFP, Dad4-CFP, and Spc110-mCherry and the same experiment procedure was carried out as in **Figure 6**. The majority of mutant cells with two SPBs exhibited detached kinetochores or had kinetochores spread along spindle microtubules. All scale bars: 2 μ m.

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Appendix B

The molecular architecture of the Dam1 kinetochore complex is define by cross-linking based structural modelling

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The molecular architecture of the Dam1 kinetochore complex is defined by cross-linking based structural modelling

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Accurate segregation of chromosomes during cell division is essential. The Dam1 complex binds kinetochores to microtubules and its oligomerization is required to form strong attachments. It is a key target of Aurora B kinase, which destabilizes erroneous attachments allowing subsequent correction. Understanding the roles and regulation of the Dam1 complex requires structural information. Here we apply cross-linking/mass spectrometry and structural modelling to determine the molecular architecture of the Dam1 complex. We find microtubule attachment is accompanied by substantial conformational changes, with direct binding mediated by the carboxy termini of Dam1p and Duo1p. Aurora B phosphorylation of Dam1p C terminus weakens direct interaction with the microtubule. Furthermore, the Dam1p amino terminus forms an interaction interface between Dam1 complexes, which is also disrupted by phosphorylation. Our results demonstrate that Aurora B inhibits both direct interaction with the microtubule and oligomerization of the Dam1 complex to drive error correction during mitosis.

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The kinetochore is a network of protein complexes that assemble on centromeric DNA and mediate the attachment of chromosomes to dynamic spindle microtubules (MTs). This attachment allows chromosomes to be equally segregated into daughter cells¹. The yeast Dam1 complex is composed of ten proteins and is essential to attach kinetochores to MTs. *In vitro*, the Dam1 complex recapitulates many functions of the kinetochore, including the ability to couple cargo to dynamic MTs, while bearing load^{2–9}. In solution, the Dam1 complex exists primarily as a 406-kDa dimer¹⁰. In the presence of MTs, however, it oligomerizes into rings encircling the MT^{3,11–14}. Such oligomerization is necessary for the Dam1 complex to form MT attachments that are robust against tension⁹.

The ten Dam1 complex proteins are as follows: Ask1p (32 kDa), Dad1p (11 kDa), Dad2p (15 kDa), Dad3p (11 kDa), Dad4p (8.2 kDa), Dam1p (38 kDa), Duo1p (27 kDa), Hsk3p (8.1 kDa), Spc19p (19 kDa) and Spc34p (34 kDa). The functions of few of the subunits are known. Biochemical and genetic evidence showed that Dam1p and Duo1p are primarily responsible for MT binding^{2,3,10,13–15}. In the absence of Hsk3p, the remaining nine components of the Dam1 complex split into two subcomplexes as follows: (A) Dam1p, Duo1p, Spc34p, Spc19p, Dad1p and Dad3p, which binds MTs but does not oligomerize, and (B) Ask1p, Dad2p and Dad4p, which does not bind MTs^{9,15}.

Errors in chromosome segregation are devastating to the cell and, in multicellular organisms, are associated with a range of diseases as well as being a hallmark of cancer¹⁶. Intricate error-correction mechanisms exist to prevent such errors and delay cell cycle progression until correct kinetochore–MT attachments are achieved¹⁷. A major component of these protective pathways is the Ipl1p (Aurora B) kinase, which phosphorylates kinetochore proteins to promote detachment of incorrectly attached kinetochores from MTs^{18,19}. As the Dam1 complex plays a central role in mediating kinetochore–MT coupling, it is an essential target of Ipl1p²⁰.

The Dam1 complex is phosphorylated by Ipl1p at six different sites: Dam1p S20, S257, S265, S292, Ask1p S200 and Spc34p T199 (ref. 20). The four Dam1p sites have been shown to play a role in regulating the ability of Dam1 complex to bind MTs and mutations that block phosphorylation at all four sites are lethal. However, either the N-terminal site alone or the three C-terminal sites are sufficient for viability, suggesting functional redundancy²⁰. Moreover, removal of the C terminus of Dam1p reduces the affinity of the Dam1 complex for MTs but does not abolish binding completely^{13,14}. We previously found that phosphorylation of Dam1p S20 decreases the apparent affinity of the Dam1 complex to MTs, whereas phosphorylation of the C-terminal Ipl1 sites together has little effect². Thus, both N- and C-terminal regions of Dam1p have been implicated in MT binding, although their specific roles remain elusive. Despite much work, key questions remain regarding how Ipl1 phosphorylation of Dam1p regulates the kinetochore–MT interface during mitosis.

One challenge that has prevented further progress is a lack of detailed structural information. Previous work culminated in a model for the layout of the Dam1 complex within a 30-Å electron microscopy (EM)-based structure, wherein five of the ten protein subunits were localized³. Subunit localization was performed in the absence of MTs, but previous studies have conflicted over whether the Dam1 complex binds MTs and can form oligomeric rings without a major conformational change^{3,10}. In addition, the specific regions involved in the MT-binding interface have not been identified and the localization of half of the subunits remains unknown.

Here we used integrative structural modelling²¹, combining information from cross-linking/mass spectrometry (MS),

previously published EM³ and biochemical data¹⁵, to determine the molecular architecture of the Dam1 complex. The work presented here is the first time a data set consisting primarily of cross-linking distance restraints has been used to produce a coarse-grained, yet biologically informative structural model of a large protein complex for which no X-ray structures nor homology models were available for any of its components. We then tested key predictions generated from this structural model, to define how Aurora B regulates the Dam1 complex during error correction.

Results

A simple accurate and high-yield cross-linking method. We benchmarked our cross-linking method using three protein complexes of known structure: the four-protein 300-kDa γ -TuSC complex, the two-protein 27-kDa BRCA1/BARD1 complex and the three-protein 120-kDa SCF^{FBXL3} ubiquitin ligase complex. Using our simple unlabelled method and a false discovery rate (FDR) of 1%, we obtained 129, 57 and 67 unique distance restraints (UDRs) from these 3 complexes, respectively. Agreement with available structures was excellent, with 91%, 81% and 94%, respectively, of cross-linked lysine C α –C α distances within the 30 Å that our cross-linker can span²² (Supplementary Fig. 1 and Supplementary Tables 1–4). A comparison of lysine C α –C α distances observed cross-linked together versus all possible lysine C α –C α distances from the structures showed a clear enrichment of distances <20 Å at 1% FDR, indicating our method accurately reports on the native structure (Supplementary Fig. 2). We chose this same conservative 1% FDR cutoff for modelling data. Cross-linker reactivity and MS detection were also good, as we detected 69–88% of lysines modified by the cross-linker (Supplementary Table 1). These data demonstrate our cross-linking method produces a high number of accurate distance restraints on complexes of various size and complexity. The number of unique cross-link identifications made by our method was greater than other methods (Supplementary Table 5). Our method also has key advantages: it performs well without isotopic labelling, post cross-linking fractionation or enrichment of cross-linked peptides. Data analysis using our Kojak cross-link identification algorithm is simple, fully automated and faster than existing methods²³.

The molecular architecture of free Dam1 complex. Our cross-linking analysis of free Dam1 complex in the absence of MTs identified 678 UDRs at 1% FDR (Supplementary Tables 1 and 6). Despite our simple method, this number (about 34 UDRs per 10 kDa of protein) compares very favourably with cross-linking analyses of other protein complexes (Supplementary Table 5)^{24–27}. The cross-linking data were specific, because several pairs of proteins show multiple cross-links over all or part of their lengths (Supplementary Fig. 3a), whereas others exhibited little or no cross-linking to each other, even though lysines modified as ‘mono-links’ were detected throughout these proteins (Supplementary Fig. 3b and Supplementary Tables 6–8). With the high information content of our cross-linking data set, we sought to expand on existing information to build a complete model of the molecular architecture of the Dam1 complex. Our modelling procedure consists of generating structural models consistent with the input experimental data plus physical and statistical considerations. This procedure has four stages: (1) gathering information, (2) selecting a suitable representation of the system and encoding experimental data into spatial restraints, (3) the generation of structural models and (4) analysis²⁸. We used a coarse-grained description of the system and we tuned the resolution based on our cross-linking data. On average, we have

1 cross-linked residue every 13 amino acid residues along a protein chain. We therefore represented each Dam1 complex component as a chain of beads, each bead representing the average volume occupied by ten residues. A granularity much smaller than ten residues per bead would have resulted in modelling a large number of beads based on no information, but chain connectivity, leading to less precise models for large fragments of each component. On the other hand, a granularity much larger than ten residues per bead would not have optimally exploited the information available from the cross-linking data.

Previous work localized single termini from five of the ten Dam1 complex protein components within the EM reconstruction of a Dam1 complex dimer³. We used the Integrative Modeling Platform (IMP)²¹, to model our cross-linking data combined with biochemical data¹⁵ plus the EM localization data (Supplementary Fig. 4; see Methods for details). We produced a complete structural model of the Dam1 complex, which localizes, for the first time, all ten components of the Dam1 complex at a resolution of ten amino acid residues per bead (Fig. 1, Supplementary Movie 1 and Supplementary File 1). This model fit 88% of the observed cross-linking data (Fig. 2, left green arrows). A probability density function representing the

variability in the location of the beads in the top-scoring 1,000 models shows that the positions of 90% of the beads are tightly constrained, in that their centres vary by 3 Å or less at half maximal height (Fig. 3a). This model for the Dam1 complex in solution was used as a basis for comparison, to understand how the Dam1 complex interacts with MTs and if this is accompanied by substantial conformational changes.

Dam1 complex–MT interactions specify a preferred orientation.

We next performed cross-linking analysis on the Dam1 complex bound to MTs. Examination of the cross-linked Dam1 complex by EM showed its assembly into rings around MTs, as expected^{3,11–14} (Supplementary Fig. 5). Of the ten proteins that make up the Dam1 complex, only the C-terminal domains of Dam1p and Duo1p cross-linked to the MT (Fig. 4 and Supplementary Table 9). No other proteins formed a single cross-link to the MT, indicating our results were highly specific. We also detected cross-links between tubulin subunits, all of which agree with published structures and provide further confidence that our cross-links accurately report on the native structure (Supplementary Fig. 6 and Supplementary Table 10).

