Kinase Cdk1 and kinesin Kar3 regulate the Dam1 complex in its role to strengthen kinetochore-microtubule attachment

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Abstract

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Cell division is successful when each new daughter cell ends up with the correct complement of DNA. To obtain this outcome, a macromolecular protein complex known as the kinetochore is required to connect chromosomes to microtubules. The inner portion of the kinetochore binds to chromosomes at a region known as the centromere while the outer structure of the kinetochore fastens onto the microtubule. Sustained attachments of kinetochores to microtubules for accurate chromosome segregation depends on a kinetochore

component, the ten-member Dam1 complex (Dam1c), in budding yeast. The Dam1c oligomerizes to encircle the microtubule and can harness the forces generated by shortening microtubules to power chromosome segregation. Previous work found that Dam1c undergoes phosphor-regulation, in which phosphorylation by the Ipl1 kinase (Aurora-B) predominantly destabilizes erroneous kinetochore-microtubule interactions, a critical step to ensure proper segregation of genetic material.

I, along with collaborators, used an optical trap-based assay to test purified native kinetochores, or its subcomplexes, from yeast or from bacterially expressed recombinant protein to examine attachment strength of these various protein complexes to microtubules. Surprisingly, I found that kinetochore-microtubule coupling is strengthened when the native Dam1c is phosphorylated. The kinase responsible for the increased in strength is Cdk1 and the key substrate is the Ask1 component of the Dam1c.

To determine how Cdk1-mediated phosphorylation strengthens attachments I tested the ability of Dam1c to: (1) bind kinetochores, (2) bind microtubules, and (3) form higher order oligomeric rings. This revealed that Cdk1 phosphorylation had no influence in microtubule binding or enhance Dam1c retention at the kinetochore, but it did enhance Dam1c function in oligomerizing around microtubules ensuring stronger kinetochore-microtubule attachments.

In addition, preliminary data suggests nuclear motor proteins, Cin8 and Kar3, have a role in kinetochore-microtubule coupling. To determine how motor proteins strengthen kinetochore-microtubule attachment, I tested whether Dam1c localization at the kinetochores required motor proteins. Indeed, we found that Kar3 had a role in retaining Dam1c at the kinetochore.

Together, my data has identified additional regulatory mechanisms that tune kinetochore-microtubule attachment strength.

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Contents

1. The cell cycle, kinetochores, microtubules, and microtubule binding proteins

1.1. Cell cycle and Cdk1

A single-cell microorganism such as yeast or a complex multi-cellular organism such as a human have noticeable differences, yet on a fundamental level both systems can exist because of their success in undergoing cell division. A human is estimated to be composed of about 37 trillion cells all of which began from a fertilized egg that underwent continuous rounds of cell division (Bianconi et al., 2013). For cell duplication to occur kinases, enzymes that phosphorylate substrates, are needed to initiate and proceed through the cell cycle. In humans, a critical component for this function is the cyclin dependent kinase (Cdk). About half of the 20 Cdks in humans have a role in the cell cycle; Cdk1 is distinguished as it can be sufficient for mammalian cells to divide, yet not enough for an organism to survive (Malumbres, 2014; Santamaría et al., 2007). Discovery as to how the cell cycle functions arose from utilizing model organism *Saccharomyces cerevisiae* or budding yeast (Sue Biggins, 2015). In *S cerevisiae* there are a total of 6 Cdks, which includes one essential kinase whose role expands throughout the cell cycle, the ortholog of Cdk1, Cdc28 (Mendenhall & Hodge, 1998). Yeast Cdc28 will be referred to as Cdk1 unless otherwise stated.

Cdk1 phosphorylates serine/threonine consensus sites (S/T-P-X-K/R) and is dependent on cyclin for its activity (Mendenhall & Hodge, 1998). As the descriptive name suggests, cyclins are cyclical as the cells expresses them and then targets them for degradation. Cdk1's association with different oscillating cyclins at different phases of the cell cycle govern its various functions

in accomplishing cell duplication (Voet, Lorson, Srinivasan, Bennett, & Heuvel, 2009). If Cdk1 was compromised, then the cell would remain stuck at a cell cycle phase.

To get the cells growing in G1 at least one of the three G1 cyclins; Cln1, Cln2, or Cln3, is required to activate Cdk1 for the cell to progress to the next phase (Cross, 1990; Richardson, Wittenberg, Cross, & Reed, 1989). In S phase, a stage important for duplicating chromosomes, in order for Cdk1 to be effective it is required to interact with Clb5 and Clb6 (J. Bloom & Cross, 2007; Schwob & Nasmyth, 1993; Toone, Aerne, Morgan, & Johnston, 1997). To initiate the process of chromosomal segregation, B-type cyclins Clb1-4 play an important role in G2/M-phase mitotic entry and mitotic exit (Angelika Amon, Tyers, Futcher, & Nasmyth, 1993; Fitch et al., 1992; Ghiara et al., 1991; Schwab, Lutum, & Seufert, 1997; U. Surana et al., 1993; Uttam Surana et al., 1991). There are redundancies among the mitotic cyclins and this is indicative of ensuring Cdk1 is operational and regulating the cell cycle.

1.2. Kinetochore

Completion of the cell cycle makes two genetically identical daughter cells. The movement of chromosomes into the newly formed cells is made possible with its recognizable region known as the centromere, which functions as an essential platform to erect yeast's ~70 protein kinetochore structure (K. Bloom, 2015; Cheeseman & Desai, 2008; Lampert & Westermann, 2011; Yamagishi, Sakuno, Goto, & Watanabe, 2014). It is the macromolecular kinetochore that is responsible for bridging the genetic material to microtubules, which is part of spindle apparatus (Asbury, 2017; Cheeseman, 2014; Scholey, Civelekoglu-Scholey, & Brust-Mascher, 2016).

In yeast, the centromere sequence is 125 base pairs, a fraction when compared to a human centromere's megabases, and it coils around histones containing an H3 variant called Cse4. It is this variant that forms a distinct chromatin region that marks where the kinetochore will locate and function. Within this short region, the point centromere, there are three identifiable gene sequences known as the centromere-determining elements (CDEI, CDEII, and CDEIII). CDEIII has an essential function in binding to the CBF3 complex that is composed of four proteins: Ndc10, Cep3, Ctf13, and Skp1 (Sue Biggins, 2013). CBF3 plays an important role in stabilizing point centromeres and allows for the successful recruitment of an array of inner kinetochores proteins termed the constitutive centromere-associated network proteins (CCAN) (Sue Biggins, 2013; Cho & Harrison, 2011; Lang, Barber, & Biggins, 2018; Lechner & Carbon, 1991). Having the inner kinetochore proteins present provides the necessary scaffold for the outer kinetochore proteins to attach (Janke, Ortíz, Tanaka, Lechner, & Schiebel, 2002).

CCAN represents multiple subunits and two of these are the Cnn1-Wip1 complex that connect to the inner kinetochore through the Ctf3 complex: Ctf3, Mcm16, and Mcm22 (Altunkaya et al., 2016). Cnn1 complex directly interacts with two of the kinetochore's essential four member protein Ndc80 complex (Ndc80c; Spc24, Spc25, Nuf2, Ndc80) via the heterodimer Spc24 and Spc25 (Altunkaya et al., 2016). Cnn1 tethered to mini-chromosomes can successfully recruit the necessary kinetochore sub-complexes to support ~65% mini-chromosome segregation (Altunkaya et al., 2016; Lampert, Hornung, & Westermann, 2010; Schleiffer et al., 2012). When tethered to the microtubule complex, Ndc80c via the Spc24 protein onto the mini-chromosome, then this resulted to an inability to segregate. This is an indication that Cnn1 subcomplex can recruit sufficient outer kinetochore proteins and perhaps have flexibility to influence the

microtubule binding subunits in an arrangement that supports proper microtubule binding for chromosomal segregation (Schleiffer et al., 2012). Despite Cnn1's role in recruiting the vastly essential Ndc80c, Cnn1 arrives noticeably late in mitosis, and in anaphase Cnn1 protein reaches its apex, over three-fold when compared to the G1 phase (Bock et al., 2012; Lang et al., 2018). *cnn1*Δ null mutant cells remain viable. This is possible due to Cnn1 being one of two arms that recruits the Ndc80c. The other arm, or pathway, is independent of Cnn1, but cells are inviable if both are compromised. This second arm is the heterotetramer Mtw1/MIS12 complex (Mtw1, Dsn1, Nnf1, Nslf1). Mtw1 pathway also binds to the Ndc80c via its heterodimeric Spc24 and Spc25 subunits (Bock et al., 2012; Schleiffer et al., 2012).

As mentioned above, the recruitment of Mtw1 complex is important as it links to the Ndc80c, but it can also bind to the complex Spc105 (also known as KNL1 and interacts with Kre28) via its C-terminus (Sue Biggins, 2013; Hemmerich et al., 2008; Maskell, Hu, & Singleton, 2010). The Spc105 complex is part of the outer kinetochore, but its primary role is not in microtubule-binding. Rather, Spc105 is involved in signaling to the cell if chromosomes lack proper bi-orientation, as discussed below.

1.3. The microtubule binding side of the kinetochore

One end of the kinetochore couples to the chromosomal centromere, while the other end couples to the microtubules of the mitotic spindle. Microtubules are active, dynamic structures that need to be secured by specialized microtubule binding kinetochore complexes. Yeast outer kinetochore subcomplexes that are responsible for microtubule binding consist of the KMN network (Spc105/KNL1, Mtw1, and Ndc80) and the Dam1 complex [Dam1c; Duo1, Dam1, Ask1,

Spc34, Spc19, Dad1, Dad2, Dad3, Dad4 (Hsk2), and Hsk3] (Kudalkar et al., 2015; Pagliuca, Draviam, Marco, Sorger, & Wulf, 2009; Pinsky, Kung, Shokat, & Biggins, 2005; Scarborough, Davis, & Asbury, 2019). The Spc105/KNL1 complex influences kinetochore-microtubule binding at its lattice, but it has not been established as to how it affects direct microtubule tip binding (Akiyoshi et al., 2010; Pagliuca et al., 2009). The Mtw1 complex encourages a conformational change in the Ndc80c that promotes its microtubule binding at the lattice by a four-fold when compared to the Ndc80c alone (Kudalkar et al., 2015; Scarborough et al., 2019; Tien et al., 2014). When Mtw1, Spc105, and Ndc80c work together they synergistically increase microtubule-lattice-binding (Cheeseman, Chappie, Wilson-Kubalek, & Desai, 2006). It's important to promote microtubule binding since in yeast the kinetochore relies stringently on the essential Ndc80c, alongside with the essential Dam1c, to interact and sustain attachment to single microtubules (Cheeseman et al., 2006; DeLuca et al., 2006; Espeut, Cheerambathur, Krenning, Oegema, & Desai, 2012; Tien et al., 2010).

The Ndc80c is conserved from yeast to animals. The Ndc80c stretches to a length of ~60 nm, and at the N-termini, the globular domain of Ndc80 and Nuf2 protein fold to a unique calponin-homology (CH) domain that carry microtubule-binding function (Tooley & Stukenberg, 2011). The Ndc80 subunit contains an N-terminus tail that's ~110 residues (~80 for humans) yet it is not essential to yeast (Demirel, Keyes, Chaterjee, Remington, & Burke, 2012). The tail does have influence in microtubule binding, it has a role in the regulation of proper kinetochore-microtubule attachment, and if absent, then in combination with a compromised Dam1c subunit cell growth is poor. This is suggestive that it may have an important role in aiding microtubule

tip binding, an important function of the Dam1c (Demirel et al., 2012; Lampert et al., 2010; Wei, Al-Bassam, & Harrison, 2006).

The Ndc80c colocalizes and serves as the receptor for the Dam1c (Janke et al., 2002, 2002; Shang et al., 2003; Wong et al., 2007). Just like the Spc105 and Mtw1 complex, the synergistic interaction between the Ndc80c and Dam1c enhances microtubule-binding (Janke et al., 2002; Lampert et al., 2010; Tien et al., 2010). Because the Dam1c localization to the kinetochore is Ndc80c dependent, compromising the Ndc80c would result in loss of the Dam1c. With a weakened Ndc80 or Dam1 complex, the only two essential microtubule-binding factors, the cell exhibits an inability for the kinetochore to carry out its function, undergoing chromosomal mis-segregation and death (Osborne, Schlenstedt, Jinks, & Silver, 1994; Pinsky et al., 2005; K. Tanaka, Kitamura, Kitamura, & Tanaka, 2007; Wigge & Kilmartin, 2001).

The Dam1c independently binds to microtubules and it is specialized to oligomerize 17 of its heterodecamers to form a ring around the microtubule (Enquist-Newman et al., 2001; Jenni & Harrison, 2018; Miranda, Wulf, Sorger, & Harrison, 2005). In the absence of microtubules, the Dam1c can continue to form rings but only at saturating concentrations. So, this suggests that this action is favorable and regulated in the presence of microtubules (Westermann et al., 2005). Whether ring formation happens prior to or after Dam1c contacts the Ndc80c is not known. Having a ring is critical in retaining the kinetochore well fastened to dynamic microtubules (see below) (Akiyoshi et al., 2010; Sarangapani, Akiyoshi, Duggan, Biggins, & Asbury, 2013; Umbreit et al., 2014). The ring is proposed to serve as a collar-like function in which the Dam1c can follow the microtubule and harness the power stroke to move the entire chromosome as the microtubules retracts to its poles (Asbury, Tien, & Davis, 2011).

In *S. cerevisiae*, the Dam1c is essential, while metazoans that lack the Dam1c carry a "mechanical homolog", Ska complex (Gaitanos et al., 2009; Hanisch, Silljé, & Nigg, 2006; Helgeson et al., 2018). Although both the Ska and the Dam1 complex serve to stabilize the Ndc80c at dynamic microtubules, the Ska complex has not been observed not form a ring around microtubules (Jeyaprakash et al., 2012). *S. cerevisiae* utilizes its point centromere to scaffold a single kinetochore that will bind one and only one microtubule. Therefore, attachment is critical, unlike other eukaryotes that can bind a few to 30 microtubules as the cell matures in the cell cycle (McEwen, Heagle, Cassels, Buttle, & Rieder, 1997). *S. cerevisiae* utilizes the Dam1c function to oligomerize into a ring enabling the kinetochore to fasten to dynamic microtubule and reduce the chance for microtubule detachment (Umbreit et al., 2014).

1.4. Dynamic microtubules mechanically aid in chromosomal segregation

Microtubules have multiple roles in eukaryotes as they are involved in: cellular structure, cell signaling, nuclear positioning, fertility, cell migration, and many other functions (Guertin, Trautmann, & McCollum, 2002; Gundersen & Worman, 2013; Ludington, Shi, Zhu, Berns, & Marshall, 2012; O'Donnell & O'Bryan, 2014). Microtubules are built from alpha-beta heterodimers that stack on top of each other to form protofilaments. Thirteen of these protofilaments will laterally interact to form a tube that we recognize as the microtubule. In yeast, the formation of a microtubule arises from the spindle pole body (SPB) that is embedded within the nucleus and the microtubule will grow outwards. At the pole, this region of the microtubule is known as the minus-end. The other end is the plus-end. The yeast microtubules are categorized as: astral microtubules, required to position the nucleus; interpolar microtubules, microtubules within the nucleus that help slide poles away from each other to gain separation

during anaphase; and kinetochore microtubules, microtubules that bind kinetochores and push and pull them via microtubule dynamics (Dumont & Mitchison, 2009).

The role of microtubules in mitosis is essential as they: seek and capture unbound kinetochores, position chromosomes at the center of the cell, provide tension across the kinetochores and fulfill kinetochore-microtubule binding occupancy to satisfy the spindle assembly checkpoint, and segregate chromosomes (Akiyoshi et al., 2010; Asbury, 2017; Etemad, Kuijt, & Kops, 2015; Heald & Khodjakov, 2015; Salmon & Bloom, 2017; T. U. Tanaka, 2010). The construction of microtubules allows the structure to execute mitotic functions based on its dynamic instability. Microtubules can grow, or polymerize; shorten, or depolymerize; and rescue, or switch from shortening to growing to "rescue" a full rapid depolymerization known as "catastrophe" (Meunier & Vernos, 2012). During dynamic instability, kinetochores will hold on to microtubules and prevent them from depolymerizing, and the kinetochore will withstand the force produced by the microtubule. The yeast kinetochore will experience the force of a single microtubule, and work initiated by Nicklas, and further refined by others, helped to estimate that a microtubule's depolymerization produces about 12 piconewtons (pN) of force (Asbury, 2017; Nicklas, 1983).

The alpha and beta tubulin subunits have unstructured C-terminal tails, known as E-hooks. E-hooks contain several negatively charged aminoacyl residues and are known to interact with proteins containing positively charged patches (Tajielyato, Li, Peng, Alper, & Alexov, 2018). Because Ndc80 and Nuf2 proteins contain positive charges at their calponin homology domain, and at the Ndc80 protein N-terminal tail, it was demonstrated that Ndc80c and microtubules have an electrostatic interaction (Alushin et al., 2012, 2010; Miller, Johnson, & Stukenberg, 2008).

Similarly, Dam1c utilizes E-hooks, along with its tilt, to bridge the gap based on the fact that ring dimensions being 540 angstrom on the outside and 320 angstrom on the inside, Duo1 and Dam1 proteins protruding from the ring favored contact with microtubules containing E-hooks to help extend their diameter of 260 angstrom, a statement that has been debated (Miranda et al., 2005; Ramey et al., 2011; Westermann et al., 2005). The ring can also help kinetochores withstand overwhelming forces by reducing the rate of depolymerization (Ekaterina L. Grishchuk et al., 2008). As mentioned, the kinetochore remains coupled to microtubules, and the essential oligomerization of Dam1c to make a collar around the peeling microtubule keeps it bound.

The Ndc80 and Dam1 complexes are essential in fastening kinetochores to dynamic microtubules, and this interaction is needed to maintain and to prevent a kinetochore from releasing during chromosome segregation that would lead to cell aneuploidy, an imbalance number of chromosomes. Such error in humans can lead to common abnormalities such as trisomy 21 and still births and is also known to be a major feature in cancer (Cimini, 2008; Hassold & Hunt, 2001). When an attachment is not made accurately, then a cell needs to survey and regulate the kinetochore to release the microtubule in attempt to properly re-bind to microtubules.

1.5. Motor proteins

Motor proteins play essential roles in microtubule dynamics and in the structure of the mitotic spindle. In yeast, there are 6 kinesins and a dynein (Cin8, Kar3, Kip1, Kip2, Kip3, Smy1, and Dyn1). Only four of those proteins (Kip1, Cin8, Kip3 and Kar3) are found within the nucleus;

this is important since yeast have closed mitosis, in which the nuclear membrane remains intact throughout the cell cycle (Hildebrandt & Hoyt, 2000). Cin8 and Kip1, part of the kinesin-5 family, function to crosslink microtubules, maintain the spindle apparatus from collapsing, and push poles apart as they walk towards the plus-end direction. The kinesins are also suggested to regulate microtubule length by promoting microtubule shortening and contribute to kinetochores clustered on microtubules (Gardner et al., 2008; Hildebrandt & Hoyt, 2000; Saunders & Hoyt, 1992). Kip3, part of kinesin-8 family, functions to regulate the length of spindles as it arrives to the plus-end of the microtubule to trigger microtubule depolymerization. In anaphase, Kip3 also functions in keeping kinetochores from lagging behind during pole separation (Arellano-Santoyo et al., 2017; Cottingham & Hoyt, 1997; Tytell & Sorger, 2006). Kar3, part of the kinesin-14 family, is a minus-end directed protein that has depolymerizing activity and despite predominantly found at the SPBs its localization to detached kinetochores allows it to transport the kinetochore towards the minus-end and facilitate its rebinding to microtubules (Cottingham, Gheber, Miller, & Hoyt, 1999; K. Tanaka et al., 2005; Tytell & Sorger, 2006).

1.6. Recognizing unbalanced kinetochore-microtubule attachments to halt anaphase onset

Humans are highly complex organisms composed of many cells with a wide range of functions, yet all cells require the same genetic material. The cell has a surveillance system to ensure that before anaphase onset chromosomes are bi-orientated, which means that each opposite spindle pole will emanate microtubules that seek and capture their respective sister-chromatid's kinetochore. When the kinetochores are bi-orientated, they are under tension as they oppose the microtubule forces and resist succumbing to splitting apart due to the resistance

of the cohesion complex, a ring-shaped structure found on newly replicated sister chromatids (Sue Biggins, 2013; Gerton, 2005; Salmon & Bloom, 2017; T. Tanaka, Cosma, Wirth, & Nasmyth, 1999). Remarkably, tension at the kinetochore-microtubule interface promotes a stronger interaction, also known as a catch bond (Akiyoshi et al., 2010; London & Biggins, 2014; Maresca & Salmon, 2010). When cells have achieved bi-orientation, the shortening of kinetochore-bound microtubules can generate energy to pull the kinetochore poleward and divide duplicated chromosomes into daughter cells (Maresca & Salmon, 2009; Mukhtar, Adhami, & Mukhtar, 2014; Suzuki et al., 2016). If there is lack of bi-orientation, then the cell will prevent anaphase onset and the cell will activate the spindle assembly checkpoint (SAC) to afford the necessary time to correct errors and avoid defects in chromosome segregation (London & Biggins, 2014; Pangilinan & Spencer, 1996; Proudfoot et al., 2019; Rieder, Schultz, Cole, & Sluder, 1994).

Failed biorientation can arise from microtubules coming from the same pole and binding to both of the sister-chromatid kinetochores, or from having only one kinetochore bound from the chromosome pair, also known as syntelic or monotelic attachments, respectively. To detect the botched arrangement, the cell's operation of the surveillance system uses: budding uninhibited by benzimidazole (BUB; BUB1 and BUB2); mitotic arrest deficient [MAD; MAD1, MAD2, and MAD3 (BUBR1)]; and kinase monopolar spindle (MPS1) (Hoyt, Totis, & Roberts, 1991; Li & Murray, 1991; London, Ceto, Ranish, & Biggins, 2012).

Kinetochores that lack proper microtubule attachment utilize kinetochore-bound Mps1 to effectively phosphorylate Spc105/KNL1. Mps1 phosphorylates the six Spc105 N-terminus MELT motifs, yet only two or three phosphorylated motifs are required to induce recruitment of the SAC proteins (Aravamudhan, Chen, Roy, Sim, & Joglekar, 2016; London et al., 2012). Following

the phosphorylation of Spc105 protein a signal system will build at the kinetochore from the recruitment and retention of: Bub3-Bub1, Bub3-Mad3, and finally Mad1 and “closed-Mad2”. Next, soluble “open-Mad2” will interact with kinetochore-bound “closed-Mad2” to convert it into free “closed-Mad2”. Free “closed-Mad2” is then authorized to neutralize cell division cycle 20 (Cdc20) by sequestering it in an assembled mitotic checkpoint complex (MCC), which is composed of closed-Mad2 and Bub3-Mad3. Briefly, Cdc20 is a cofactor of the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C) that is responsible for degrading securin (homolog of yeast Pds1), a protein that inhibits separase (homolog of yeast Esp1) from cleaving the cohesion complex (Mcd1/Scc1). The cascade will inhibit the cell from entering anaphase and give the cell time to correct any inappropriate attachments; the responsibility to reverse incorrect attachments is heavily burdened on the kinase, Ipl1.

1.7. Ipl1-dependent phosphorylation in error correction

Ipl1(Ipl1, Sli15, Nbl1 and Bir1; homolog of Aurora B complex) is a heterotetrameric serine/threonine kinase that regulates kinetochores lacking tension by helping maintain cells arrested in mitosis and cause kinetochores to dissociate from microtubules (Sue Biggins & Murray, 2001; Kalantzaki et al., 2015; T. U. Tanaka et al., 2002). Ipl1 works by disrupting the microtubule binding function of the Ndc80 and Dam1 complex (S Biggins et al., 1999). Ipl1-dependent phosphorylation of proteins Ndc80 and Nuf2 at its CH domain, and the phosphorylation of Ndc80’s N-terminal “tail” disrupts its interaction with microtubules (Ciferri et al., 2008; Wimbish & DeLuca, 2020; Zaytsev et al., 2015). Also, Ipl1 phosphorylates the Dam1c to disrupt its function in microtubule binding and oligomerization, which is an important feature that increases its interaction with microtubules and helps retain the Ndc80c at microtubules

(Cheeseman et al., 2002; Gestaut et al., 2008; Umbreit et al., 2014; Zelter et al., 2015). The association of Dam1 and Ndc80c will synergistically withstand the forces generated by microtubules, but that activity also depends on their interaction, which is negatively regulated upon Ipl1 phosphorylation (Cheeseman et al., 2002; Kalantzaki et al., 2015; Kim et al., 2017; Maure et al., 2011; Shang et al., 2003; Tien et al., 2010). Ipl1 does affect the kinetochore-microtubule coupling, but it important that binding is not permanent and can be regulated to create detachments at the kinetochore-microtubule interface. This is necessary to allow abnormal attachments to undergo trials of catch and release until the cell definitely establishes bi-orientation.

1.8. Silencing the SAC and exiting the cell cycle

After cells have achieved bi-orientation, the SAC can be silenced. The Spc105/KNL1 protein has an RVSF motif that serves as the site for recruitment of Protein Phosphatase 1 (PP1; Glc7 in yeast). PP1 will reverse phosphorylation on the Spc105 that leads to loss of SAC protein and ultimately the inhibition of CDC20 will stop (Aravamudhan et al., 2016; London et al., 2012; Pinsky, Kotwaliwale, Tatsutani, Breed, & Biggins, 2006; Rosenberg, Cross, & Funabiki, 2011). PP1 function is also essential in reversing Ipl1 phosphorylation, which would lead to the stabilization of the Dam1 and Ndc80c on newly formed microtubule attachments (Rosenberg et al., 2011; Suzuki et al., 2018).

Another step as the cell exits mitosis is the downregulation of CDK1 activity. Disrupting CDK1 can begin with CDK1 phosphorylating APC at an inhibitory loop to free it so that it can interact with CDC20 (Primorac & Musacchio, 2013; Qiao et al., 2016; Rudner & Murray, 2000).

With free Cdc20 and APC uninhibited, APC^{cdc20} negatively targets CDK1 activity by ubiquitinating mitotic cyclins, such as Clb2, for its degradation (Irniger, 2002). APC^{cdc20} can signal securin for degradation, which would allow separase to degrade cohesin and begin the separation of sister chromatids. In addition, the fourteen early anaphase release (FEAR) and mitotic exit network (MEN) pathways help to diminish the nuclear inhibition of Cfi1/Net1 against Cdc14, a yeast phosphatase (Sullivan & Morgan, 2007). Active Cdc14 is present in late mitosis to safely reverse CDK1 phosphorylated protein, such as de-phosphorylating Sic1 protein. Sic1 can avoid detection from the ubiquitin ligase and serve to inhibit CDK1 activity, as it is no longer required and its ongoing activity would inhibit the cell from exiting anaphase (A Amon, 1997; J. Bloom & Cross, 2007; Stegmeier & Amon, 2004).

1.9. Research Questions

The Dam1c can oligomerize, fastens around the microtubule, and can withstand the force generated by dynamic microtubules to retain kinetochores at microtubule to facilitate chromosome segregation. Research has identified that phosphorylation of Dam1c, in the form of negative regulation, is important for correcting kinetochore-microtubule attachment errors. In apparent contradiction to this idea, there is an isolated gem that suggests phosphorylation is important in Dam1c function (Li & Elledge, 2003). In Chapter 2 and Chapter 3 we investigate how kinetochore-microtubule coupling results in a stronger interface when the Dam1c is phosphorylated. Thus, the ability of the Dam1c to enhance attachment strength depends upon specific phosphorylation. We seek to elucidate as to whether the improvement in binding can be due to 1) Dam1c having stronger interaction with the kinetochore, 2) increase affinity to

microtubules, 3) or promoting more efficient oligomerization. Also, in Chapter 2 and we seek to determine the kinase and phosphorylation site(s) that lead to enhanced Dam1c function.

One key factor in the process of chromosome segregation is the kinesin family of motor proteins. Motor proteins mechanically move along microtubules and have many conserved functions in mitosis, such as regulating microtubule dynamics, linking microtubules for spindle assembly, sliding nuclear microtubules to push dividing cells apart, and chromosome positioning (Hildebrandt & Hoyt, 2000). Previous work in *S. cerevisiae* shows that kinesins localize to kinetochores and that they interact with the centromere by chromatin immunoprecipitation (Gardner et al., 2008). However, although there has been much progress in understanding the mechanisms of cell division, the role of kinesins in regulating kinetochore-microtubule attachment is not fully understood. In Chapter 3, using purified kinetochores I seek to determine (1) which nuclear motor proteins can bind to the kinetochore and (2) elucidate as to whether it can enhance kinetochore-microtubule coupling.

2. Chapter 2: CDK1 phosphorylation of Dam1c does not influence its interaction with the Ndc80 complex

2.1 Summary

Here, I used a reconstitution system to discover that Cdk1, the major mitotic kinase that drives the cell cycle, phosphorylates the Ask1 component of the Dam1c to enhance kinetochore-microtubule attachment strength. I asked whether phosphorylated Dam1c plays an important role in its interaction with the kinetochore, or its kinetochore's receptor, the Ndc80c. After exhaustive efforts investigating whether phosphorylation of Dam1c aids in rebinding to kinetochores by bulk assays, single molecule TIRF, and genetic interactions, I, along with collaborators, identify that Cdk1 phosphorylation does not aid in the Dam1c binding to the kinetochore.

2.2 Introduction

To ensure the faithful inheritance of DNA, the macromolecular kinetochore sustains the connection between chromosomes and force-generating dynamic microtubules during cell division. One of the major microtubule binding activities in the kinetochore is mediated by the conserved Ndc80 complex (Ndc80c) (Cheeseman et al., 2006; DeLuca et al., 2006; Wigge & Kilmartin, 2001). In budding yeast, the retention of kinetochores on dynamic microtubule tips also depends on the essential heterodecameric Dam1 complex (Dam1c), which binds to Ndc80c (Asbury, Gestaut, Powers, Franck, & Davis, 2006; Doodhi et al., 2019; E L Grishchuk et al., 2008; Ekaterina L. Grishchuk et al., 2008, 2008; Lampert et al., 2010, 2010; K. Tanaka et al., 2007, 2007; Tien et al., 2010, 2010; Westermann et al., 2005, 2005, 2006).

In yeast, the Dam1c and Ndc80c are both essential and by collaborating they synergistically work to bind and track microtubules (Kim et al., 2017; Tien et al., 2010). Ipl1-dependent phosphorylation of Dam1c causes its interaction with Ndc80c to be weakened and in turn it negatively affects Ndc80c's ability to track depolymerizing microtubules and the coupling force at the kinetochore-microtubule interface, therefore leading to chromosomal segregation defects (Kim et al., 2017; Lampert et al., 2010; Lampert, Mieck, Alushin, Nogales, & Westermann, 2013). Ipl1 activity in error correction is reversed as the cell progresses in the cell cycle and the kinetochore is under tension (Keating, Rachidi, Tanaka, & Stark, 2009). In addition to the reversing the negative regulation on the Dam1c, I was fascinated as to whether the balance of a weakened Dam1c shifts to a strengthened Dam1c function to ensure kinetochores withstand dynamic microtubules as the cell readies to segregate chromosomes.

With the use of an optical trap-based assay, I was able to test native kinetochores and their Dam1c, as well as recombinant Dam1c, and examine the kinetochore-microtubule attachment strength. Kinetochore-microtubule coupling is strengthened by phosphorylation that was dependent on Cdk1. Cdk1 has consensus sites Ask1 (S216 and S250), Dad3 (S6 and S49) and Spc19 (S116). In testing the Cdk1 sites on Ask1, its phosphorylation is sufficient to promote strong kinetochore-microtubule attachment.