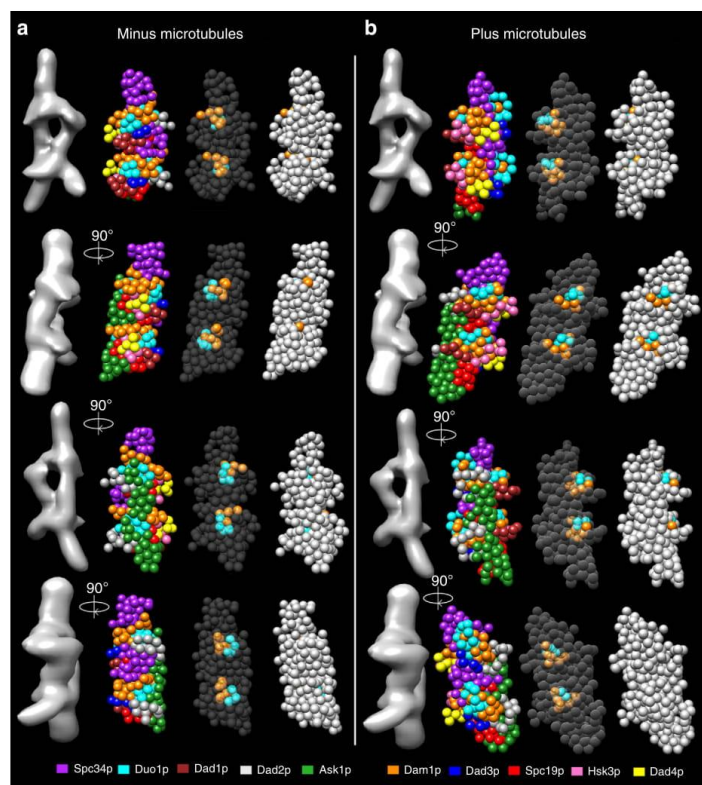


Figure 1 | Structural model of the Dam1 complex. Structural model of the Dam1 complex in the (a) absence and (b) presence of MTs. Each model represents the centre of the single cluster found in the top 1,000 models. Four orientations are shown. Each panel shows the Dam1 EM structure EMD-1372 for reference (left) followed by 3 differently coloured versions of the Dam1 structural model. The left version shows all the beads coloured according to protein (key given at the bottom of the figure). The centre version highlights only those residues that cross-link to MTs. Transparency is used to make the MT-binding region visible. The right-hand version removes the transparency so that the proximity of the MT-binding region to the model's surface can be clearly seen. It is noteworthy that in the absence of MTs, the MT-binding region is in the interior of the structure.

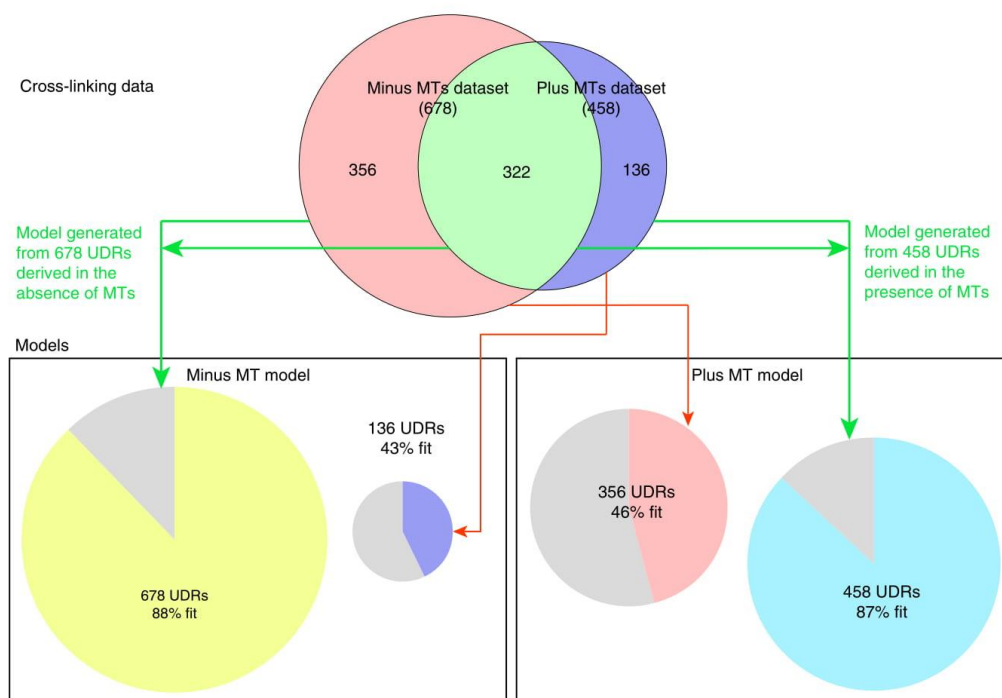


Figure 2 | The number and overlap of distance restraints generated by cross-linking experiments on the Dam1 complex in the presence and absence of MTs. Per cent fit of distance restraint data with the minus MT and plus MT models generated is shown in boxes. Data are shown for peptides with Percolator⁴⁹ assigned q -values ≤ 0.01 . A distance restraint is considered a 'fit' if the modelled bead surfaces of the two beads that included the cross-linked residues are within 10 Å. The minus MT model was generated using the 678 UDRs observed in the absence of MTs. The plus MT model was generated using the 458 UDRs observed in the presence of MTs. Per cent fit is shown for the data used to generate the models (green arrows) and for the data unique to the alternate condition (red arrows).

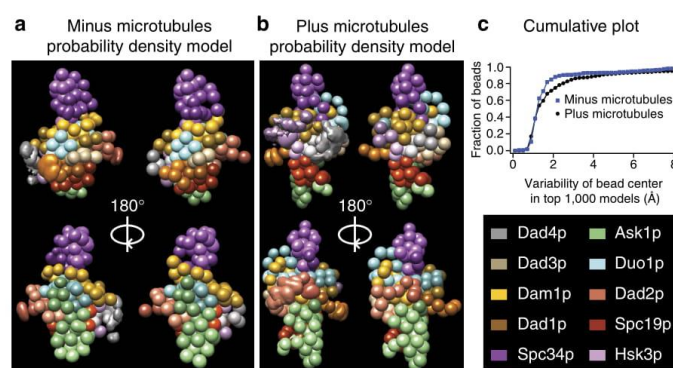


Figure 3 | The precision of our modelling approach in determining the positions of all beads. (a,b) Density maps expressing the probability that a given region of space is occupied by a bead in the top 1,000 scoring models. The space was discretized in bins of 2 Å size. The density maps of each bead are shown at half their maximum value. It is noteworthy that almost all beads are well determined and therefore look similar in the probability density map and the representative model. An exception would be the Dad2p beads (salmon color) in the presence of MTs (bottom right image). Only a Dam1 complex monomer is shown for clarity. (c) A cumulative plot of bead variability.

At 1% FDR, this analysis identified 458 UDRs within the Dam1 complex, with 90% of lysines modified by the cross-linker at least once (Supplementary Tables 1 and 6). As described above, we used IMP to generate a structural model for the Dam1 complex in the presence of MTs (Fig. 1b, Supplementary Movie 2 and Supplementary File 2). Eighty-seven per cent of the observed

distance restraints were satisfied by this model (Fig. 2, right green arrows). The probability density function for the top-scoring 1,000 models reveals that 86% of the beads are tightly constrained (their positions vary by 3 Å or less at half maximal height), indicating this model is also well determined (Fig. 3b).

We then used a flexible-docking procedure to fit this model of the Dam1 complex dimer onto a MT, based on the cross-links observed between the Dam1 complex and tubulin (Figs 4, 5, Supplementary Movie 3 and Supplementary File 3). A good fit was obtained, as 76% of the observed Dam1 complex to tubulin cross-links were satisfied (Supplementary Table 11). Furthermore, the top 3,264 models produced a single preferred orientation with respect to MT polarity (as depicted in Fig. 5), while alternative orientations were only found in low-scoring models. The orientation observed is compatible with ring formation, as the Dam1 dimer is arranged perpendicular to the protofilaments. Moreover, the orientation is consistent with recently published *in vivo* fluorescence resonance energy transfer (FRET) data²⁹ (Fig. 5c).

The Dam1 complex adopts a distinct MT-bound conformation.

Of the 458 UDRs observed in the presence of MTs, 322 were shared with the data set obtained in the absence of MTs, suggesting that many interactions remained unchanged (Supplementary Table 7 and Supplementary Fig. 3a). However, 136 distance restraints were specific to Dam1 complex in the

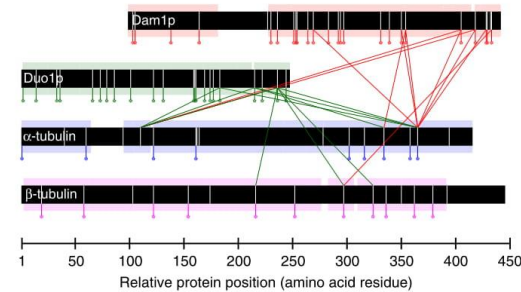


Figure 4 | Dam1 complex to MT cross-links. Peptide sequence coverage (coloured boxes), mono-links (coloured vertical lines and circles) and lysines (vertical white lines) are also shown. Dam1p to Duo1p and tubulin to tubulin cross-links are hidden for clarity. Data are shown for peptides with Percolator⁴⁹ assigned *q*-values ≤ 0.01 .