Because the Dam1c has many functions, in this chapter I wanted to elucidate if phosphorylated Dam1c was able to promote its interaction with the kinetochore. As mentioned, the interaction between the two microtubule binding components, Ndc80c and Dam1c, is essential for chromosomal integrity. In using TIRF microscopy and *in vivo* re-binding assays, I did not find that phosphorylation of Dam1c influenced its kinetochore or Ndc80c binding.

In addition to investigating Ask1 phosphorylation, its phospho-mimetic version had an advantage in its *in vivo* genetic interaction with the Cnn1-Ndc80c pathway, an alternative to the Mtw1 pathway. Cnn1 is incorporated into the kinetochore at late metaphase, early anaphase. This is suggestive that as the cell progresses through the cell cycle, modifications are made to adjust the Ndc80c and Dam1c to be equipped to facilitate kinetochore-microtubule coupling.

2.3 Results

2.3.1 Phosphorylation of the native Dam1c strengthens kinetochore-microtubule attachments

To measure the contribution of the Dam1c to the strength of tip-bound kinetochore-microtubule attachments, I used an *in vitro* reconstitution system. Wild-type kinetochore particles were purified from budding yeast cells by immunoprecipitating Dsn1, a conserved kinetochore protein (Supplemental Figure 2.1) (Akiyoshi et al., 2010). To obtain kinetochore particles lacking the Dam1c, we used *dad1-1* cells that contain a temperature sensitive mutant in the Dam1c component Dad1 (Supplemental Figure 2.1) (Enquist-Newman et al., 2001). We confirmed that kinetochore particles purified from *dad1-1* cells (referred to as Dad1-1 kinetochores) grown at the non-permissive temperature lack the Dam1c, but otherwise retain the major core kinetochore components (Supplemental Figure 2.1). To test the biophysical coupling strength of the Dad1-1 kinetochores to microtubule tips, we used an optical trap to exert tension on kinetochore-microtubule attachments. Kinetochore particles were linked to polystyrene microbeads that were captured in a laser trap and then placed at the plus ends of dynamic microtubules that were grown from microtubule seeds tethered to a slide (Figure 2.1A). Once a kinetochore-microtubule interaction was established, tensile force was applied and then

gradually increased until the kinetochore ruptured from the microtubule tip (Akiyoshi et al., 2010; Sarangapani et al., 2013; Umbreit et al., 2014). To compare Dad1-1 versus wild-type kinetochores, we measured rupture forces for many individual kinetochore particles of each type. For each population, the fraction of attachments that survived up to a given level of force was plotted and the mean rupture force was also calculated. Wild-type kinetochores ruptured over a range of forces, with a mean strength of 9.4 piconewtons (pN). Dad1-1 kinetochores were substantially weaker. Their rupture force distribution was shifted to lower values and their mean strength was only 3.4 pN, consistent with previous measurements (Figure 2.5) (Akiyoshi et al., 2010; Umbreit et al., 2014). To determine whether the reduced strength of the Dad1-1 kinetochores was solely due to the lack of the Dam1c, we purified recombinant Dam1c (rDam1c) from bacteria and performed add-back experiments (Umbreit et al., 2014). When we introduced soluble rDam1c into the optical trapping assay with the kinetochore-bound beads, it partially rescued the strength of Dad1-1 kinetochores, increasing the average rupture force from 3.4 to 5.6 pN (Figure 2.1C and 2.1D), as observed previously (Umbreit et al., 2014). One possible reason for the lack of a full rescue was that the recombinant complex lacked key post-translational modifications. To test this, we purified native Dam1c (nDam1c) from yeast cells and performed the same experiment. In contrast to the rDam1c, the addition of the nDam1c to Dad1-1 kinetochores fully restored kinetochore-microtubule attachment strength, increasing the mean rupture force to 9.1 pN (Figure 2.1C and 2.1D). This observation suggests that the native complex carries key modifications that contribute to its ability to hold microtubule tips under force and that are lacking on the rDam1c.

Because the Dam1c is known to have multiple activities regulated by phosphorylation (Cheeseman et al., 2002; Gestaut et al., 2008; Kalantzaki et al., 2015; Kang et al., 2001; Keating, Rachidi, Tanaka, & Stark, 2009; Lampert et al., 2010; Li et al., 2002; Li & Elledge, 2003; Pinsky, Kotwaliwale, Tatsutani, Breed, & Biggins, 2006; Sarangapani et al., 2013; Shah et al., 2018; Shimogawa et al., 2006; Tien et al., 2010; Zelter et al., 2015), we tested whether phosphorylation was responsible for the higher kinetochore-microtubule attachment strength observed with nDam1c relative to rDam1c. We treated purified nDam1c with phosphatase (λ) to remove phosphorylation or with phosphatase plus inhibitors (λ + inh) as a control. The migration of the second band, Ask1 protein increased after phosphatase treatment (Figure 2.1E, silver-stained SDS-PAGE), indicating successful dephosphorylation. We then tested the activity of the dephosphorylated nDam1c in add-back assays as described above. As expected, the control-treated nDam1c fully rescued the Dad1-1 kinetochore particles, bringing their rupture strength up to 8.8 pN on average. In contrast, the phosphatase-treated nDam1c only partially restored the mean rupture force to 5.7 pN, similar to the rDam1c (Figure 2.1E and 2.1F). Taken together, these data indicate that Dam1c phosphorylation promotes stable kinetochore-microtubule attachments. Although this result was surprising given that Ipl1 phosphorylation of the Dam1 component of the complex is known to weaken the interaction between kinetochores and microtubules (Cheeseman et al., 2002; Gestaut et al., 2008; Kalantzaki et al., 2015; Kang et al., 2001; Keating et al., 2009; Lampert et al., 2010; Li et al., 2002; Pinsky et al., 2006; Sarangapani et al., 2013; Shah et al., 2018; Tien et al., 2010; Zelter et al., 2015), our kinetochore and native Dam1c purifications have little detectable phosphorylation on these sites (Sarangapani et al., 2013).

2.3.2 Cdk1-mediated phosphorylation of Ask1 enhances kinetochore-microtubule attachment strength

To further assess the role of Dam1c phosphorylation, we sought to identify the specific phosphorylation sites on nDam1c that contribute to kinetochore-microtubule attachment strength. The predominant migration shift on the purified Dam1c on silver-stained SDS-PAGE gels occurs on the Ask1 protein (Figure 2.1E). Ask1 phosphorylation increases as cells proceed into mitosis due to the major mitotic kinase Cdk1 (Cdc28 in *S. cerevisiae*), although the function of this phosphorylation is not known (Cheeseman, Brew, et al., 2001; Li & Elledge, 2003; Ubersax et al., 2003). We therefore tested whether Cdk1 activity is required for nDam1c activity in the optical trap assay. To do this, we treated wild-type cells or those containing an analog-sensitive allele of Cdk1 (*cdc28-as1*) with the analog 1-NM-PP1 to specifically inhibit Cdk1 in the *cdc28-as1* cells prior to purifying nDam1c (Figure 2.2A, right panel; Supplemental Figure 2.3) (Bishop et al., 2000; Ubersax et al., 2003). Visualization of Ask1 on silver-stained SDS-PAGE showed that it migrated further when purified from the analog-sensitive strain compared to wild-type, indicating that the inhibitor worked and was specific to *cdc28-as1* cells. In parallel, the mutant allele was functional in phosphorylating Ask1 in the absence of its inhibiting drug (Supplemental Figure new 2.3). We assayed the activity of Dam1c purified from these two strains in add-back assays and found that the control nDam1c fully rescued Dad1-1 kinetochore particles to an average rupture force of 9.2 pN, but the nDam1c purified from *cdc28-as1* cells only partially rescued the mean rupture force to 7.1 pN (Figure 2.2A and 2.2B). We obtained similar results when nDam1c was purified from a temperature-sensitive mutant Cdk1 strain, *cdc28-13* (Lörincz

& Reed, 1986) (Supplemental Figure 2.4). Taken together, these data suggest that Cdk1 phosphorylation is required for the full contribution of Dam1c to kinetochore-microtubule attachment strength. However, additional phosphorylation on Dam1c may contribute to the interaction strength because the Dam1c complexes lacking Cdk1-mediated phosphorylation strengthened attachments more than rDam1c.

We suspected that Ask1 might be the relevant substrate because it was previously shown that Ask1 is phosphorylated by Cdk1 at the consensus sites S216 and S250 (Li & Elledge, 2003; Ubersax et al., 2003). To test the importance of the Cdk1 sites in Ask1, we mutated both residues to alanine (Ask1-2A) to block Cdk1-mediated phosphorylation. We then purified nDam1c from yeast cells expressing *ask1-2A* as the sole copy of the gene and tested this mutant complex (nDam1c$^{Ask1\text{-}2A}$) in our add-back optical trap assay. The nDam1c$^{Ask1\text{-}2A}$ partially rescued the rupture force of Dad1-1 kinetochores to 6.9 pN (Figure 2.2C and 2.2D), which is similar to the strength measured with nDam1c purified from cells lacking Cdk1 activity. These results indicate that Cdk1 phosphorylation of one or both Ask1 sites is required for full Dam1c-dependent strengthening, and that additional Cdk1-independent phosphorylation of the Dam1c partially contributes to its function. Consistent with this idea, Ask1 phospho-mutants retained some Ask1 phosphorylation because phosphatase treatment increased their mobility on SDS-PAGE (Supplemental Figure 2.5).

To further analyze the role of Cdk1 phosphorylation of Ask1, we tested whether nDam1c containing phospho-mimetic mutations, serines mutated to aspartic acid, in the Cdk1 target sites on Ask1 (nDam1c$^{Ask1\text{-}2D}$) could rescue the rupture force of the Dad1-1 kinetochores. Dam1c was purified from *ask1-2D* cells and added back to the optical trap assay. The nDam1c$^{Ask1\text{-}2D}$ fully

rescued the rupture force to the wild-type level of 9.4 pN (Figure 2.2C and 2.2D). To test whether phosphorylation on the Cdk1 sites are sufficient for full Dam1c activity, we phosphatase-treated the nDam1c$^{Ask1\text{-}2D}$ prior to analysis in the optical trap assay. This would remove the three other potential Cdk1 phosphorylation sites on Spc19 and Dad3, along with any other phosphorylation on the Dam1c. Remarkably, the phosphatase-treated nDam1c$^{Ask1\text{-}2D}$ was fully functional and rescued Dad1-1 kinetochores to 8.8 pN (Figure 2.2C and 2.2D). Therefore, although additional phosphorylation on the nDam1c appears to contribute to its activity, Cdk1-mediated phosphorylation of Ask1 appears to be sufficient for Dam1c to support kinetochore-microtubule attachments.

2.3.3. Targeted Cdk1 phosphorylation of recombinant Dam1c^{Ask1} rescues wild-type kinetochore-microtubule attachment.

To further test whether Cdk1 phosphorylation of the Dam1c is sufficient to fully restore the strength of kinetochore-microtubule attachments to Dad1-1 kinetochores, we directly phosphorylated rDam1c with Cdk1. There was a resulting shift in the migration of Ask1 on the silver-stained SDS-PAGE gel, indicating the kinase reaction worked (Figure 2.3A, right panel). We added the phosphorylated rDam1c to Dad1-1 kinetochores and performed the optical trap assay. Phosphorylated rDam1c fully rescued the rupture force to 9.3 pN (Figure 2.3A and 2.3B), suggesting that Cdk1 phosphorylation is sufficient to fully restore rDam1c activity. To test whether the Cdk1 phosphorylation of Ask1 was the only relevant substrate, we assayed the phospho-mimetic rDam1c (rDam1c$^{Ask1\text{-}2D}$) and found that it fully rescued the rupture force to 8.6 pN (Figure 2.3A and 2.3B). To verify there are no additional contributing Cdk1 phosphorylation sites on Dam1c, we phosphorylated rDam1c$^{Ask1\text{-}2D}$ with Cdk1. There were no further migration

shifts on the Ask1 protein after the Cdk1 kinase reaction when compared to Ask1-2D. Consistent with this, the Cdk1 phosphorylated rDam1c$^{Ask1\text{-}2D}$ rupture force was 8.8 pN, which is similar to untreated rDam1c$^{Ask1\text{-}2D}$ (Figure 2.3A and 2.3B). Together, these data strongly suggest that even though other phosphorylation on Dam1c might strengthen kinetochore-microtubule attachments, Cdk1 phosphorylation of the Ask1 protein is sufficient to fully restore the strength of kinetochore-microtubule attachments to Dad1-1 kinetochores.

2.3.4. Ask1 phosphorylation does not alter Dam1c interaction with immobilized kinetochores

We sought to address how Dam1c phosphorylation enhances Dam1c function to promote kinetochore-microtubule attachment strength. It was previously shown that Dam1c interacts with the kinetochore microtubule-binding component Ndc80 to promote its association with microtubules, at both the lattice and at the plus-end tips (Doodhi et al., 2019; Lampert et al., 2010; Miranda, Wulf, Sorger, & Harrison, 2005; Tien et al., 2010). In addition, Cdk1 phosphorylation of the vertebrate Ska complex directly promotes its interaction with the Ndc80c (Kalantzaki et al., 2015; Zhang et al., 2017). We therefore tested whether phosphorylation enhances the interaction between Dam1c and the kinetochore bound Ndc80c. To approach this, we set off to re-bind free nDam1c that was pre-treated with either phosphatase or phosphatase plus inhibitors and then added it to immobilized Dad1-1 kinetochores. Dam1c successfully bound to Dad1-1 kinetochores and was retained following multiple rounds of washes, and there was no non-specific binding to the beads (Figure 2.4B and Supplemental Figure 2.6). We immobilized *dad1-1* kinetochores on beads and added increasing amounts of either de-phosphorylated or phosphorylated nDam1c and monitored Dam1c levels by immunoblotting and fluorescence

imaging (Figure 2.4B). Similarly, independent of the phosphorylation state, preliminary data of free nDam1c binding to Dad1-1 kinetochores followed with high salt washes did not alter binding of the nDam1c (Supplemental Figure 2.7). This suggests that phosphorylation does not promote Dam1c from binding to the kinetochore.

2.3.5. Ask1-2D does not alter Dam1c interaction with the Ndc80c

Because either immobilized *dad1-1* kinetochores or phosphorylated nDam1c may retain phosphorylation outside of the desired site we wished to study, we used a complementary assay to look at binding by using recombinant Dam1c and Ndc80c. It was previously shown that the interaction of recombinant Dam1c with Ndc80c promotes the association of Ndc80c with microtubules (Kim et al., 2017). We therefore monitored the interaction between the Dam1c and Ndc80c and asked whether Ask1 phosphorylation alters the ability of Ndc80c to interact with microtubules. To do this, we purified recombinant Dam1c containing either WT Ask1 or the phospho-mimetic Ask1-2D protein. We then used single-molecule total internal reflection fluorescence (TIRF) microscopy (Figure 2.4C) to measure the residence time of fluorescently labeled Ndc80c-GFP on taxol-stabilized microtubules in the presence of rDam1c^{Ask1} or rDam1c$^{ask1\text{-}2D}$. In the absence of Dam1c, Ndc80c transiently interacts with microtubules for an average residence time of 2.3 seconds (Figure 2.4D and 2.4E). With the addition of rDam1c^{Ask1}, the residence time increased to 5.3 seconds, as previously shown (Kim et al., 2017). We obtained a similar result with rDam1c$^{ask1\text{-}2D}$, suggesting that phosphorylation does not alter the interaction between Dam1c and Ndc80c.

2.3.6. Phosphorylation of Ask1 protein can withstand induced depolymerization using the Ndc80c-Cnn1 pathway, *in vivo*.

Phosphorylation of Dam1c did not alter its binding to kinetochores purified from asynchronous cells or to purified rNdc80c. What is known is: the Ask1 protein becomes fully phosphorylated in mitosis and its interacting partner, the Ndc80c, also adapts as it progresses in mitosis by altering its structure, copy number, and its kinetochore receptor as the cell transitions to anaphase. The Ndc80c interaction with the microtubule is dynamic and this will cause the Ndc80c to undergo multiple conformational changes; at the microtubule lattice and under low tension, it is stretched to a length of 44 nm; during metaphase, and under high tension, the Ndc80c is stretched to 55 nm; in anaphase, under low tension, it will shorten to 34 nm (Joglekar, Bloom, & Salmon, 2009). In addition, depending on the cell cycle, the Ndc80c begins to change in copy number ranging from ~ 6 to ~ 10 copies at the kinetochore (Dhatchinamoorthy et al., 2017, 2019; Joglekar, Bouck, Molk, Bloom, & Salmon, 2006). Finally, as the cell enters anaphase, the Ndc80c utilizes an additional pathway from the Mtw1/MIS12 complex, a second arm, the Cnn1 complex (Lang, Barber, & Biggins, 2018; Schleiffer et al., 2012). With the Ask1 protein fully phosphorylated in late mitosis and the Ndc80c obtaining a second scaffolding arm, then it's compelling to hypothesize that these cellular modifications will lead to phosphorylated Dam1c (Ask1-2D) favoring the Cnn1 pathway. I used the Ask1 mutant in combination with a second mutant from one of the Mis12 complex's protein, Dsn1-S240A, S250A, S264A (*dsn1-3A*). The allele is proposed to disrupt the Mtw1 arm and forces the kinetochore to rely on the Cnn1 arm to scaffold the Ndc80c (Lang et al., 2018). To approach this hypothesis, an assay looking at cellular growth of a serial dilution in the absence or presence of microtubule destabilizing drug

benomyl was done. In addition to WT, non-phosphorylatable Ask1-2A and phosphomimetic Ask1-2D were used to determine if there was a preference for the Cnn1 pathway. There was no significant difference in growth when comparing the single *ask1-2A* alone or in combination with *dsn1-3A ask1-2A* (Figure 2.5A). This was the same result at this concentration for *dsn1-3A ask1-2D*. Despite the Ndc80c lacking the Mis12 arm, at these conditions the Cnn1 pathway is sufficient to support viability and does not favor the phosphorylated Ask1 mutant. The story changes when cells are grown at a higher benomyl concentration to further disrupt microtubules. This modification is detrimental to WT and a*sk1-2A*, but *ask1-2D* cells can survive (Supplemental Figure 2.8). In these same conditions, the combination of *dsn1-3A ask1-2D* causes the cells to be mildly sensitive to the microtubule drug when compared to *ask1-2D* alone (Supplemental Figure 2.9). This is suggesting that the selection in outer kinetochore scaffold from the Cnn1 pathway that is rescued by Cdk1-dependent Ask1 phosphorylation may have an advantage to stabilizing kinetochores to microtubules. Alternatively, phosphorylated Ask1 may have an intrinsic function outside of the kinetochore that leads to better stabilization of microtubules and Dsn1-3A pathway. Despite being mildly sicker, the Cnn1 pathway benefits from Ask1-2D function. Regardless, it is difficult to impose benomyl induced microtubule depolymerization and the cell cannot depend only on the Cnn1 pathway but requires both Cnn1 and Mtw1.

The Mtw1 pathway has a role in helping phosphorylated Ask1 cells resist benomyl (Supplementary Figure 2.9). So, we decided to test whether the pathway can overcome the benomyl in the presence of phosphorylated Dam1c. In using Ask1 mutants with a deleted Cnn1 strain, *cnn1Δ* (*cnn1^*), the cell's Ndc80c was dependent on the Mtw1 arm. We assayed serial dilutions for growth alone or with the addition of benomyl. There was a decrease in growth in all

the strains containing *cnn1Δ* allele (Figure 2.5). This suggests that the Cnn1 pathway is an important scaffold for the outer kinetochore microtubule binding components and the cell neglects the phosphorylation state of Dam1c.

We reasoned that Cdk1 phosphorylation of Ask1 would increase the Dam1c interaction at the kinetochore via its receptor, the Ndc80c, but that was not the case (Figure 2.4). This then led us to hypothesize that a temperature sensitive Ndc80c, *ndc80-1* allele, would be unaffected upon the phosphorylation state of Ask1. Preliminary data of *ask1-2A ndc80*-1 assayed in a serial dilution at room temperature had zero affect in growth (Supplemental Figure 2.10). Surprisingly, the combination of *ask1-2A ndc80*-1 at semi-permissive temperature were healthier when compared to *ndc80-1* allele alone (Supplemental Figure 2.10). It is baffling how a compromised Ndc80c and a weakened oligomeric Dam1c genetically interact to overcome microtubule stress. The mechanism remains to be solved.

2.4 Discussion

Dam1c promotes chromosome segregation through multiple functions that include directly binding to microtubules, interacting with the Ndc80 kinetochore complex, and oligomerizing around the microtubule to ensure processive tracking of dynamic microtubule tips (Cheeseman, Enquist-Newman, et al., 2001; Ekaterina L. Grishchuk et al., 2008; Hofmann et al., 1998; Janke, Ortíz, Tanaka, Lechner, & Schiebel, 2002; Jin & Wang, 2013; Kiermaier, Woehrer, Peng, Mechtler, & Westermann, 2009; Lacefield, Lau, & Murray, 2009; Lampert, Mieck, Alushin, Nogales, & Westermann, 2013; Li et al., 2002; Tien et al., 2010). Here, we identify a key phospho-regulatory mechanism that does not affect the ability of Dam1c to bind to Ndc80c or to

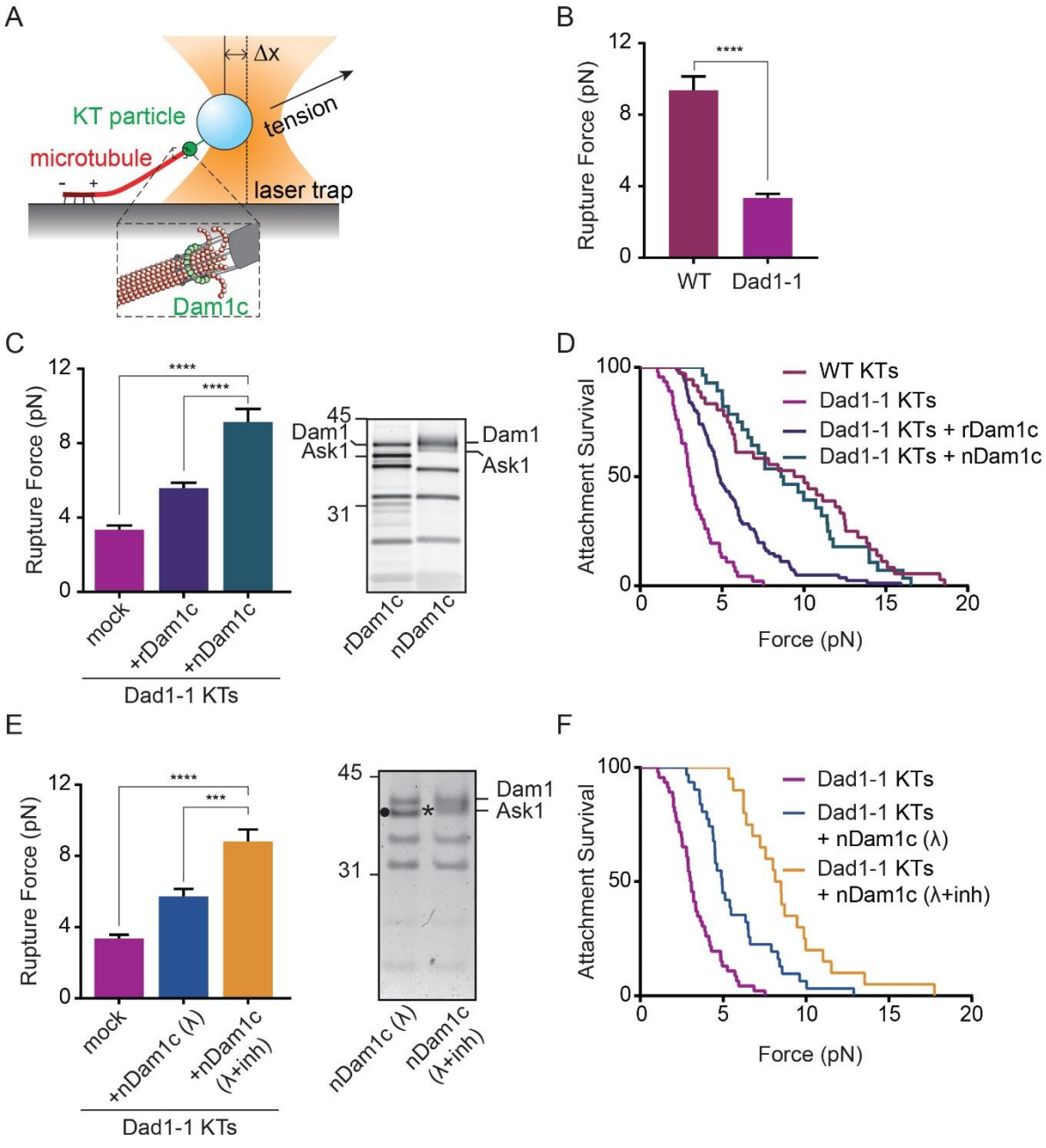
kinetochores. Cdk1 phosphorylation of the Ska complex (Veld, Volkov, Stender, Musacchio, & Dogterom, 2019; Zhang et al., 2017) is also known to promote kinetochore function, consistent with it having functions analogous to Dam1c (Hooff et al., 2017; Zhang, Chen, Yang, & Liu, 2018). However, the underlying mechanism is different because the Cdk1 phosphorylation of the Ska complex enhances its direct interaction with the Ndc80c (Veld et al., 2019; Zhang et al., 2017). Although a high-resolution structure of the Dam1c was recently solved using cryoEM (Jenni & Harrison, 2018), the C-terminal portion of Ask1 that contains the Cdk1 phosphorylation sites was not present in the structure. Despite this information from crosslinking of C-terminal Ask1 to the Ndc80 and Nuf2 coil-coiled region, it is important to know that the phosphorylation of a protein can induce conformations that we don't understand for Ask1 (Zelter et al., 2015; Jae ook Kim et al., 2017). Future structural work remains to be done in the field and to determine how phosphorylated Ask1 behaves.

In vitro, compromising of the Mtw1 pathway (*dsn1-3A* allele) makes it difficult for kinetochores to recruit or assemble the Ndc80c in comparison to kinetochores lacking the Cnn1 pathway (Lang et al., 2018). In addition, *in vivo*, the loss of Cnn1, which is added in late mitosis, is suggested not to be essential in recruiting additional Ndc80c to the kinetochore during anaphase (Dhatchinamoorthy et al., 2017). It is argued that Cnn1 is not vital to recruiting the outer kinetochore component. Therefore, when Cnn1 and Mtw1 were tested, Cnn1's survival (*dsn1-3A*) on benomyl, with phosphorylated Ask1 (Ask1-2D) was interesting (Figure 2.5A and Supplemental Figure 2.9). Cnn1 alone (using *dsn1-3A*) is suggested to be unfavored, yet *cnn1^* strains were sicker when compare to *dsn1-3A*. In focusing solely on the Cnn1 pathway, the Cnn1 arm may favor phosphorylated Dam1c. In the serial dilution (Supplemental Figure 2.9) when

using benomyl to stress the cell only *ask1-2D* and *ask1-2D dsn1-3A* can survive. In addition to *ask1-2D* having an upper hand in the presence of microtubule drug, *ask1-2D* can rescue *dsn1-3A*. This may suggest that the interaction of Dam1c with Cnn1c-Ndc80c has a greater importance to the cell than with Mtw1 when under microtubule stress. In the absence of Cnn1, all cells are equally unhappy, again bolstering the idea that Cnn1 has important function with microtubules, and as to whether this is specifically at the tip remain to be solved.

Alternatively, Lang et al., 2018, showed that Dsn1-3A combined with Cnn1 had residual Ndc80c recruitment. If the Cnn1 was deleted and the Mtw1 complex was depleted, then the kinetochore could not recruit the Ndc80c. In the serial dilution (Figure 2.5A and Supplemental Figure 2.9) because we are using Dsn1-3A, there may be some residual Dsn1-3A Ndc80c recruitment aiding the Cnn1 pathway and this is why cells are more resistant to the microtubule drug. Regardless, alleles *dsn1-3A ask1-2D* have an advantage in growing in comparison to *dsn1-3A ask1-2A*. Despite this serving as preliminary data, Cnn1 does have an important role *in vivo*, and it may favor the Cdk1 phosphorylation of Dam1c.

2.5 Figure Legend



2.5.1 Figure 2.1. Phosphorylation of the native Dam1c promotes kinetochore-microtubule attachment strength.

A) Schematic of laser trap assay. Anchored microtubule seeds (dark red) are fixed to coverslips and dynamic microtubules (red) are grown from the plus ends. A bead (light blue sphere) linked to purified kinetochores (green dot) is attached to the tip of a microtubule using a laser trap and force is subsequently applied to the kinetochore-microtubule interface. The zoom-in (boxed) depicts an oligomerized Dam1c (green ring) around the microtubule that is connected to the rest of the kinetochore (grey).

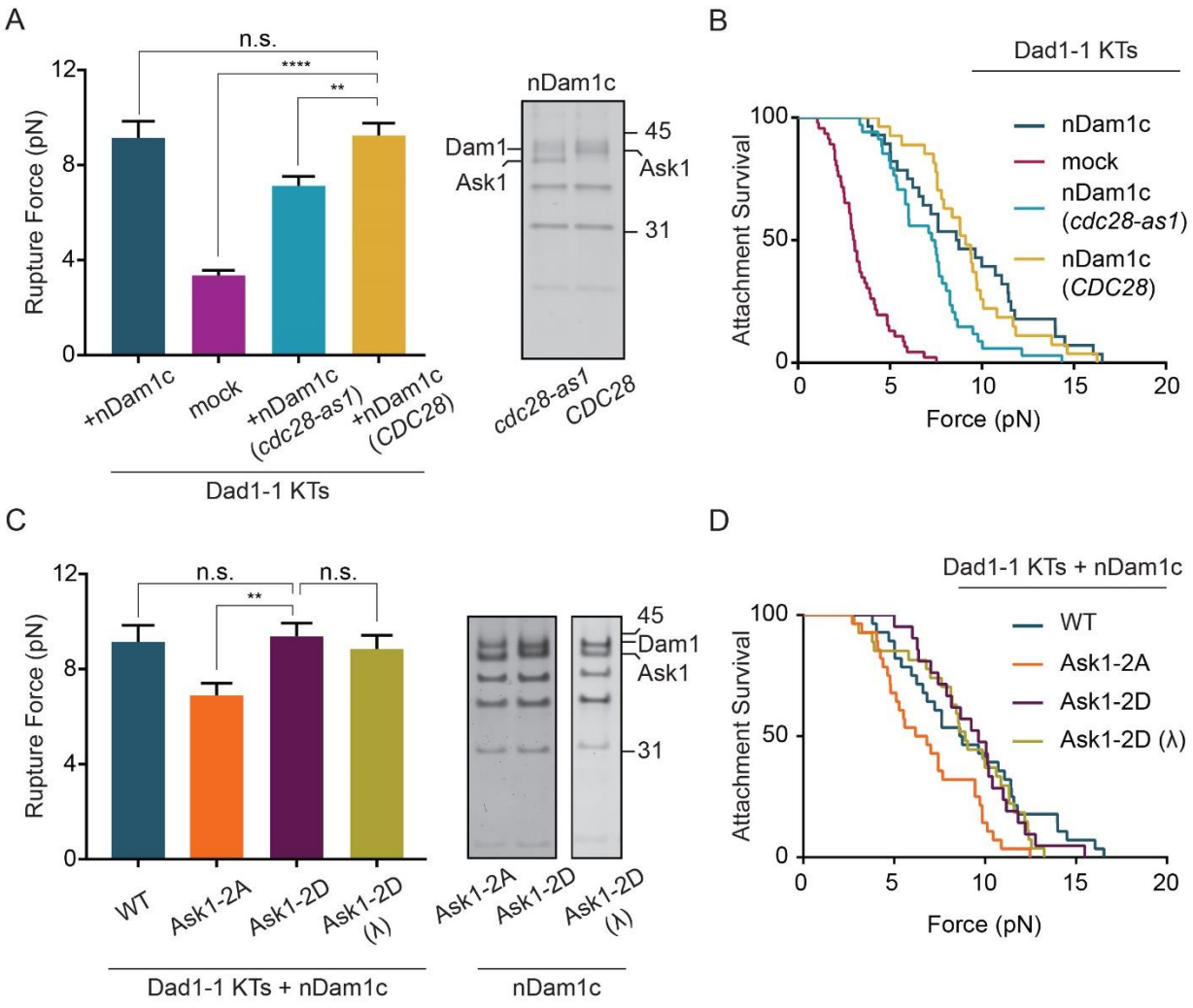
B) Mean rupture forces of wild-type (WT; SBY8253) and Dad1-1 (SBY8944) kinetochores. Error bars represent standard error of the mean (SEM; n = 36–46 events). P-values were determined using a two-tailed unpaired t test (****= $p<0.0001$).