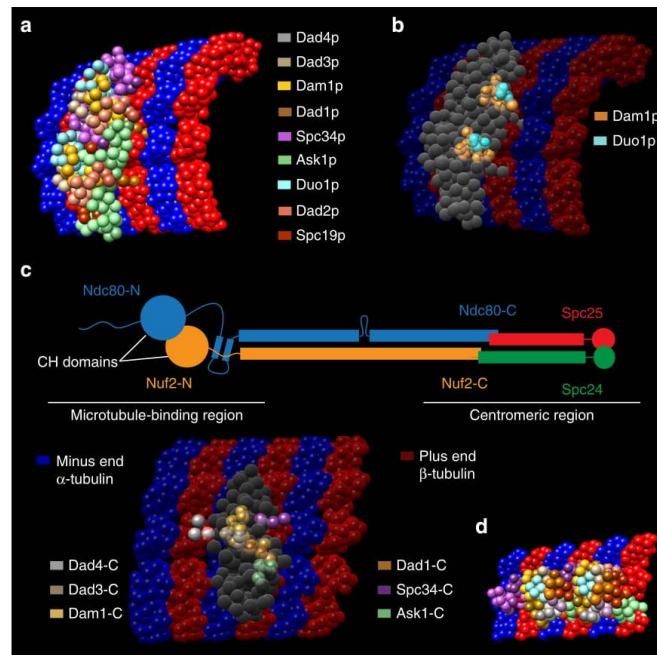


Figure 5 | Cross-link based flexible docking of the Dam1 complex model onto the MT specifies a preferred orientation. (a) The model of the Dam1 complex on the MT with beads coloured by protein (Hsk1p is not visible at this angle). (b) Only those beads found cross-linked to the MT are highlighted. (c) The orientation of the Dam1 complex on the MT with respect to the orientation of the Ndc80 complex. The C-terminal MT-binding region of the Ndc80 complex is nearer the minus end of the MT. Aravamudan *et al.*²⁹ measured FRET between N-Nuf2 and six Dam1 complex C termini²⁹. The proteins in the key to the left and right of the model represent these results, which indicates proximity between the C termini of Dam1p, Dad4p and Dad3p to the N terminus of Nuf2, while suggesting that the C termini of Spc34p, Ask1p and Dad1p are further away. Only a Dam1 complex monomer is shown for clarity. (d) Attempts to dock the model of the Dam1 complex in the minus MT conformation resulted in a poor fit and an orientation incompatible with a ring.

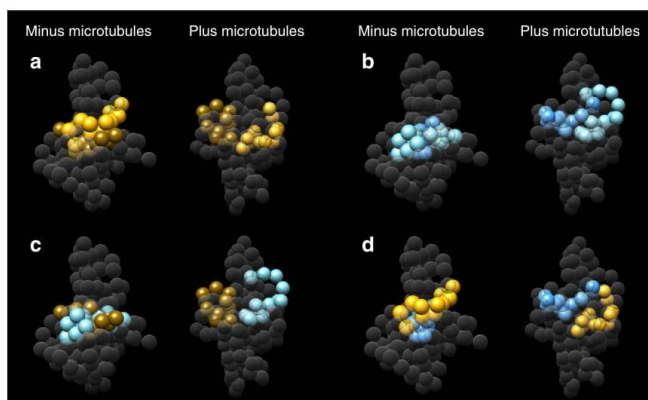


Figure 6 | The Dam1 complex MT-binding domain. Structural models show that the C-terminal regions of Dam1p and Duo1p interact with the N-terminal regions of Dam1p and Duo1p in the absence of MTs. On binding MTs, these interactions are lost, freeing the C-terminal regions of Dam1 and Duo1, and allowing them to bind the MT. **(a)** Dam1N (light yellow) to Dam1C (dark yellow); **(b)** Duo1N (turquoise) to Duo1C (blue); **(c)** Dam1C (dark yellow) to Duo1N (turquoise); **(d)** Dam1N (light yellow) to Duo1C (blue). The Dam1 complex is shown as a monomer.

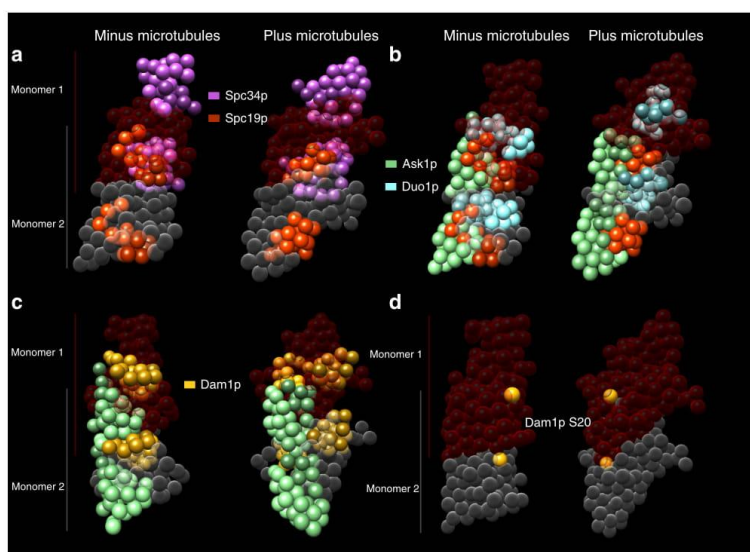


Figure 7 | The Dam1 complex to Dam1 complex interface. In all panels, the Dam1 complex is shown as a dimer, with one monomer in red and the other in grey. **(a)** The interface between the two monomers is formed by multiple interactions between Spc19p and Spc34p in both the presence and absence of MTs. **(b)** In the presence of MTs, Duo1p more than doubles its interactions across the interface by binding to Spc19p and Ask1p. **(c)** On binding to MTs, Dam1N gains interactions with Ask1p and Dam1M, and Dam1C lose interactions with Ask1N. **(d)** The Aurora B kinase phosphorylation site Dam1p S20 lies at the interface between the two Dam1 complex monomers. Dam1p S20 beads are coloured yellow.

presence of MTs and, conversely, this data set did not contain 356 that were observed in the absence of MTs (Fig. 2). Of the 136 MT-specific cross-links, only 43% are satisfied by the model for Dam1 complex in the absence of MTs (Fig. 2, left red arrow). The reverse is also true: only 46% of the 356 cross-links found exclusively in the absence of MTs are satisfied by the model for the Dam1 complex in the presence of MTs (right red arrow). Attempts to dock the model of the Dam1 complex made in the

absence of MTs onto a MT resulted in a poor fit and an orientation incompatible with a ring: just 29% of the observed Dam1 complex to tubulin cross-links were fit (Fig. 5d and Supplementary Table 11). These findings indicate that several interaction interfaces are strongly affected on MT binding and suggest that the Dam1 complex undergoes significant conformational changes (Supplementary Table 8 and Supplementary Fig. 3c).

To examine these conformational changes in more detail, we analysed the interactions at a coarse structural level. We divided each protein into 'macro-regions' of ~ 100 amino acid residues (Supplementary Table 12). For example, Ask1p was divided into

three macro-regions: Ask1N is the N-terminal 97 amino acids, Ask1M is the middle 97 amino acids and Ask1C is the C-terminal 98 amino acids. Proteins under 100 amino acids, such as Dad1p, Dad2p and so on consisted of just one macro-region: the whole

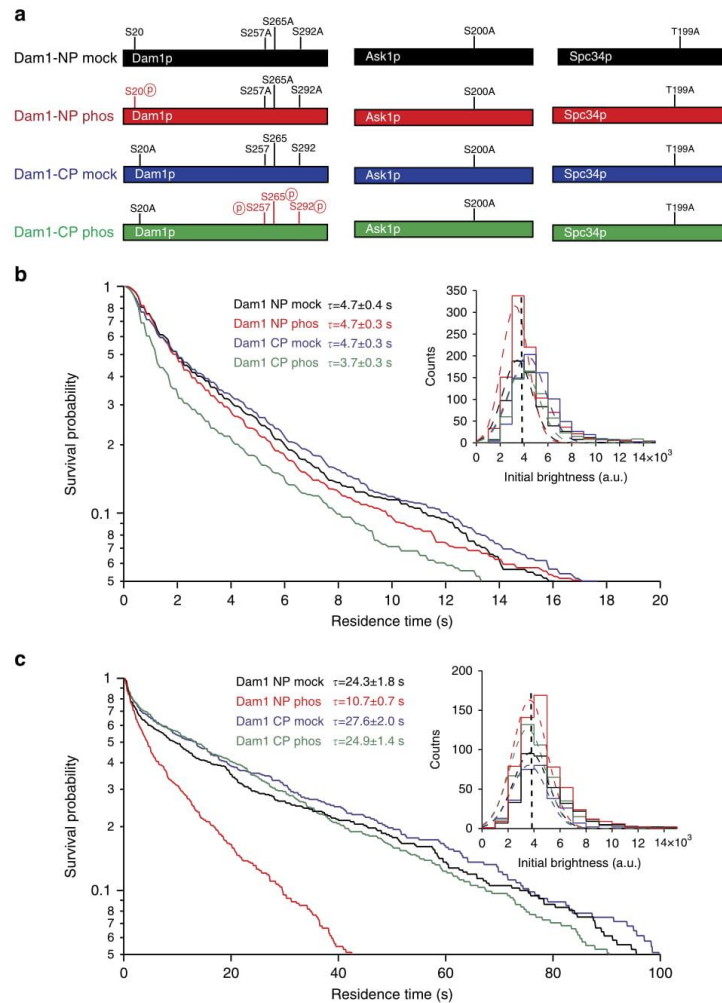


Figure 8 | Aurora B kinase (Ipl1) phosphorylation controls both MT binding and oligomerization of the Dam1 complex. (a) Schematic of phospho-blocking Dam1 complex constructs. Both constructs were phosphorylated by Ipl1 kinase in the presence of ATP. Mock-treated Dam1 complex had ATP substituted with distilled water. (b) Survival curve summary of single-molecule Dam1-GFP TIRF experiments. Dam1 NP mock ($n = 638$), Dam1 NP phos ($n = 1009$), Dam1 CP mock ($n = 777$) and Dam1 CP phos ($n = 646$) were each incubated at 40 pM for single-molecule Dam1-GFP complex imaging. Inset: GFP fluorescence (A.U.) distributions for Dam1 NP mock ($3,500 \pm 1,200$), Dam1 NP Phos ($3,300 \pm 1,100$), Dam1 CP mock ($4,300 \pm 1,500$) and Dam1 CP phos ($3,900 \pm 1,400$). (c) Survival curve summary of tracer Dam1-GFP TIRF experiments. GFP-tagged Dam1 NP mock (20 pM; $n = 360$), Dam1 NP phos ($n = 587$), Dam1 CP mock ($n = 294$) and Dam1 CP phos ($n = 454$) constructs were incubated with 2 nM corresponding non-tagged Dam1 constructs. Inset: GFP fluorescence (A.U.) distributions for Dam1 NP mock ($3,700 \pm 1,300$), Dam1 NP phos ($3,700 \pm 1,300$), Dam1 CP mock ($3,600 \pm 1,400$) and Dam1 CP phos ($3,500 \pm 1,400$) in tracer experiments. Inset panels show initial brightness of Dam1-GFP complex are similar across the whole TIRF data set, indicating that all data are from imaging single molecules of Dam1-GFP complexes either alone or incorporated into oligomers of unlabelled Dam1 complexes (in the tracer experiments). Vertical dashed line in inset panels represents the average height of single-step photobleach events under identical conditions ($3,900 \pm 1,900$ A.U., $n = 287$). Average single-step photobleach duration under identical conditions is 130.9 ± 5.7 s ($n = 287$). All errors are s.d. of the mean.

protein. We then compared the interactions of each macro-region in the presence and absence of MTs (Supplementary Table 13). A striking difference is seen for Dam1C and Duo1C. In the absence of MTs, Dam1C and Duo1C interact with Dam1N and Duo1N. When bound to MTs, these interactions are not present (Fig. 6 and Supplementary Table 13, grey highlighting). Instead, Dam1C and Duo1C move to the surface of the complex and bind to the MT (Supplementary Movie 4).