C) Mean rupture forces of Dad1-1 (SBY8944) kinetochores alone, or with the addition of soluble recombinant Dam1 complex (rDam1c) or with native Dam1 complex (nDam1c; purified from SBY13538). The Dam1 complexes were analyzed by silver-stained SDS-PAGE (right panels). Molecular weight markers (kDa) are indicated and only the portion of the gel with the largest and most prominent Dam1c components is shown. Note that the whole gel is displayed, yet it is difficult to observe all ten of the Dam1c proteins by silver stain. (See Supplemental Figure 2.2). Error bars represent standard error of the mean (SEM; n = 28–81 events). P-values were determined using a two-tailed unpaired t test (****= $p<0.0001$).

D) Attachment survival probability versus force for data in B) and C).

E) Mean rupture forces of Dad1-1 kinetochores with the addition of soluble nDam1c (purified from SBY13538) that was either treated with phosphatase (λ) or phosphatase with inhibitors (λ + inh). Error bars for standard error of the mean (SEM; n = 20–46 events). P-values were determined using a two-tailed unpaired t test (***= $p<0.0005$; ****= $p<0.0001$). The Dam1 complexes that were added back were visualized by silver stained SDS-PAGE. De-phosphorylated Ask1 migrates more quickly and is indicated by the black circle while phosphorylated Ask1 is indicated by the black asterisk. Molecular weight markers (kDa) are indicated on the left.

F) Attachment survival probability versus force for data in E) for Dad1-1 kinetochores alone or with an add-back of nDam1c that was treated with phosphatase (λ) or phosphatase with inhibitors (λ+ inh).



2.5.2 Figure 2.2. Cdk1 mediated phosphorylation of native Ask1 is required for wild-type kinetochore-microtubule attachment strength.

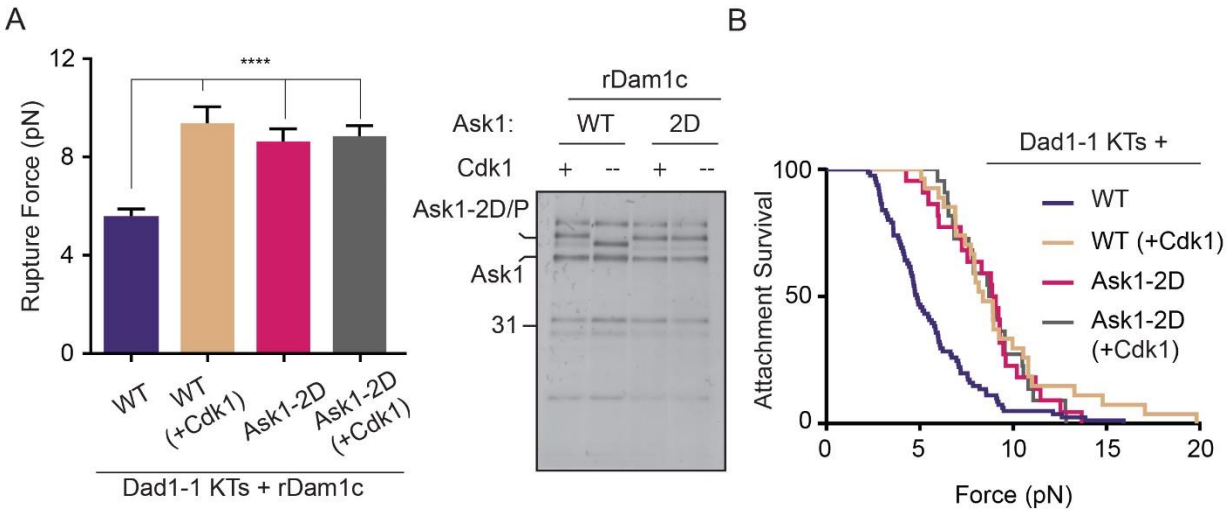
A) Mean rupture forces for Dad1-1 kinetochores with the addition of soluble nDam1c that was purified from strains containing either analog-sensitive *cdc28-as1* (SBY13509) or wild-type *CDK1* (SBY12464) that had been treated with the analog 1-NM-PP-1 (left graph). Error bars for standard error of the mean (SEM; n = 27-46 events). P-values were determined using a two-tailed unpaired t test (n.s.= not significant; **= p<0.005; ****= p<0.0001). The mean rupture force data for Dad1-

1 alone (mock) or with the addition of nDam1c comes from Figure (2.1C). Purified Dam1c was visualized by silver stained SDS-PAGE (right panel). Molecular weight markers (kDa) are indicated on the right.

B) Attachment survival probability versus force for data in A).

C) Mean rupture forces for Dad1-1 kinetochores in the presence of soluble nDam1c that was purified from strains containing Ask1-S216A, S250A (Ask1-2A; SBY17831) or Ask1-S216D, S250D (Ask1-2D; SBY17831) that were untreated or phosphatase-treated. Error bars represent the standard error of the mean (SEM; n = 21-28 events). P-values using a two-tailed unpaired t test (n.s.= not significant; **= $p<0.005$). The mean rupture forces for Dad1-1 with nDam1c (WT) added back are from figure (2.1C) (left graph). The purified nDam1c was analyzed by silver stain SDS-PAGE (right panel).

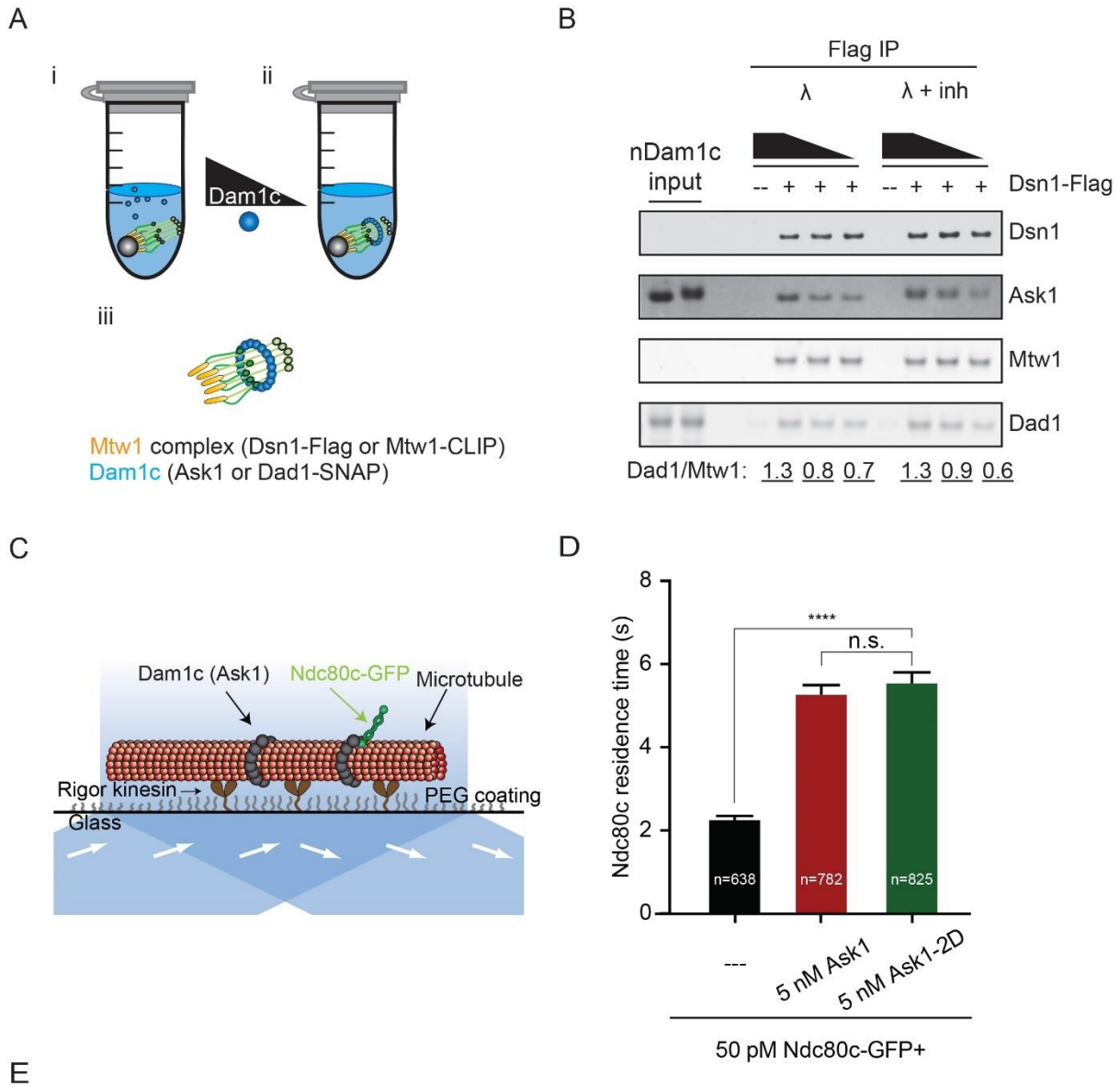
D) Attachment survival probability versus force for data in C).



2.5.3 Figure 2.3. Cdk1 mediated phosphorylation of recombinant Dam1c reconstitute wild-type kinetochore-microtubule attachment strength.

A) Mean rupture forces for Dad1-1 kinetochores in the presence of soluble rDam1c^{Ask1} or rDam1c$^{Ask1\text{-}2D}$ with or without Cdk1 phosphorylation. Error bars represent the standard error of the mean (SEM; n = 22-27 events). P-values using a two-tailed unpaired t test (****= p<0.0001). The mean rupture forces for Dad1-1 with non-phosphorylated rDam1c^{Ask} is from figure (2.1C). The purified rDam1c was analyzed by silver stain SDS-PAGE (right panel).

B) Attachment survival probability versus force for data in A).


A
i
ii
iii
Dam1c
Mtw1 complex (Dsn1-Flag or Mtw1-CLIP)
Dam1c (Ask1 or Dad1-SNAP)
B
Flag IP
λ
λ + inh
nDam1c input
-- + + + -- + + + Dsn1-Flag
Dsn1
Ask1
Mtw1
Dad1
Dad1/Mtw1: 1.3 0.8 0.7 1.3 0.9 0.6
C
Dam1c (Ask1)
Ndc80c-GFP
Microtubule
Rigor kinesin
PEG coating
Glass
D
Ndc80c residence time (s)
****
n.s.
n=638
n=782
n=825
---
5 nM Ask1
5 nM Ask1-2D
50 pM Ndc80c-GFP+
E
(%) Survival Probability
100
10
50 pM Ndc80-GFP +
--- --- ---
5nM Ask1
5nM Ask1-2D
0
5
10
15
20
Ndc80c residence time (s)

2.5.4 Figure 2.4. Dam1c phosphorylation does not enhance its interaction with the kinetochore's Ndc80c.

A) Schematic of the rebinding assay. Immobilized, (i) magnetically bound kinetochores via Mtw1 complex (Dsn1-Flag, orange oval; ii) with the addition of gradient Dam1c (blue sphere, concentration gradient: 9, 3 or 1 ng; iii). The ratio of Dam1c to Mtw1 was analyzed via immunoblot and fluorescently imaged with a Typhoon Trio to uncover if phosphorylation influenced Dam1c-kinetochore binding.

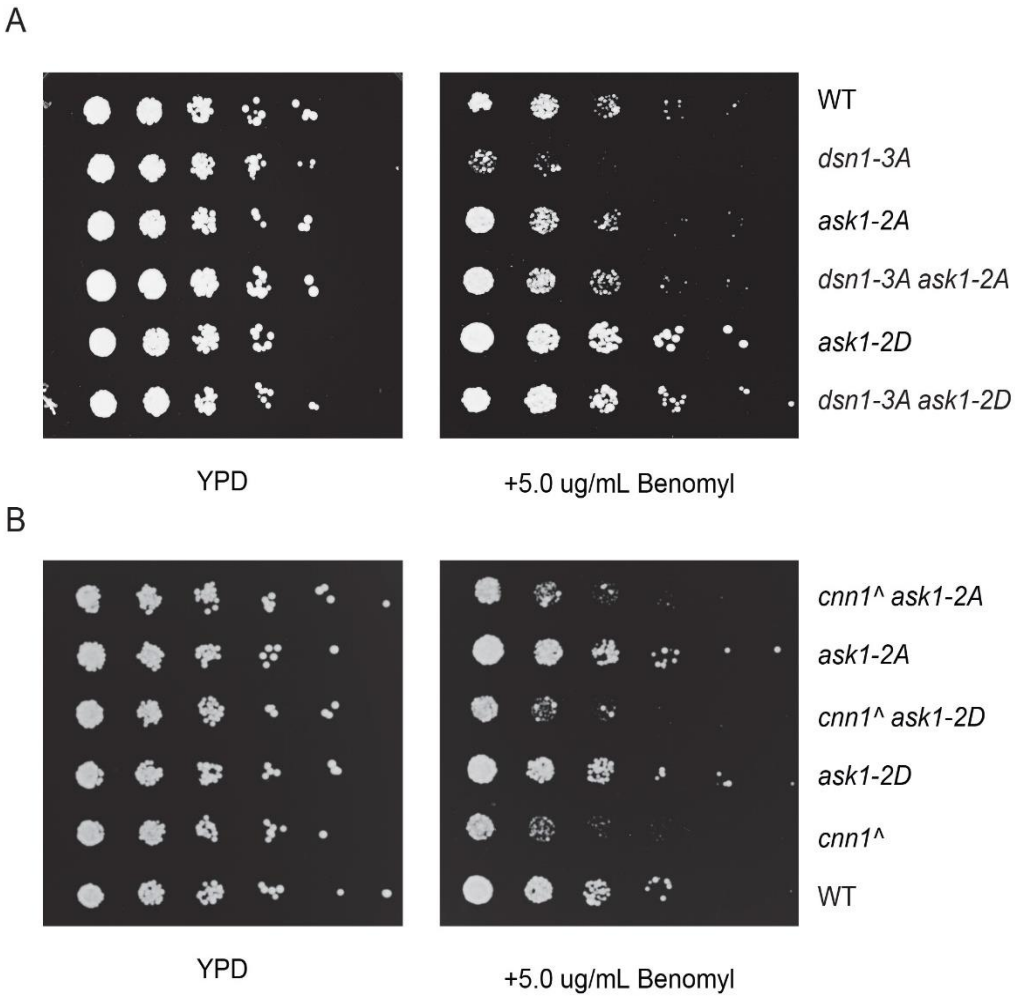
B) Kinetochores were purified from *dad1-1* cells via Dsn1-Flag immunoprecipitation (MTW-CLIP; SBY16828), dyed and retained on beads. Purified nDam1c (Dad1-SNAP-3V5; SBY16766) that was dyed and pre-treated with either phosphatase (λ) or phosphatase plus inhibitors (λ+inh) was incubated at 3-fold dilutions with the immobilized kinetochores. After excess nDam1c was removed, the kinetochores were eluted using α-flag peptide and then analyzed via immunoblot to detect Dsn1 and Ask1 using Flag and Dam1c antibody, respectively. Dad1-SNAP-3V5 and Mtw1-CLIP were fluorescently imaged with a Typhoon Trio. The Dad1/Mtw1 ratio was calculated for each sample and did not vary significantly between the phosphatase-treated and mock samples at any concentration.

*Phosphorylated Ask1 is spread in the immunoblot because it has bands that are phosphorylated and non-phosphorylated running proximal to one another. This issue is absent when phosphatase treated. Therefore, the Dam1c was quantified using the Dad1 protein of the Dam1c.

C) Schematic of the TIRF assays. Wild-type rDam1c (rDam1c^{Ask1}; grey sphere, 5 nM) or mutant rDam1c Ask1-S216D, S250D (rDam1c$^{Ask1\text{-}2D}$; grey sphere) were added with (50 pM) single molecule Ndc80c-GFP (green intertwining rods; Nuf2-GFP). Time was recorded once samples were residing on taxol stabilized microtubules.

D) Average residence time of Ndc80c-GFP on the microtubule by itself or with either recombinant rDam1c^{Ask1} or mutant rDam1c$^{Ask1\text{-}2D}$ Dam1c. Bars represent average residence time ± error of the mean (****= $p<0.0001$, n.s.= not significant).

E) Attachment survival probability versus residence time for data in D).

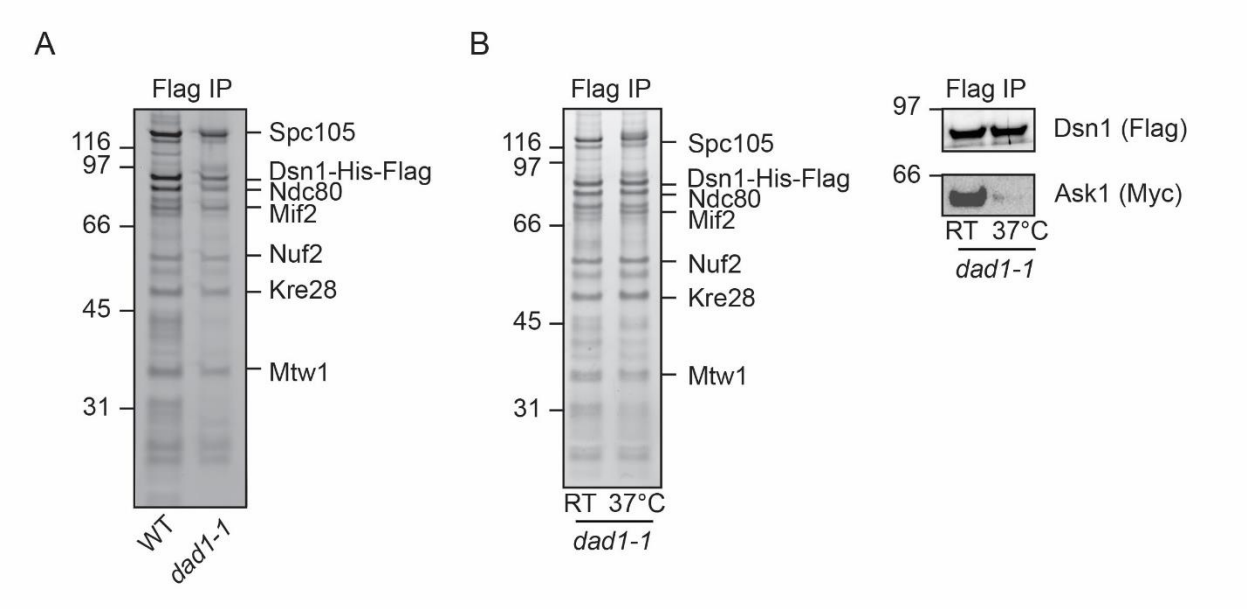


2.5.5 Figure 2.5. Phosphorylation of Ask1 protein can mildly withstand induced depolymerization when dependent on the Ndc80c-Cnn1 pathway, *in vivo*.

A) Five-fold serial dilutions of WT (SBY18086), *dsn1-3A* (SBY18460), *ask1-2A* (SBY18088), *ask1-2A dsn1-3A* (SBY18462), *ask1-2D* (SBY18089) and *ask1-2D dsn1-3A* (SBY18464) cells were plated onto yeast media. Samples were grown at 23 °C (left) or 30 °C with benomyl (right).

B) Five-fold serial dilutions of *ask1-2D cnn1^* (SBY17869), *cnn1^* (SBY17831), *ask1-2D cnn1^* (SBY17874), *ask1-2D* (SBY17833), *cnn1^* (SBY17934) and WT (*Ask1*, SBY17823) cells were plated onto yeast media. Samples were grown at 23 °C alone (left) or with benomyl (right).

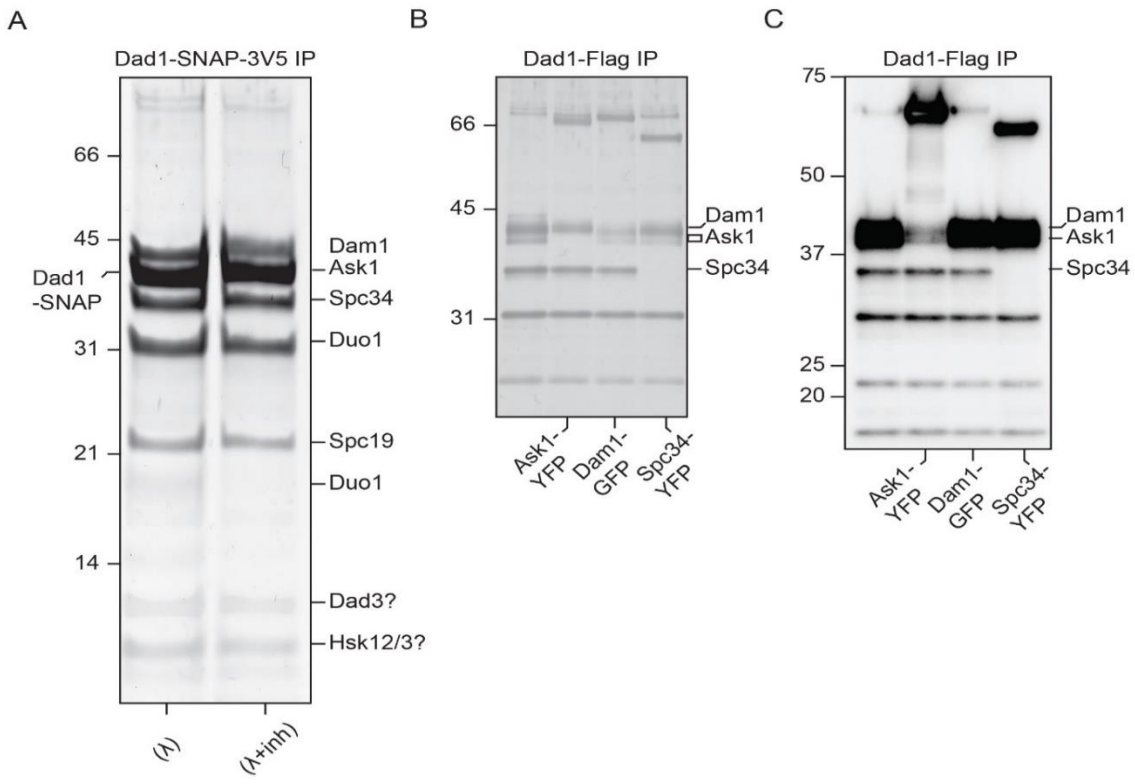
2.6 Supplemental Figure Legend



2.6.1 Supplemental Figure 2.1. Kinetochores purified from *dad1-1* cells lack kinetochore-associated Dam1c but retain core kinetochore components.

A) Kinetochores purified by immunoprecipitation of Dsn1-Flag from wild-type cells (SBY8253) or Dam1c temperature-sensitive *dad1-1* mutant cells (SBY8944). Cells were grown at room-temperature (RT) to compare the two. Proteins were analyzed by silver stained SDS-PAGE. Molecular weight markers (kDa) are indicated on the left of the SDS-PAGE.

B) Cells pulled by Dsn1-Flag and the temperature-sensitive *dad1-1* mutant with *ask1-12MYC* (SBY13486) were grown at room-temperature (RT) or the non-permissive temperature, 37 °C and kinetochores were purified. The associated proteins were analyzed by silver stained SDS-PAGE (left) and by immunoblot (right). The core kinetochore proteins are retained but the Dam1 complex is absent when cells were grown at 37 °C.

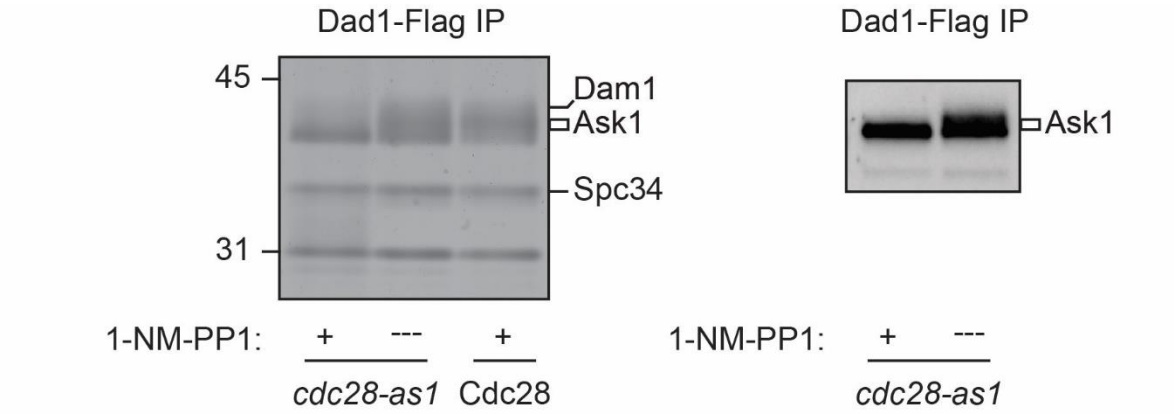


2.6.2 Supplemental Figure 2.2. Identifying the Dam1c components by protein gel mobility shift in epitope-tagged proteins

A) Visualizing the smaller subunits of the Dam1c by purifying nDam1c from cells via Dad1-SNAP-3V5 (SBY16766) by using fivefold greater volume of sample when compared to other silver stained gel images. nDam1c was eluted with V5 peptide and analyzed by silver stain SDS-PAGE. The lower subunits of the Dam1c are not determined by silver stain, but by mass spectrometry all ten subunits were determined to be present on the gel.

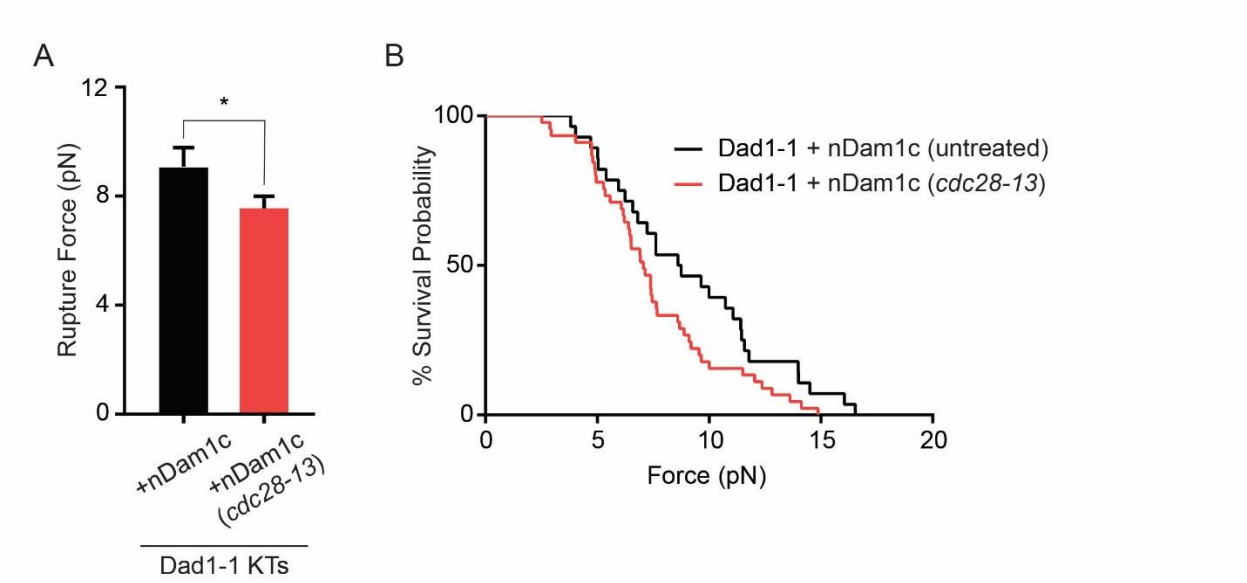
B) The identity of some Dam1c components was determined by purifying nDam1c from cells via Dad1-3Flag (SBY12464) alone or with the addition of epitope tags on Ask1 (Ask1-YFP; SBY19661), Dam1 (Dam1-GFP; SBY1659) or Spc34 (Spc34-YFP; 19657). nDam1c was eluted with flag peptide and analyzed by silver stain SDS-PAGE

C) Immunoblot sample of Dad1-3Flag (SBY12464) alone or with the addition of epitope tags on Ask1 (Ask1-YFP; SBY19661), Dam1 (Dam1-GFP; SBY1659) or Spc34 (Spc34-YFP; 19657). nDam1c was eluted with flag peptide and analyzed by silver stain SDS-PAGE and probed with polyclonal α-Dam1c antibodies that recognize multiple Dam1 complex components, specifically Ask1 protein.



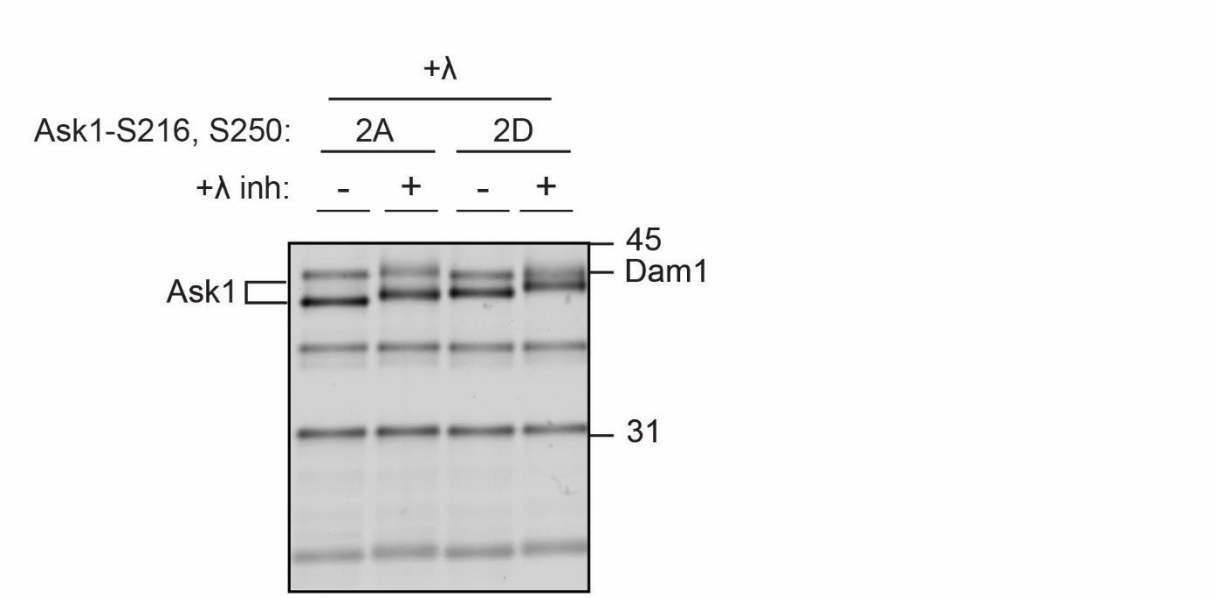
2.6.3 Supplemental Figure 2.3. *cdc28-as1* strain is specifically sensitive to analog 1-NM-PP1 and only then can it no longer phosphorylate Ask1.