Binding to MTs also coincides with rearrangement of the interface between monomers of the Dam1 complex. As the Dam1 complex exists primarily as a dimer in solution, interactions between proteins within the complex can be within a monomer or between the monomers that make up the dimer. Both in the presence and absence of MTs, the interface between monomers is coordinated largely by Spc34p, which interacts with Spc19p (~100 interactions; Supplementary Table 13, blue highlighting, Fig. 7a and Supplementary Movie 5), as well as with Dad2p and Dad3p (Supplementary Table 13, lilac highlighting). However, in the presence of MTs, Duo1p more than doubles its interactions across the interface by binding to Spc19p and Ask1p from the other monomer (Fig. 7b and Supplementary Movie 5). Dam1p interactions are also extensively remodelled in the presence of MTs. Dam1M and Dam1C both lose interactions with Ask1N in the other monomer, whereas Dam1N gains interactions with Ask1p (Fig. 7c, Supplementary Movie 5 and Supplementary Table 13, green highlighting). We hypothesize that these interactions, which cross the interface between monomers of the Dam1 complex and are remodelled in the presence of MTs, participate directly in the assembly and regulation of oligomeric rings.

Aurora B kinase regulates Dam1 complex oligomerization. The models presented here make testable predictions about the MT-binding and oligomerization interfaces of the Dam1 complex. In our models, the Dam1p S20 Aurora B phosphorylation site lies at the interface between the two Dam1 complex monomers (Fig. 7d and Supplementary Movie 5). Thus, we hypothesized that phosphorylation of Dam1p at S20 should specifically affect oligomerization of the complex rather than the interface between Dam1p and the MT. By contrast, phosphorylation of the three C-terminal sites of Dam1p should regulate the interface with the MT. We tested these predictions by measuring the binding of Dam1 complexes to MTs, both at the single-molecule level and when assembled into oligomers. First, we constructed two phospho-blocking mutants of the Dam1 complex (Fig. 8a): (1) 'Dam1 CP', in which only the Dam1p C-terminal sites can be phosphorylated (Dam1p S20A, Ask1p S200A and Spc34p T199A) and (2) 'Dam1 NP', in which only the Dam1p N-terminal S20 site can be phosphorylated (Dam1p S257A, S265A, S292A, Ask1p S200A, Spc34p and T199A). We used total internal reflection fluorescence (TIRF) microscopy to test the effects of phosphorylation on MT binding at the single-molecule level (40 pM Dam1-GFP)⁹. Consistent with our model, phosphorylation of the three C-terminal sites of Dam1p (Dam1 CP) decreased the residence time of single Dam1 complexes on MTs by 20%, whereas phosphorylation of the N-terminal S20 site (Dam1 NP) had no effect (Fig. 8b). To measure the effects of phosphorylation on oligomerization, we performed the TIRF assay at concentrations that support the formation of oligomeric rings (2 nM total Dam1 complex) wherein small 'tracer' quantities of green fluorescent protein (GFP)-tagged Dam1 complex (1:100) were included to measure the behaviour of individual oligomeric particles, as previously described⁹. Relative to single molecules, the average residence time of non-phosphorylated Dam1-GFP complexes increased >5-fold,

indicating their incorporation into oligomers of untagged Dam1 complexes (Fig. 8c). Tracer Dam1 CP complexes similarly exhibited increased residence time in the presence of excess untagged Dam1 CP complexes, indicating that phosphorylation of the C-terminal sites of Dam1p does not block oligomerization. However, Dam1 NP was defective in oligomerization, as indicated by a residence time increased by only twofold. These results confirm the predictions based on our model that phosphorylation of the Dam1p N terminus inhibits the ability of the Dam1 complex to form oligomers, whereas phosphorylation of the C-terminal sites regulates the interface with the MT.

Discussion

Chemical cross-linking/MS has great potential for rapidly generating structural information and accurately defining large protein interaction networks³⁰. Great progress in the cross-linking field has been made over the past few years^{22,25,26,31,32} and recent integrative work has begun to make use of such data^{33,34}. The degree of success is dependent on the yield and accuracy of the data obtained. Given a large data set, such as presented here, the utility of the data can be dramatically increased through integrative structural modelling. This approach allowed us to combine cross-linking data with previously published EM and biochemical data, to determine the molecular architecture of the Dam1 complex. We further show that this approach produces biologically important structural models, allowing the formulation of clear and testable predictions.

Our data show that the C-terminal domains of Dam1p and Duo1p, exclusively, are cross-linked to the MT. Structural modelling shows these regions form a single compact domain in both the presence and absence of MTs. When bound to MTs, the complex adopts a distinct structural arrangement in which the C-terminal domains of Dam1p and Duo1p move to the surface of the complex and interact with the MT. A recent FRET study on the Dam1 complex in living cells placed the C-terminal region of Dam1p proximal to the MT²⁹; however, two sets of EM studies conflict as to whether the C-terminal region of Dam1p lies proximal¹⁰ or distal³ to the MT. They differ in how the dimer Dam1 complex is docked into the EM reconstructions of the rings. As a C-terminal truncation of Dam1p (Δ C-Dam1) caused loss of a protrusion domain that was also missing in the reconstruction of the rings, Wang *et al.*¹⁰ concluded that the C-terminus of Dam1p was facing the MT. This required that dimer Dam1 complex undergo a conformational change to form a ring. Ramey and coworkers³ later proposed an alternative fit of the dimer Dam1 complex into the ring structure: one that did not require a conformational change for a good fit to be made but had the N-terminal domain of Dam1p facing the MT.

Our results strongly argue that the EM densities facing towards the MT are the Dam1p and Duo1p C-terminal regions in agreement with the first EM reconstruction¹⁰. We also observe that the conformation of the Dam1p complex changes on formation of rings around the MT. Finally, our data explain observations that deletion of (or phosphomimetic mutations in) the C-terminal domain of Dam1p decrease but do not abolish MT binding, as we show that the C-terminal region of Duo1p also interacts with the MT.

We used the cross-links observed between the Dam1 complex and tubulin, to fit our model of the Dam1 complex dimer onto a MT. During the docking procedure, Dam1 complex dimers were initialized in random orientations and thus sampled orientations incompatible with a ring (that is, parallel to the MT protofilaments). Despite this, the single preferred orientation determined

was perpendicular to the MT and thus compatible with ring formation. Previous work found no evidence of a preferred orientation of Dam1 rings for facing either the plus or minus end of the MTs *in vitro*¹⁴. In contrast, our model predicts that the Dam1 complex does have a preferred orientation. Although our model is in agreement with previously published *in-vivo* FRET data²⁹, the novel structural information presented in this paper lends weight to a different, but not contradictory, interpretation. As N-Nuf2 contains the MT-binding calponin homology domain, Aravamudan *et al.*²⁹ hypothesized that proteins showing FRET with N-Nuf2 are proximal to the MT lattice. By the same token, a lack of such FRET would indicate a protein's localization distal to the MT lattice. Although this may be true, a protein facing the minus end of the MT would have closer proximity with N-Nuf2 than a protein facing the plus end, as the Ndc80 complex is aligned along the MT with the calponin homology domains towards the minus end³⁵ (Fig. 5c). The proteins that Aravamudan *et al.*²⁹ observed to have FRET with N-Nuf2 are also those that face the minus end in our model, that is, Dad4-C and Dad3-C. Moreover, the proteins that do not FRET with N-Nuf2 (Dad1-C, Spc34-C and Ask1-C) face the plus end of the MT in our model (Fig. 5c). Dam1-C takes a more central position in the complex proximal to the MT lattice. The agreement between our model and the FRET data is important, as it increases confidence in both independent studies.

Dam1p and Duo1p cross-link to multiple residues on the MT as does human Skl1, which is structurally unrelated but proposed to be functionally analogous to the Dam1 complex³¹. Similar to the Dam1 complex, the Skl1 complex forms oligomers³⁶ and is regulated by Aurora B kinase³⁷. Both the Dam1 complex and the Skl1 complex cross-link extensively to residues on the outside of the MT, although the interfaces do not obviously overlap. Whereas the majority of the cross-links between the Dam1 complex and the MT occur with α -tubulin (Supplementary Table 9), the majority of the Skl1 cross-links to the MT occur with β -tubulin³¹. Further work is necessary to resolve the significance of these differences.

The position of the Dam1 protein within the complex as presented in this work have important ramifications on the regulation of kinetochore attachment by Aurora B kinase. *In vivo*, mutation of the three C-terminal phosphosites in Dam1p confers mild defects such as sensitivity to benomyl, a MT depolymerizing drug, and synthetic lethality with mutations in *IPL1* (ref. 20). Here we find that phosphorylation of the C-terminal sites of Dam1p also has mild effects and decreases the affinity of the Dam1 complex for MTs, but only under conditions where the complex is a monomer. The same phosphorylation does not detectably affect the MT-binding affinity of the complex under oligomeric conditions, probably because the loss of affinity for the MT is largely overcome by the cooperativity of forming rings. Consistent with our results, the Dam1p C terminus is not required for Dam1 complex assembly nor for its accumulation at the MT tip^{14,18}.