The function of Cdc28 was disrupted with analog-sensitive strain, *cdc28-as1* (SBY13509) when in the presence of analog 1-NM-PP-1 but not when mock treated, DMSO (left panel). Wild-type CDK1 (SBY12464) that had been treated with analog 1-NM-PP-1 did not affect its kinase activity as observed by a retarded shift on Ask1 protein, suggesting phosphorylation is occurring. Purified Dam1c was visualized by silver stained SDS-PAGE (left panel) and by immunoblot with polyclonal α-Dam1c antibodies (right panel). Molecular weight markers (kDa) are indicated on the left.



2.6.4 Supplemental Figure 2.4. Cdk1 activity is required for wild-type kinetochore-microtubule attachment strength.

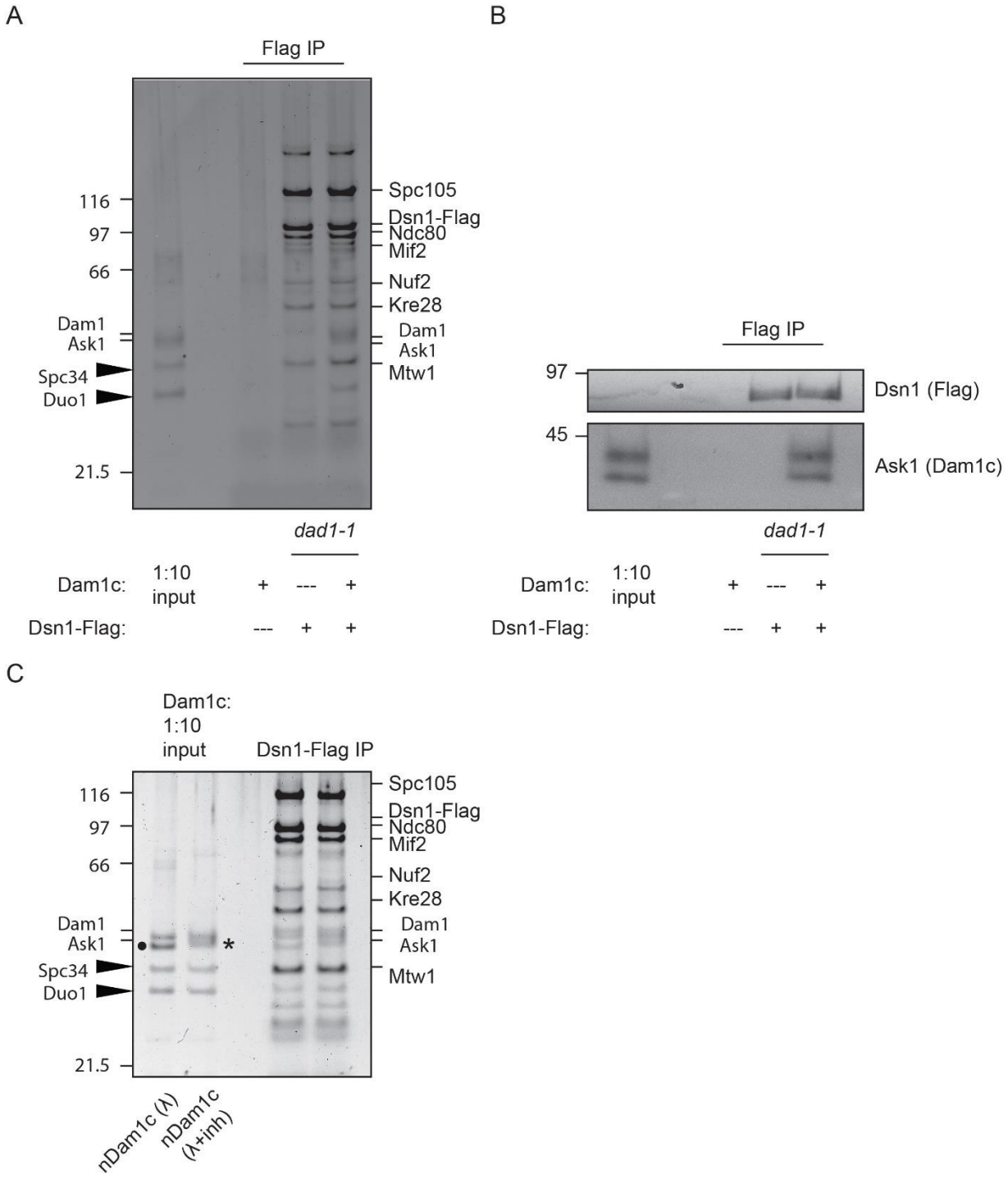
A) To test the role of Cdk1 in Dam1c function, the Dam1c was purified from wild-type (SBY8253) and *cdc28-13* (SBY13507) cells that had been shifted to 37 °C for 2.5 hrs. The soluble purified nDam1c was added back to Dad1-1 kinetochores (SBY8944) and the mean rupture force was measured. Error bars represent standard error of the mean (SEM; n = 36–45 events). P-values were determined using a two-tailed unpaired t test (*= $p<0.05$).



B) Attachment survival probability versus force.

2.6.5 Supplemental Figure 2.5. There are Ask1 phosphorylation sites in addition to the Cdk1 sites.

nDam1c with protein Dad1-3Flag was purified from cells containing *ask1-S216A, S250A* (2A; SBY17831) or *ask1-S216D, S250D* (2D; SBY17833) alleles and immobilized via α-flag magnetic beads. The complexes were incubated with phosphatase in the absence or presence of phosphatase inhibitors. nDam1c was washed, eluted by flag peptide and analyzed by silver stain SDS-PAGE. Phosphatase treatment alters the migrations of the mutant proteins, indicating there are additional phosphorylation sites on Ask1.


A
Flag IP
116
97
66
Dam1
Ask1
Spc34
Duo1
21.5
Spc105
Dsn1-Flag
Ndc80
Mif2
Nuf2
Kre28
Dam1
Ask1
Mtw1
dad1-1
Dam1c:
1:10 input
+

+
Dsn1-Flag:

+
+
B
Flag IP
97
45
Dsn1 (Flag)
Ask1 (Dam1c)
dad1-1
Dam1c:
1:10 input
+

+
Dsn1-Flag:

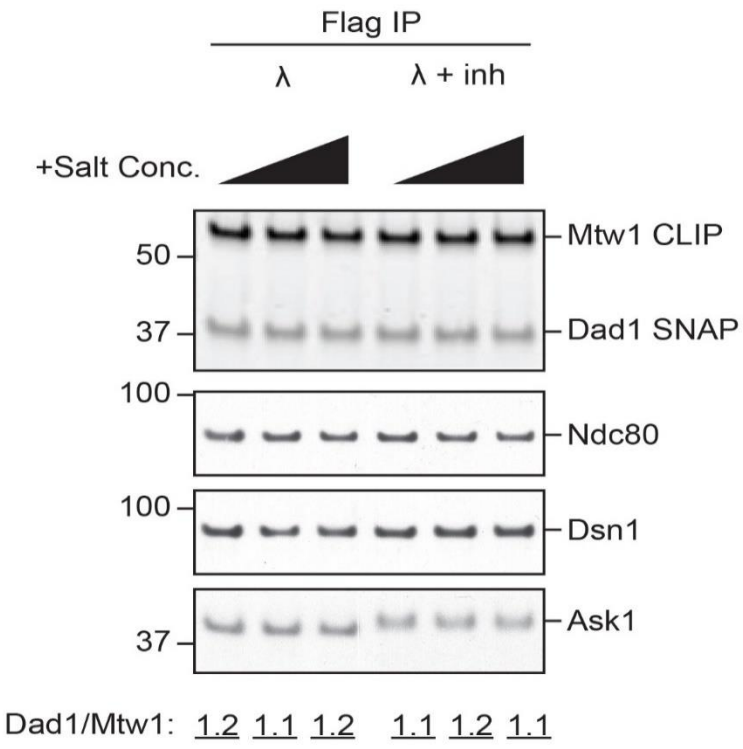
+
+
C
Dam1c: 1:10 input
Dsn1-Flag IP
116
97
66
Dam1
Ask1
Spc34
Duo1
21.5
Spc105
Dsn1-Flag
Ndc80
Mif2
Nuf2
Kre28
Dam1
Ask1
Mtw1
nDam1c (λ)
nDam1c (λ+inh)

2.6.6 Supplemental Figure 2.6. nDam1c, phosphorylated or de-phosphorylated, can rebind to Dad1-1 kinetochores

A) α-flag magnetic beads were used to purify Dsn1-Flag and retain kinetochores. WT (Dsn1; SBY3) or *dad1-1* (Dsn1-3Flag *dad1-1*; SBY8944) cells were incubated and retained by Dsn1-Flag. Purified nDam1c (Dad1-3V5; SBY13538) was untreated and incubated with the immobilized kinetochores. After excess nDam1c was removed, kinetochores were eluted using α-flag peptide. kinetochores were analyzed by silver stained SDS-PAGE

B) Immunoblot of A) to detect associated proteins. Dsn1 and Ask1 proteins were detected by using Flag and polyclonal Dam1c antibody, respectively. There was no non-specific binding.

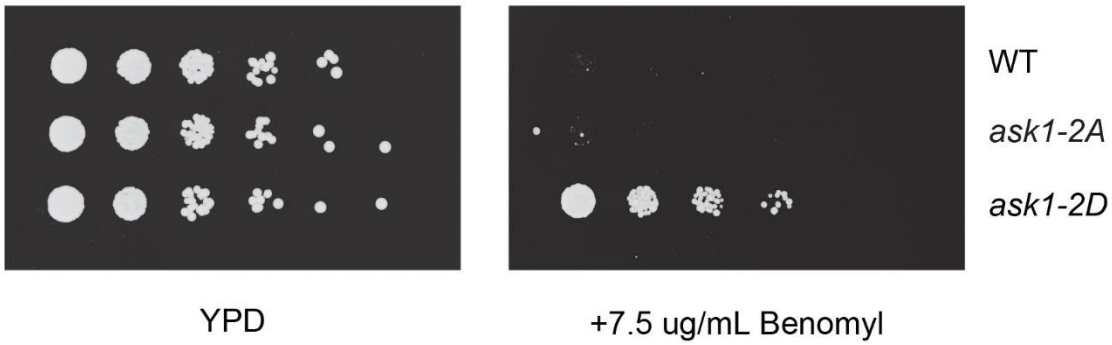
C) Kinetochores were purified from *dad1-1* cells (Dsn1-3Flag *dad1-1*; SBY8944) via Dsn1-Flag immunoprecipitation and retained on beads. Purified nDam1c (Dad1-3V5; SBY13538) was pre-treated with either phosphatase (λ) or phosphatase plus inhibitors (λ+inh) and incubated with the immobilized kinetochores. The associated proteins were analyzed by silver stained SDS-PAGE.



2.6.7 Supplemental Figure 2.7. Phosphorylated Dam1c does not enhance its interaction with the kinetochore under high salt washes.

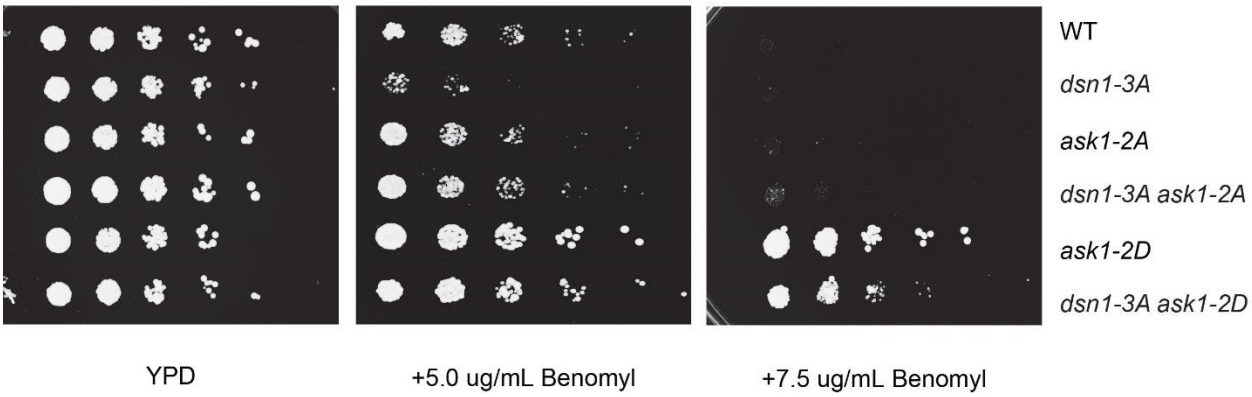
Kinetochores were purified from *dad1-1* cells via Dsn1-Flag immunoprecipitation (MTW-CLIP; SBY16828), dyed and retained on beads. Purified nDam1c (Dad1-SNAP-3V5; SBY16766) that was dyed and pre-treated with either phosphatase (λ) or phosphatase plus inhibitors (λ+inh) was incubated at 9 ng with the immobilized kinetochores and received a high gradient salt wash (0.25 0.275 and 0.3 M KCl) before the kinetochores were eluted using α-flag peptide. Kinetochores were analyzed on SDS-PAGE, and immunoblotted to detect associated proteins, Dsn1 and Ask1, by using Flag and polyclonal Dam1c antibody, respectively. Samples were also detected by fluorescent imaging via Dad1-SNAP-3V5 and Mtw1-CLIP. The Dad1/Mtw1 ratio was calculated for

each sample and did not vary significantly between the saturated phosphatase-treated and mock samples at any salt concentration.



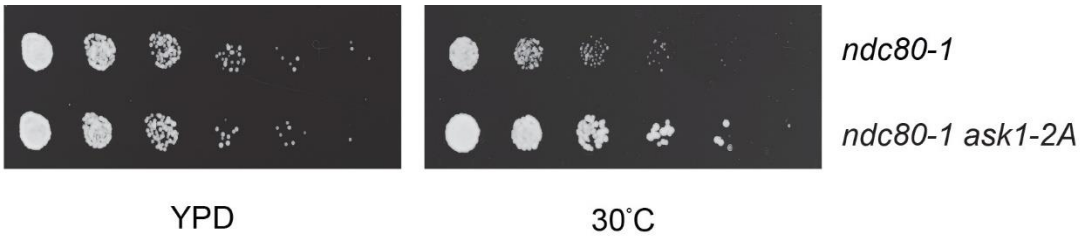
2.6.8. Supplemental Figure 2.8. Ask1-2D is resistant to microtubule depolymerizing drug, benomyl.

Five-fold serial dilutions of WT (SBY18086), ask1-2A (SBY18088), and ask1-2D (SBY18089) cells were plated onto in YPD (left) or with benomyl (right).



2.6.9 Supplemental Figure 2.9. Phosphorylation of Ask1 protein can withstand highly induced depolymerization when dependent on the Ndc80c-Cnn1 pathway, *in vivo*.

Five-fold serial dilutions of WT (SBY18086), *dsn1-3A* (SBY18460), *ask1-2A* (SBY18088), *ask1-2A dsn1-3A* (SBY18462), *ask1-2D* (SBY18089) and *ask1-2D dsn1-3A* (SBY18464) cells were plated onto yeast media. Samples were grown in YPD at 23 °C alone (left) or with 5.0 (center) or 7.5 ug/mL of benomyl (right).