We have previously shown that phosphorylating the Dam1p C terminus, Ask1 S200 and Spc34 T199 together fully disrupt the interaction between the Dam1 and Ndc80 complexes³⁸. We did not determine which phosphorylation site is responsible for disrupting this interaction, leaving the possibility that Dam1p C terminus may interact with the Ndc80 complex. The Dam1p C terminus was recently shown to interact with the Ndc80 complex through a yeast two-hybrid assay¹⁸. As phosphorylating the Dam1p C terminus mildly decreases the Dam1 complex's affinity to MTs, it is likely to be that this domain contains multiple functions that can be deciphered by further investigation.

Our previous work showed that oligomerization of the Dam1 complex is critically important to support stable bipolar

attachment of sister chromatids to the mitotic spindle⁹. This is probably caused by a requirement for oligomerization of the Dam1 complex to couple cargo to disassembling MTs under tension. Oligomerization seems like an obvious target for stringent regulation of kinetochore attachments, but which site(s) was responsible for this regulation was unknown. Previous work showed that individual phospho-site mutations in the Dam1 complex have mild phenotypic consequences. However, several different phospho-site mutations confer severe phenotypes when combined with Dam1p S20A, suggesting that S20 is an important regulatory site. We previously showed that phosphorylation of Dam1p S20 reduces the apparent affinity for MTs but could not distinguish between an effect on affinity, cooperativity or both (see Supplementary Note 1). The new results presented here unite these observations. Our model of the molecular architecture of the Dam1 complex places S20 of Dam1p right at the interface between two monomers (far from the MT) and thus strongly predicts that phosphorylation of S20 should inhibit oligomerization. We show here that S20 phosphorylation has no effect on the direct interaction with the MT but a pronounced effect on oligomerization. Considering the importance of oligomerization for maintaining attachments to dynamic MTs, this presents a mechanism by which Aurora kinase B could potentially detach kinetochores by phosphorylation at a single site.

Methods

Reagents. Disuccinimidyl suberate (DSS) was from Pierce (Rockford, IL). Trypsin was from Promega (Madison, WI). Other reagents were from Sigma-Aldrich.

Reference protein sequences and structures. Distance restraint location numbering is based on amino acid sequences listed in Supplementary Table 14. Reference structures for the proteins analysed were as follows: 1JM7.pdb (model 1) for BRCA1/BARD1 and 4I6I.pdb for SCF^{FBXL3}. The MT structure was previously published^{39,40} as was the pseudo-atomic structure of the γ -TuSC³⁹. For the Dam1 complex, EMDataBank file 1,372 was used. Structures generated in the study are presented in RMF format²¹ and are viewable in version 1.10.1 or higher of the UCSF Chimera⁴¹. Figures and movies based on these structures were made using Chimera.

Protein expression and purification. γ -TuSC was expressed in Sf9 cells and purified using published methods^{42,43}. Wild-type Spc97p and Spc98p plus Tub4p with an N-terminal 6 $\times$ His tag and tobacco etch virus cleavage site were used to produce γ -TuSC. Cell pellets from Sf9 cells grown in shaking flasks were lysed in HB101 lysis buffer: 40 mM HEPES, pH 7.5, 100 mM NaCl, 25 mM imidazole, 1% NP-40, 1 mM GTP, 0.1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride. Complete mini-EDTA-free protease inhibitor cocktail tablets were added to the lysis buffer (Roche Applied Sciences, Indianapolis, IN). Purification was by nickel affinity using Bio-Scale Mini Profinity IMAC Cartridges (Bio-Rad, Hercules, CA) followed by anion exchange on a Mono Q 5/50 GL column (GE Healthcare Biosciences, Pittsburgh, PA). Peak fractions from the Mono Q eluted in 500 mM NaCl and were combined and stored at -80°C in 40 mM HEPES, pH 7.5, 500 mM NaCl, 0.1 mM EGTA and 10% glycerol without further processing. Final γ -TuSC concentration was $\sim 1\text{ mg ml}^{-1}$. All ten Dam1 complex subunits were expressed in *Escherichia coli* (BL21 Rosetta; Novagen, Madison, WI) from a single polycistronic vector⁴⁴. The complex was affinity purified using a C-terminal His6 $\times$ tag on Spc34p. The eluate was purified on a size-exclusion column (Superdex 200 10/300GL; Amersham Biosciences, Piscataway, NJ), equilibrated with 20 mM phosphate at pH 7.0, 500 mM NaCl. SCF^{FBXL3} ubiquitin ligase complex was a gift from Xing *et al.*⁴⁵ BRCA1/BARD1 RING-domain heterodimer was a gift from Kleit and colleagues⁴⁶.

Cross-linking. For γ -TuSC, 146 μg protein was diluted with HB500 buffer (40 mM HEPES, 100 mM NaCl, pH 8) to a final volume of 331.2 μl . Cross-linker concentration was brought to 0.86 mM by adding 14.5 mM DSS. The reaction was allowed to proceed for 2 min at 25°C before quenching with 26 μl 500 mM NH_4HCO_3 . For BRCA1/BARD1 complex, 40 μg protein was diluted with PBS buffer to a final volume of 100 μl . Cross-linker concentration was brought to 0.86 mM by adding 14.5 mM DSS. The reaction was allowed to proceed for 2 min at 25°C before quenching with 10 μl 500 mM NH_4HCO_3 . For SCF^{FBXL3} ubiquitin ligase complex, protein was first buffer exchanged into HB200 buffer (40 mM HEPES, pH 7.5, 100 mM NaCl) using protein desalting spin columns (Pierce). Ten per cent glycerol was added to the desalted protein. Twenty micrograms protein

was diluted with HB200 buffer to a final volume of 46.5 μ l. Cross-linker concentration was brought to 0.06 mM by adding 2.9 mM DSS. The reaction was quenched with 7 μ l 500 mM NH_4HCO_3 after 30 min at 25 °C. For Dam1 complex, 40 μ g protein in 93 μ l 500 mM NaCl 50 mM phosphate at pH 6.9 was brought to 0.48 mM DSS by adding 14.5 mM DSS. The reaction was quenched with 10 μ l of 500 mM NH_4HCO_3 after 2 min cross-linking at 25 °C. For Dam1 complex on MTs, 20 μ M tubulin was polymerized in BRB80 (80 mM PIPES, 1 mM MgCl_2 , 1 mM EGTA, adjusted pH to 6.9 using KOH) containing 1 mM GTP, 6 mM MgCl_2 , 3.8% dimethyl sulfoxide at 37 °C (total volume 100 μ l). After 30 min polymerization, 100 μ l BRB80 containing 10 μ M taxol was added. MTs were then pelleted in a TLA100 rotor at 58,000 r.p.m. (130,000g) for 10 min at 37 °C. The pellet was resuspended with 200 μ l warm BRB80 containing 10 μ M taxol. For cross-linking, 10 μ l MTs were mixed with 20 μ g Dam1 complex in 100 μ l BRB80 at 25 °C. The reaction was mixed by gentle flicking and was allowed to stand for 5 min. Three microlitres of 14.5 mM DSS in dimethyl sulfoxide was added and the mixture was allowed to cross-link for 2 min at 25 °C before quenching by addition of 10 μ l of 0.5 M NH_4HCO_3 . The quenched reaction was spun at 58,000 r.p.m. (130,000g) in a TLA100 rotor for 10 min at 37 °C and the resulting pellet was resuspended in 100 μ l ice-cold HB500 buffer. After quenching, all reactions (except Dam1 on MTs) were buffer exchanged to HB500 using protein desalting spin columns (Pierce).

Digestion. Cross-linked proteins were reduced with 10 mM dithiothreitol at 37 °C for 30 min, followed by 30 min alkylation at room temperature with 15 mM iodoacetamide. For Dam1 complex in the absence of MTs, 25% volume of heavy (H_2^{18}O) or light (unlabelled) water was added to the reactions before digestion with trypsin at a substrate-to-enzyme ratio of 60:1 overnight at room temperature, with shaking. Owing to a higher yield of cross-links obtained without isotopic labelling, only unlabelled reactions were performed for all other samples. Digested samples were acidified with 5 M HCl before being stored at -80 °C until analysis.

Mass spectrometry. MS was performed on a Q-Exactive or Q-Exactive HF (Thermo Fisher Scientific). Sample digest (1.5 μ g) was loaded by autosampler onto a 150- μ m Kasil fritted trap packed with Jupiter C12 90 Å material (Phenomenex) to a bed length of 2 cm at a flow rate of 2 μ l min $^{-1}$. After loading and desalting using a total volume of 8 μ l of 0.1% formic acid plus 2% acetonitrile, the trap was brought online with either a pulled fused-silica capillary tip (75- μ m i.d.) or an empty Pico-Frit column (New Objective) that was self-packed with 30 cm of Reprosil-Pur C18-AQ (3- μ m bead diameter, Dr Maisch) mounted in an in-house constructed microspray source and placed in line with a Waters Nanoacuity binary UPLC pump plus autosampler. Peptides were eluted off the column using a gradient of 2–35% acetonitrile in 0.1% formic acid over 120 min, followed by 35–60% acetonitrile over 10 min at a flow rate of 250 nl min $^{-1}$.

The Q-Exactive mass spectrometer was operated using data-dependent acquisition where a maximum of six tandem MS (MS/MS) spectra were acquired per MS spectrum (scan range of m/z 400–1,600). The resolution for MS and MS/MS was 70,000 and 35,000, respectively, at m/z 200. The automatic gain control targets for both MS and MS/MS were set to 1e6 and the maximum fill times were 20 and 128 ms, respectively. The MS/MS spectra were acquired using an isolation width of 2 m/z and a normalized collision energy of 25. The underfill ratio was set to 0.3%, which corresponded to the requirement that the precursor ion threshold intensity be above 2.3e4, to trigger an MS/MS acquisition. MS/MS acquisitions were prevented for +1, +2 or undefined precursor charge states. Dynamic exclusion (including all isotope peaks) was set for 10 s. The Q-Exactive HF was operated similarly, except that repeat analyses employed slight variations in MS/MS resolution (30,000 and 60,000) or automatic gain control targets (2e4 and 1e5).

Analysis of MS data. Mass spectra were converted into mzML using msconvert from ProteoWizard⁴⁷. All proteins detectable were identified by searching high-resolution MS/MS spectra against whole-proteome databases using Comet⁴⁸. Peptide identifications were processed with Percolator⁴⁹ and used to create a smaller protein database for cross-link searches. These smaller databases consisted of all proteins identified at 1% FDR and contained 114, 64, 105, 302 and 159 proteins, respectively, for the search databases for γ TuSC, BARD1/BRCA1, SCF^{FBXL3}, free Dam1 complex and Dam1 complex on MTs. MSDataP was used to visually inspect the Comet results⁵⁰. Cross-linked peptides were identified using the Kojak version 1.0 cross-link identification software²³ following the author's instructions (<http://www.kojak-ms.org>) and using the search parameters outlined in Supplementary Methods. Kojak results were exported to Percolator, to produce a statistically validated set of cross-linked peptide identifications at the desired FDR threshold (described in detail in Supplementary Methods). All distance restraint location numbering is based on the sequences in Supplementary Table 14.

A relational database, data processing software and a web application were developed to interrogate and visualize the data. All data presented in the current work are available at <http://prox.yeastrc.org/dam1-zelter-2015>. In addition, this web application makes it possible to rapidly explore every peptide and peptide spectrum match associated with every distance restraint reported in this study. Furthermore, it is possible to view the annotated spectrum for each peptide spectrum match through an integrated spectrum viewer. Multiple other features are

also available via this viewer to help the reader make a detailed exploration of our raw MS data and subsequent cross-link identifications.

Dam1 complex phosphorylation. Expression of GST-Ipl1 was from plasmid pSB196 (Sue Biggins, Fred Hutchinson Cancer Research Center, Seattle, WA) at 23 °C for 2 h. GST-Ipl1 was purified using GSTrap HP, according to the manufacturer's instructions (GE Healthcare Biosciences), except that the elution buffer was 50 mM, 250 mM KCl, 10 mM glutathione, pH 8.0. pSB503 (Sue Biggins) was used to express GST-Sli15 (residues 554–698) at 37 °C. GST-Sli15 was purified using glutathione-Sepharose 4B resin, following the manufacturer's instructions, except that the elution buffer was 20 mM Tris buffer 200 mM NaCl, 1 mM β -mercaptoethanol, 1 mM EDTA, 10 mM glutathione, pH 8.0.

Ipl1p phosphorylation of recombinant Dam1 complex was carried out in a 50- μ l reaction containing 4 μ M Dam1 complex, 0.5 μ M GST-Ipl1, 0.5 GST-Sli15 (residues 554–698), 200 mM NaCl, 10 mM ATP, 25 mM MgCl_2 and 50 mM HEPES buffer, pH 7.4. Reactions were incubated for 90 min at 30 °C. Glycerol was added (5% final) before snap-freezing and storing at -80 °C. The mock/non-phosphorylated controls were carried out by substituting distilled water for the ATP. Under these conditions, we achieve nearly stoichiometric phosphorylation of the complex².

TIRF microscopy. Flow chambers were constructed using glass slides and functionalized coverslips as reported before^{5,9,38}. Coverslip was adhered to a glass slide with double-sided tape, to form individual flow channels between two adjacent strips of tape. 'Rigor' kinesin was added to each channel to nonspecifically bind to the coverslip. This allowed for the addition and immobilization of taxol-stabilized MTs. Single-molecule imaging experiments were carried out by incubating 40 pM phosphorylated or mock-treated Dam1-GFP NP (Dam1p S257A, S265A, S292A, Ask1p S200A and Spc34p T199A) or Dam1-GFP CP (Dam1p S20A, Ask1p A200A and Spc34p T199A) with Alexa-647-labelled MTs. GFP and Alexa-647 fluorescence channels were simultaneously recorded using a custom TIRF imaging system⁵.

In 'tracer' assays, GFP-tagged and unlabelled versions of the mutant Dam1 complex were mixed to 1:100 ratio to a total concentration of 2 nM. Next, this solution was incubated with MTs. All TIRF assays were carried out in BRB80 in the presence of 0.8 mg ml $^{-1}$ α -casein, 50 mM KCl and an oxygen scavenger system (200 μ g ml $^{-1}$ glucose oxidase, 35 μ g ml $^{-1}$ catalase, 25 mM glucose and 5 mM dithiothreitol).

Single particle tracking and analysis was carried out with custom software (available on request) developed in Labview (National Instruments) and Igor Pro (Wavemetrics)^{9,38}. Mean residence times were carried out through bootstrapping analysis⁹. Each residence time data set was randomly resampled with replacement 1,000 times. All of the data sets presented formed normal distributions. Gaussian fits to these distributions yielded estimates of mean residence time and s.d.

Computational structural modelling. Analysis of the cross-linking data and structural modelling were carried out using IMP (<http://integrativemodelling.org/>)^{21,51}. IMP is an open source platform for integrative structure determination based on EM data, FRET spectroscopy, cross-linking/MS, small angle X-ray scattering profiles and various proteomics data^{33,34,52–58}.

Modelling the Dam1 complex in the absence of MTs. As no crystallographic data is available for any components of the Dam1 complex, we used a coarse-grained representation in which one spherical bead was used to represent ten residues. Total volume of beads was estimated from known masses of proteins and standard assumptions about their densities⁵⁹. Beads representing protein fragments adjacent in sequence were restrained by a harmonic function of the distance between their surfaces, with equilibrium value and spring constant equal to 0 Å and 1.0, respectively. An excluded volume restraint with intensity set at 1.0 was used to avoid steric clashes. As EM data are available only for Dam1 complex in dimeric form³, two identical copies of the complex were modelled. A symmetry constraint was used to lock the second copy in the symmetric position, which was determined from the EM map using UCSF Chimera's⁴¹ 'Fit Map to Map' tool.

Cross-linking data were encoded as upper-bound harmonic restraints acting on the distance between surfaces of the two beads that included the cross-linked residues. We used our cross-linking analysis of the SCF^{FBXL3} complex to determine an appropriate value for this upper bound was 5 Å. This value fits a similar proportion of cross-linking data as an atomic C α -C α cutoff of 21 Å (Supplementary Table 15). We chose the more restrictive value, because the restraint can be marginally exceeded during modelling. As we modelled two copies of the complex, each cross-linking data point can be explained by a pair of residues belonging either to the same copy of the Dam1 complex (intra-complex) or to two different copies (inter-complex). We did not arbitrarily decide whether a cross-link should be satisfied by inter- or intra-complex pairs of beads. Instead, for each model generated, we calculated both distances and then applied the restraint to the smallest. A total of 678 UDRs were used in the modelling (Supplementary Table 6).

As the termini of half of the components were localized by previous studies³, the EM map was manually segmented using UCSF Chimera and the positions of Ask1p, Spc19p, Duo1p, Dam1p and Spc34p were confined into bounding

boxes (Supplementary Fig. 4). The boxes used were purposefully larger than the EM localization data, to account for the location of whole proteins rather than the individual protein termini that were localized in the published study. We did not apply a hard constraint, but allowed each component to extend beyond its pre-assigned bounding box, to satisfy our cross-linking data. This was implemented by using harmonic restraints on the distance between the centre of each bead and the wall of the bounding box, acting only when a bead exited the box. In principle, each restrained Dam1 component can extend outside its bounding box by any given amount, provided that the violation of the restraint is balanced by the satisfaction of a sufficient number of cross-linking restraints. Deletion of the gene encoding Hsk3p causes the remaining nine components of the Dam1 complex to split into two subcomplexes: (A) Dam1p, Duo1p, Spc34p, Spc19p, Dad1p and Dad3p, and (B) Ask1p, Dad2p and Dad4p¹⁵. We therefore encoded a connectivity restraint among Ask1p, Dad2p and Dad4p. We ensured that each of the three proteins were in contact, that is, the distance between two beads was zero, with at least one of the other two. Sampling of the conformational space was carried out using a Monte Carlo algorithm coupled with the parallel tempering method⁶⁰. To accelerate diffusion in temperature, parallel tempering was carried out in the well-tempered ensemble⁶¹. Thirty-four replicas were distributed in a temperature range between 1.0 and 3.0 $k_B T$. Each bead was moved by a random translation of at most 1.0 Å. The initial positions of localized proteins were randomized inside the corresponding bounding box, those of the other components inside the union of all the bounding boxes. The top 1,000 scoring models obtained in 10^8 Monte Carlo steps were clustered using the Daura *et al.*⁶² clustering algorithm, with a cutoff of 1.7 nm. To visualize the precision of our modelling approach in determining bead positions, we constructed density maps, which quantify the probability that a given region of space is occupied by a bead in the top 1,000 scoring models. The space was discretized in bins of 2 Å size. Density maps of each bead were then visualized at half their maximum value.

The models obtained were validated by data jackknifing tests. The Dam1 complex was modelled using three different cross-linking data sets, which were constructed by randomly choosing 95% of the data points. The resulting models conserved 91, 88 and 92% of the interactions of the model built with the entire data set.

Modelling the Dam1 complex in the presence of MTs. To model the Dam1 complex in the presence of MTs, we used 458 UDRs (Supplementary Table 6) and applied the methods described above. At this stage, tubulin-to-Dam1 complex cross-links were not used in the modelling. The models obtained were validated by data jackknifing tests as before. The resulting models conserved 88, 87 and 91% of the interactions of the model built with the entire data set.

Determining Dam1 complex-MT orientation. To identify the orientation of Dam1 complex on the MT, we used a flexible-docking procedure onto a coarse-grained (ten residues per bead) structural model of the MT based on atomic model of the MT^{39,40}. A 6×6 lattice of α - and β -tubulin subunits was constructed. We added 21 Dam1 complex to MT cross-links (Supplementary Table 10) encoded as distance restraints acting on both Dam1 complex monomers and on the closest bead belonging to all α - and β -tubulin subunits. The 20 α - and β -tubulin subunits at the edge of the 6×6 lattice were excluded from the restraints calculation. All the other restraints used for the modelling of the Dam1 complex alone were maintained.

The flexible-docking procedure first positioned on the MT the Dam1 complex previously obtained, while optimizing positions of each bead. The coarse-grained tubulin structure was initialized in a random position and orientation 100 nm from the centre of the Dam1 complex. In a second stage, the relative orientation of the two Dam1 monomers was optimized by removing the symmetry constraints from the EM map. In this study, we discussed only the initial model of the Dam1 complex structurally aligned to the final model from the flexible-docking procedure, as it fit the data equally well. A similar approach was used to position the model of the Dam1 complex built with the cross-linking data collected in absence of MT on the MT.

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Author contributions

A.Z., N.T.U., M.B., J.K. and T.N.D. conceived the experiments. A.Z. and N.T.U. performed the cross-linking experiments. R.J. and A.Z. carried out the MS experiments. J.K. did the TIRF experiments and associated analysis. A.Z., M.R.H., R.J. and M.R. performed the MS data analysis. M.B. performed the computational modelling. M.R. and D.J. constructed the website for the cross-linking data. A.Z., M.B., J.K., N.U. and T.N.D. co-wrote the manuscript. R.M. and M.M. provided conceptual advice and assisted in the preparation of the manuscript. All authors discussed the results and commented on the manuscript.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

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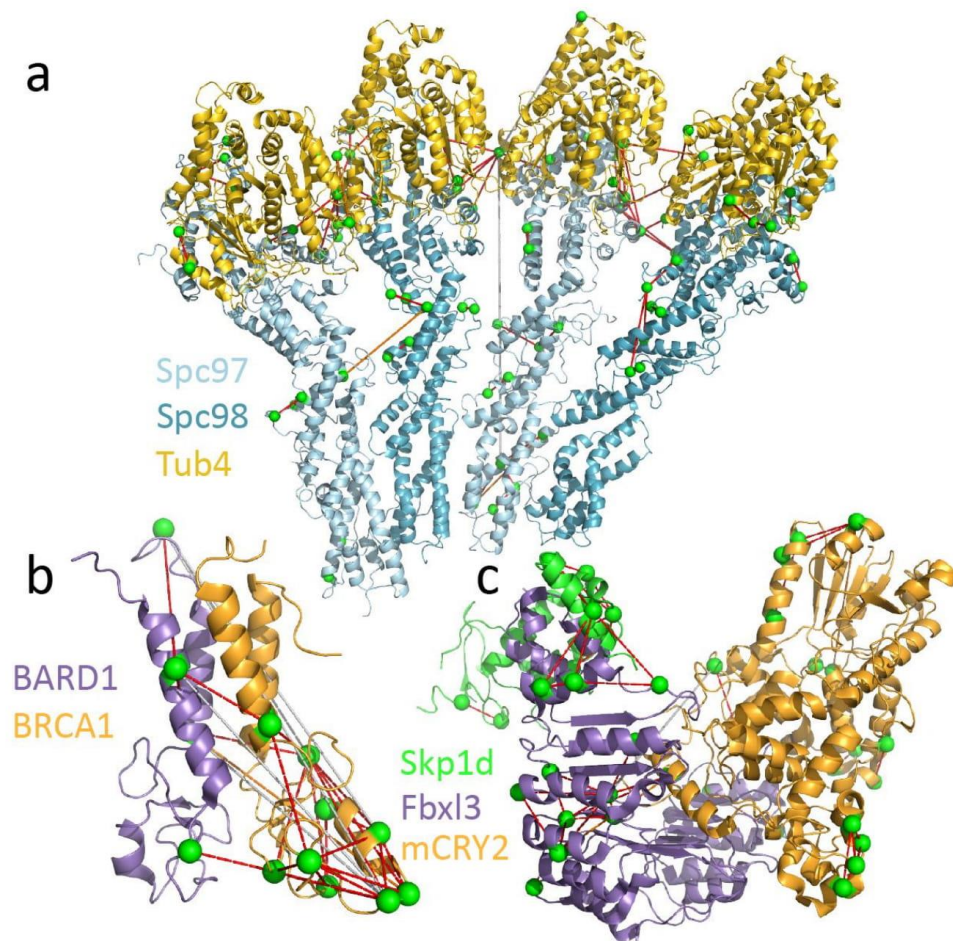
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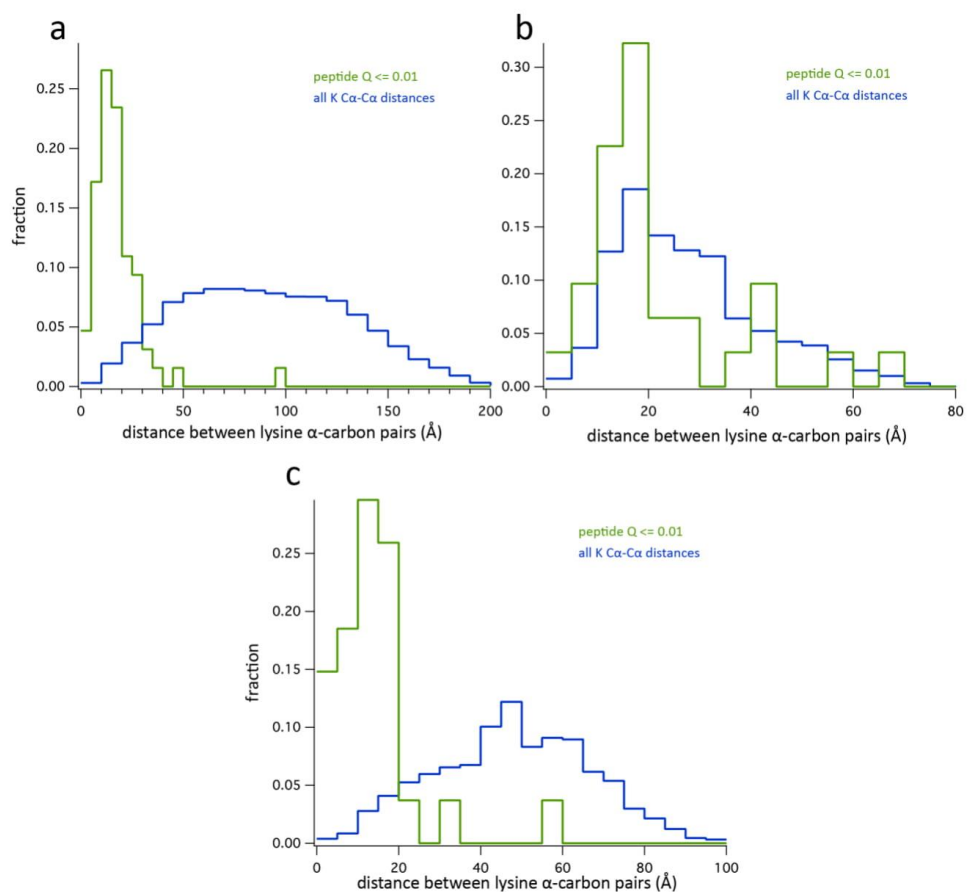
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Supplementary Information:

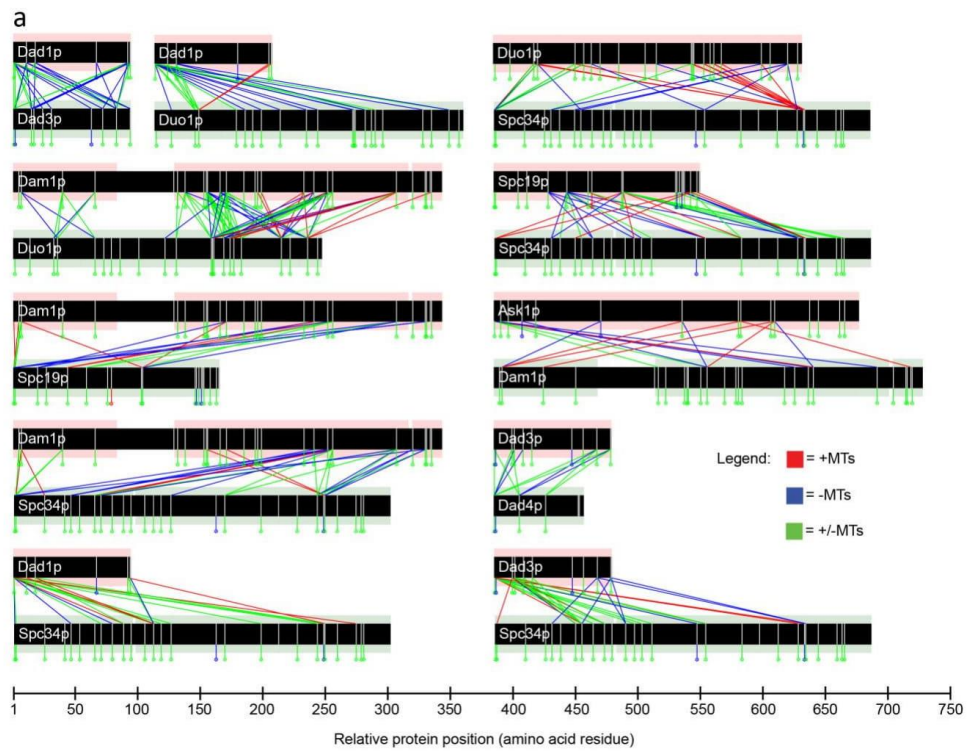
Supplementary Figures

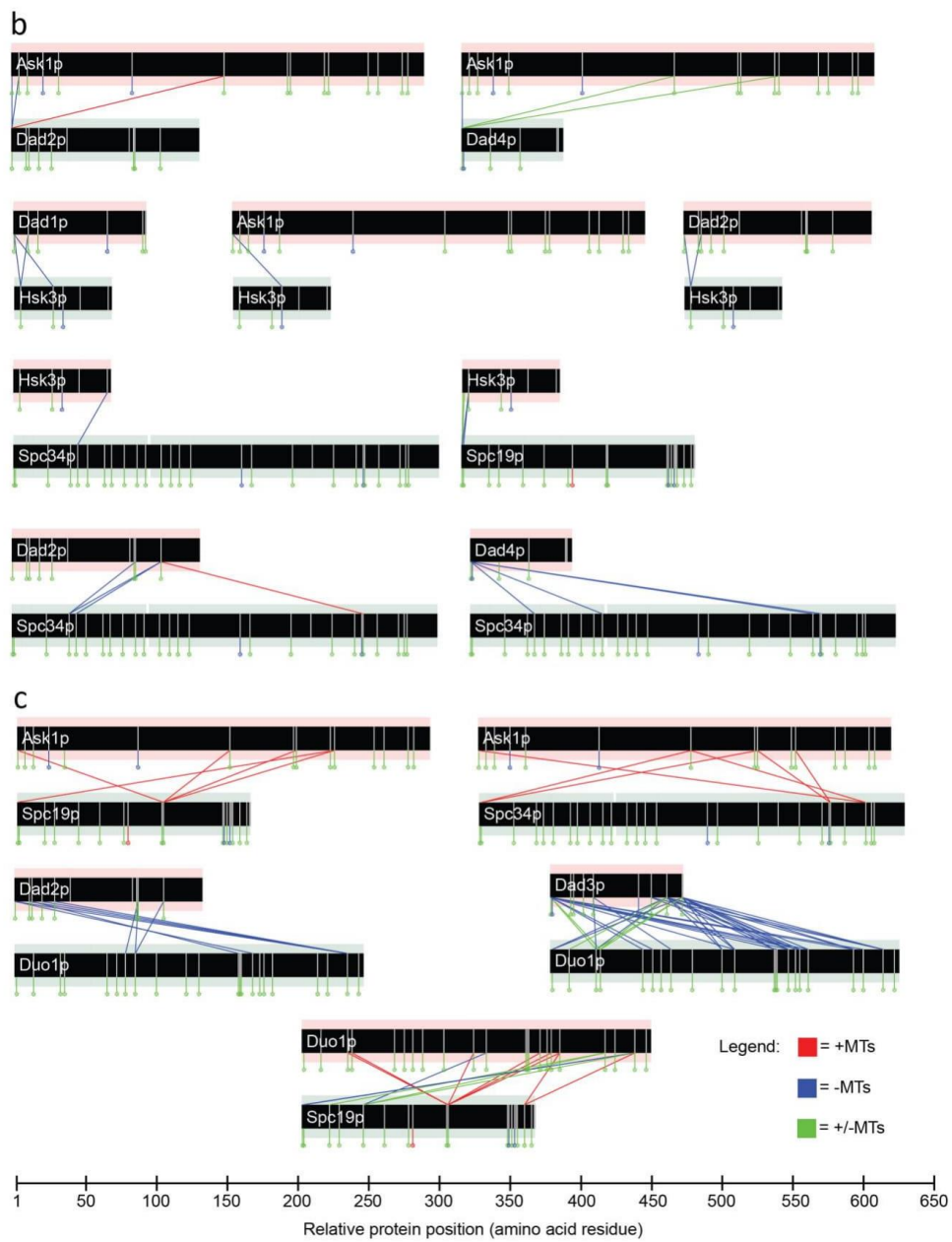


Supplementary Figure 1: Cross-linking data plotted on available structures. (a) Pseudo-atomic structure of the γ -TuSC; (b) Atomic structure of the BRCA1/BARD1 heterodimer; (c) Atomic structure of the SCF^{FbxL3} complex. Lines representing cross-links and loop-links are: red (C α -C α distance <30 Å); orange (C α -C α distance 30 to 40 Å); and grey (C α -C α distance >40 Å). Cross-linked lysine α -carbons are depicted as green spheres. Data is shown for peptides with Percolator¹ assigned q values ≤ 0.01 .

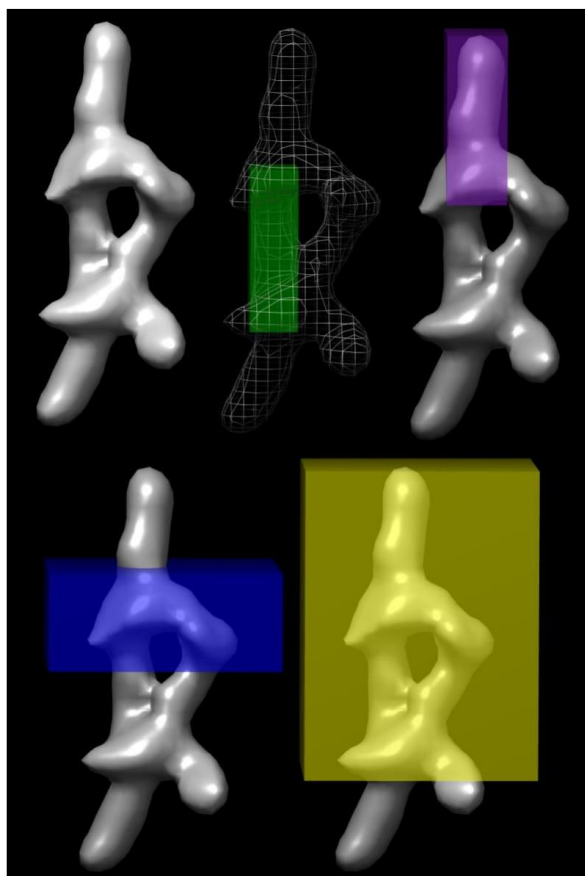


Supplementary Figure 2: Cross-linking method benchmarking. Histograms of lysine Cα-Cα distances observed cross-linked together versus all possible pairs of lysine Cα-Cα distances based on atomic structures. Data is shown for peptides with Percolator¹ assigned q values ≤ 0.01 . (a) γ -TuSC; (b) BRCA1/BARD1; and (c) SCF^{FBXL3}. Hits to peptides not present in reference PDBs are not shown.





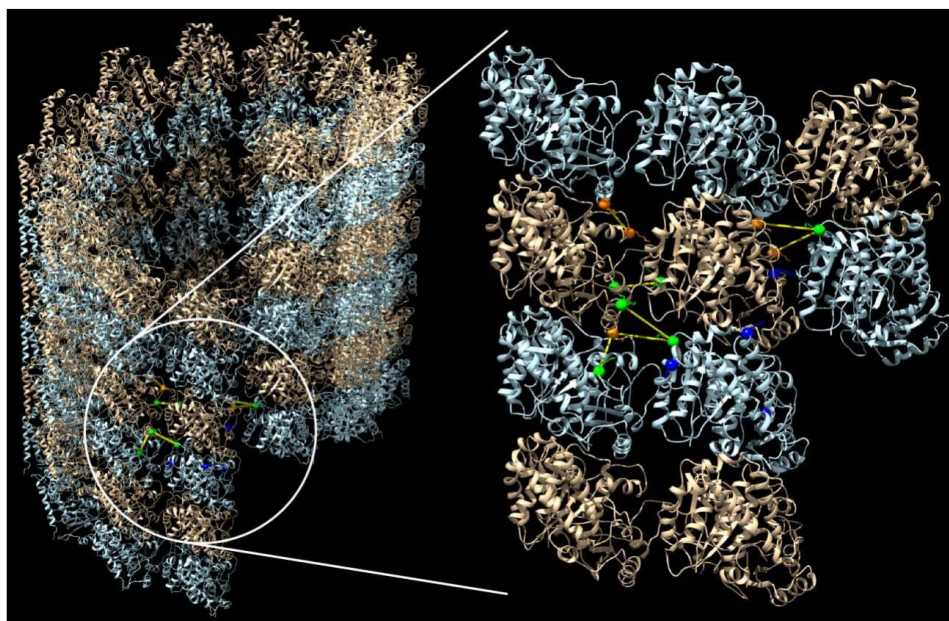
Supplementary Figure 3: Dam1 complex cross-links observed in the presence and absence of microtubules. (a) Protein pairs with multiple cross-links detected in the presence and absence of microtubules. (b) Protein pairs exhibiting minimal cross-links. (c) Protein pairs showing different cross-links in the presence and absence of microtubules. Peptide sequence coverage (colored boxes), mono-links (colored vertical lines and circles) and lysines (vertical white lines) are also shown. Red lines represent cross-links found only in the presence of microtubules. Blue lines represent cross-links found only in the absence of microtubules. Green lines represent cross-links found both in the presence and absence of microtubules. Data is shown for peptides with Percolator¹ assigned q values ≤ 0.01 .



Supplementary Figure 4: Previously published EM localization data was converted into bounding boxes, which were used as restraints during modeling. For orientation, the Dam1 EM structure (EMDB #1373) is shown top left in grey (not used in the modeling). Ask1p/Spc19p (green), Dam1p/Duo1p (blue), Spc34p (purple) and “no localization” (gold) boxes are shown. Boxes apply to a single Dam1 monomer (the upper portion of the structure depicted). The boxes used were purposefully larger than the EM localization data to account for the location of whole proteins rather than the individual protein termini that were localized in the published study. Models run using localization data made use of the blue, green and purple boxes to constrain their respective proteins. All other proteins were constrained to the gold box. We did not apply a hard constraint, but allowed each component to extend beyond its pre-assigned bounding box to satisfy our cross-linking data. This was implemented by using harmonic restraints on the distance between the center of each bead and the wall of the bounding box, acting only when a bead exited the box. In principle, each restrained Dam1 component can extend outside its bounding box by any given amount, provided that the violation of the bounding box restraint is balanced by the satisfaction of a sufficient number of cross-linking restraints.



Supplementary Figure 5: Negative stain EM image of Dam1 complex cross-linked to microtubules.
Scale bar is 100 nm.



Supplementary Figure 6: Pseudo-atomic structure of a microtubule with tubulin to tubulin cross-links depicted in yellow. All cross-linked C α -C α distance are less than 30 Å. Cross-linked lysine α -carbons are depicted as spheres. Green spheres were observed in tubulin to tubulin cross-links only. Blue spheres were observed in tubulin to Dam1 complex cross-links only. Orange spheres were observed in both tubulin to tubulin cross-links and tubulin to Dam1 complex cross-links. Alpha-tubulin is colored light blue. Beta-tubulin is colored copper. Data is shown for peptides with Percolator¹ assigned q values $\leq$ 0.01.