2.6.10 Supplemental Figure 2.10. Ask1-2A rescues temperature-sensitive ndc80-1 *in vivo*

Preliminary data of five-fold serial dilutions of *ndc80-1* (SBY18063) and *ndc80-1 ask1-2A* (SBY18016). Cells were plated onto yeast media. Samples were grown in YPD at 23 °C (left) or 30 °C (right).

2.7 Materials and Methods

Strain construction: Yeast Strains and Plasmids

Saccharomyces cerevisiae strains used in this study are derived from or backcrossed to SBY3 to be isogenic with the W303 background and are listed in Supplementary Table 1. All strains containing the following epitope-tagged genes are generated by standard PCR-based integration techniques at the endogenous loci as described in (Longtine et al., 1998) and are fully functional. Primer sequences are listed in Supplementary Table 2. *DSN1-6His-3Flag* is described in (Akiyoshi et al., 2010). *DAD1-3V5* was made with primers SB4448 and SB4449 and template plasmid pSB2047. *DAD1-3Flag* was made with primers SB4214 and SB4215 and template plasmid pSB1265. *MTW1-CLIP* was made with primers SB3109 and SB3111 and template plasmid pSB1824. *DAD1-SNAP-3V5* as made with primers SB5091 and SB5092 and template plasmid pSB2402. *CDC28-3V5* was made with primers SB5892 and SB5893 and template plasmid pSB2068. The *dam1-S20D* mutant originates from David Drubin's lab (DDY2486) (Cheeseman et al., 2002) and was backcrossed to SBY3. *Dam1-S20D* strains were derived from SBY3720 and were used in previous work (Sarangapani et al., 2013). *ASK1* plasmids were constructed as follows. *ASK1* was amplified by PCR using a primer containing sequence upstream of the *ASK1* gene and a recognition site for the restriction enzyme XhoI (SB5468) and a primer downstream of the *ASK1* stop codon with a recognition site for the restriction enzyme EagI (SB5469). The PCR product and a *LEU2* integrating vector (pSB2223) were digested with XhoI and Eag1 and ligated to create pSB2900. This plasmid was used as a template to make site directed mutations at *ASK1-S216* and *S250*, switching serine to alanine (pSB2904) by site directed mutagenesis with primers SB5602 and SB5604 or altering serine to aspartic acid (pSB2905) with primers SB5603 and SB5605. All

plasmids were verified by sequencing. The non-essential yeast chromosome fragment strain was a gift of the Kim Nasmyth (YPH278) (Spencer, Gerring, Connelly, & Hieter, 1990) and backcrossed to W303. *Cdc28-as1* was a gift from the Shokat lab and backcrossed into the W303 strain and used in (Akiyoshi et al., 2010).

Construction of *ASK1* mutant strains at the *LEU2* locus:

The following steps created strains in which *ask1* mutants were integrated at the *LEU2* locus and the endogenous *ASK1* gene was deleted. First, a heterozygous *ASK1/ask1* diploid strain was generated (SBY17818) by deleting one copy of *ASK1* by PCR integration with PCR product produced with primers SB5523 and SB5524 and template plasmid pSB54 as described in (Longtine et al., 1998). The *ASK1* deletion was verified by PCR with primers SB5532 and SB5121 and then subsequently by tetrad dissection that resulted in 2:2 viability. The various *ASK1* plasmids (plasmids pSB2900, pSB2904, and pSB2905, described above) were digested with SwaI and transformed into SBY17818 to integrate them at the *LEU2* locus and then dissected to identify the correct genotypes.

Protein biochemistry

Native kinetochores and kinetochore subcomplexes were purified from either conditionally treated or asynchronously grown cells. Cells containing *cdc28-as1* were grown to OD_{600} 0.6-1.0 and then arrested in G1 with alpha factor (1 µg/mL in DMSO) for 3 hr, and at 2.5 hr the compound 1-NM-PP1 (0.5 µM in DMSO; Toronto Research Chemical, A603003) was added. Cells were washed twice to remove alpha-factor with equal volumes of YPD containing 0.5 µM of 1-NM-PP1. Cells were then resuspended in YPD with 5.0 µM of 1-NM-PP1 for 2-2.5 hr before cells were

harvested. Cells from the *cdc28-13* temperature sensitive strain were shifted to the non-permissive temperature (37 ˚C) for 2.5 hr before harvesting. Protein lysate was prepared by lysing cells with a magnetic impact bar submerged in liquid nitrogen using a Freezer/Mill (SPEX SamplePrep). Lysed cells were resuspended in buffer H (BH) (25 mM HEPES pH 8.0, 2 mM MgCl2, 0.1 mM EDTA, 0.5 mM EGTA, 0.1% NP-40, 15% glycerol with 150 mM KCl for native kinetochores) containing protease inhibitors (at 20 µg/mL final concentration for each of leupeptin, pepstatin A, chymostatin and 200 µM phenylmethylsulfonyl fluoride) and phosphatase inhibitors (0.1 mM Na-orthovanadate, 0.2 µM microcystin, 2 mM β-glycerophosphate, 1 mM Na pyrophosphate, 5 mM NaF) followed by ultracentrifugation at 24,000 RPM for 90 min at 4 °C. Lysates were incubated with α-Flag or α-V5 conjugated dynabeads for 3 hr with constant rotation at 4 °C (Akiyoshi et al., 2010). For kinetochore purification, Dsn1-6His-3Flag strains were used,for the yeast Dam1 complex, Dad1-3Flag or Dad1-3V5, for recombinant Dam1 complex, Spc34-Flag (used in "kinase assay" below), and for yeast Cdk1, Cdc28-3V5 was used. Samples were washed three times with BH containing protease inhibitors, phosphatase inhibitors, 2 mM dithiothreitol (DTT) and either 150 mM KCl (for kinetochore purifications) or 400 mM KCl (for Dam1 complex purifications). Beads were further washed twice with BH containing 150 mM KCl and protease inhibitors. Associated proteins were eluted from the beads by gentle agitation of beads in elution buffer (0.5 mg/mL 3Flag peptide or 0.5 mg/ml 3V5 peptide in BH with 150 mM KCl and protease inhibitors) for 20-30 min at room temperature. Eluted samples were used or snap-frozen in liquid nitrogen and stored at -80 °C.

Phosphatase treatment

Yeast lysates were incubated with Dynabeads conjugated with α-Flag or α-V5 antibodies to immobilize Dam1c and separate it from the cell lysate (described above in Protein biochemistry). Prior to elution from the beads, the Dam1c was incubated for 30 min with lambda phosphatase at 30 ˚C (BH 0.15, 1 mM MnCl2, 22 units-Lambda Protein Phosphatase; New England Biolabs, P0753L). Control samples contained phosphatase inhibitors. The Dam1c was then washed and eluted as described above.

Immunoblot and silver stain analysis

For immunoblot analysis, cell lysates were prepared as described above (Protein biochemistry section). Protein samples were separated in pre-cast 4-12% or 10% Bis Tris Protein Gels (Thermo-Fisher) and standard procedures for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were followed as described in (Akiyoshi et al., 2010). A 0.45 µm nitrocellulose membrane (BioRad) was used to transfer proteins from polyacrylamide gels. Commercial antibodies used for immunoblotting were: α-Flag, M2 (Sigma-Aldrich) 1:3,000; α-GFP, JL-8 (Living Colors) 1:5,000; α-Myc, 9E10 (Covance) 1:10,000; A polyclonal α-Dam1c antibody was generated by injecting recombinant Dam1c into rabbits at Pacific Immunology (Ramona, CA). The serum was then used 1:500. The secondary antibodies used were a sheep α-mouse antibody conjugated to horseradish peroxidase (HRP) (GE Life sciences) at a 1:10,000 dilution or a donkey α-rabbit antibody conjugated to HRP (GE Life sciences) at a 1:10,000 dilution. Antibodies were detected using the Super Signal West Dura Chemiluminescent Substrate (Thermo Scientific). For analysis by silver stain, the gels were stained with Silver Quest Staining Kit (Invitrogen).

In vitro binding assays

To examine the binding of Dam1c to Dad1-1 kinetochores, Dam1c was first fluorescently labeled. Dam1c (Dad1-SNAP-3V5) was purified as described above and then incubated in buffer containing SNAP-Surface 647 dye (10 μM in BH 0.15 M KCl and phosphatase and protease inhibitors) for 30 min with continuous gentile agitation while immobilized on beads. The excess dye was washed away with three washes of BH 0.15 M KCl and protease inhibitors and then the Dam1c was eluted. To test the interaction of Dam1c with kinetochores, the Dad1-1 kinetochores was also fluorescently labeled using CLIP-surface 547 dye and purified as described above and immobilized on α-Flag dynabeads. They were incubated with three-fold dilutions of purified Dam1c (starting at 9 ng) in buffer BH 0.15 M KCl and phosphatase and protease inhibitors for 30 min at room temperature with continuous gentile agitation. Beads were then washed three times with BH containing 150 mM KCl and protease inhibitors. Kinetochore bound to beads were eluted as described above. In the case of Supplementary Figure 2.7, nDam1c was constant at 9 ng but high gradient salt washes were used (0.25, 0.275 and 0.3 M KCl a protease inhibitor as described above. Kinetochore bound to beads were eluted as described above.

Phosphorylation kinase assays

Yeast lysates were incubated with Dynabeads conjugated with α-V5 antibodies to immobilize Cdk1 (Cdc28-3V5) and separate it from the cell lysate to then be eluted (described above in Protein biochemistry). Soluble Cdk1 was then incubated for 30 min with soluble rDam1c (Spc34-Flag) in MOPS Assay Buffer (MAB) for 30 min at room temperature with continuous agitation (MAB, 25 mM MOPS, 15 mM MgCl2, 5 mM EGTA) containing protease inhibitors and phosphatase

inhibitors with 0.8 mM ATP. Control samples excluded Cdk1. The rDam1c was then immobilized, washed and eluted as described above.

Recombinant protein expression and purification

All ten *S. cerevisiae* Dam1 complex subunits were expressed in *E. coli* (BL21 Rosetta 2; Novagen, Madison, WI) from a single polycistronic vector (reference miranda I think). The complex was affinity purified using a C-terminal 6xHis-tag or Flag-tag on Spc34p, and subjected to gel filtration, as previously described (Franck et al., 2007; Gestaut et al., 2008; Kim et al., 2017; Tien et al., 2010). For TIRF microscopy experiments, the Dam1 complex was tagged with a C-terminal GFP tag on Dad1 protein. The *S. cerevisiae* Ndc80 complex was expressed in *E. coli*, using a C-terminal 6xHis-tag before being subjected to gel filtration, as previously reported (Kim et al., 2017; Powers et al., 2009; Tien et al., 2010; Wei, Sorger, & Harrison, 2005).

TIRF microscopy

Flow chambers were constructed using glass slides and functionalized coverslips as reported before (Gestaut et al., 2008; Tien et al., 2010). Coverslips were 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. Testing the interaction between the Dam1 and Ndc80 complexes, 50 pM GFP-tagged Ndc80 complex was incubated alone or in the presence of 5 nM untagged Dam1c ^{Ask1} or Dam1c $^{Ask1\text{-}2D}$ to ensure Dam1 complex oligomerization. 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, 25mM

glucose and 5mM dithiothreitol). 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 and developed in LabVIEW (National Instruments) and Igor Pro (Wavemetrics) (Gestaut, Cooper, Asbury, Davis, & Wordeman, 2010; Gestaut et al., 2008; Tien et al., 2010; Umbreit et al., 2014). Mean residence times were carried out through bootstrapping analysis (Kim et al., 2017; Umbreit et al., 2014). Each residence time data set was randomly resampled with replacement. All the data sets presented formed normal distributions; mean and standard deviation of the bootstrapped dataset are reported.

Optical trap assays

Optical trap-based bead motility assays were performed as in (Akiyoshi et al., 2010; Miller et al., 2019). Streptavidin-coated 0.56-µm polystyrene beads (Spherotech) were functionalized with biotinylated anti-penta-His antibody (Qiagen) and stored in BRB80 containing 8 mg/ml BSA and 1 mM DTT at 4 °C with continuous rotation. Beads were decorated with purified kinetochores (via Dsn1-6His-3Flag) in a total volume of 20 µl incubation buffer (BRB80 containing 1.5 mg/mL κ-casein). Kinetochore particles were diluted such that the concentration of Dsn1-6His-3Flag was ~0.4 ng/uL, and then incubated with 6 pM beads for 1 h at 4 °C. For native Dam1c add back, kinetochores received a final concentration of ~2 nM. The concentration of rDam1c and nDam1c were obtained by comparing the intensity of Spc34 and Duo1 to dilutions of BSA standards on silver-stained SDS-PAGE using a standard curve. The BSA standards and its derived equation were run on the same gel for the calculated Dam1c concentrations. Kinetochores were incubated in

the microtubule growth buffer (see below). Dynamic microtubule extensions were grown from coverslip-anchored GMPCPP-stabilized microtubule seeds in a microtubule growth buffer consisting of BRB80, 1 mM GTP, 250 µg/ml glucose oxidase, 25 mM glucose, 30 µg/mL catalase, 1 mM DTT, 1.4-1.5 mg/mL purified bovine brain tubulin and 1 mg/mL κ-casein. Assays were performed at 23 °C. Rupture force experiments were performed as in (Akiyoshi et al., 2010; Miller et al., 2019). Briefly, an optical trap was used to apply a force of ~1-4 pN in the direction of microtubule assembly. Once beads were observed to track with microtubule growth for a distance of ~100-300 nm (to ensure end-on attachment), the applied force was increased at a constant rate of 0.25 pN/s until bead detachment. Records of bead position over time were generated and analyzed using custom software (Labview and Igor Pro, respectively) and used to determine the rupture force, which was marked as the maximum force sustained by the attachment during each event.

2.8 Acknowledgement of contribution of collaborators and funding

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3. Chapter 3: CDK1 phosphorylation of Dam1c promotes oligomerization

3.1 Summary

With the interesting result of phosphorylation helping kinetochore-microtubule coupling, and in determining that it was not through Dam1c-kinetochore interaction, we investigated whether phosphorylated Dam1c positively regulates its function at the microtubule. To elucidate how Cdk1-phosphorylation of Ask1 is mechanistically functioning, we used TIRF microscopy, the optical-trap, and genetics and found that it does not impact Dam1c binding as a monomer, but it does have a role in oligomerization. Using genetic interactions, we were also able to support that phosphorylation of Dam1c is a tightly regulated system, an important aspect that plays a role in the accuracy of chromosomal segregation.

3.2 Introduction

In the previous chapter we discovered Cdk1 phosphorylation of Dam1c's Ask1 was responsible for the strength in kinetochore-microtubule attachments. Despite literature to suggest that this mechanism would be via its interaction to the Ndc80c/kinetochore, this was not the case. There are two additional known mechanisms of the Dam1c: 1) binding to microtubules and 2) oligomerizing around the microtubule.

Dam1c microtubule binding is facilitated via microtubule's negatively charged, C-terminal tail, also known as the E-hook for its numerous glutamic acid residues (Ramey H V et al., 2010). When the Dam1c is incubated *in vitro* it has an attraction to microtubules at GMPCPP islands, sites that mimic the GTP lattice of microtubules (Westermann et al., 2005). This is a trait of Dam1c setting itself at the plus-end of microtubules, where it resides to function with the

dynamic environment of growing and shrinking tips. Dam1c can reside to the microtubule dependent on two of its ten proteins, Dam1 and Duo1, which are essential to tracking microtubules dynamics. Following its time at the microtubule, oligomerization formation can begin to occur.

With Dam1c monomers beginning to interact with each other what follows is the process of the complexes wrapping around the microtubule. Oligomerization of Dam1c is essential as it is proposed to function as a collar around the microtubule and can continue to track microtubule plus-ends under great tension, a mechanism that would ensure kinetochores are retained at the spindle (Asbury, Gestaut, Powers, Franck, & Davis, 2006; Doodhi et al., 2019; E L Grishchuk et al., 2008; Lampert, Hornung, & Westermann, 2010; Tanaka, Kitamura, Kitamura, & Tanaka, 2007; Tien et al., 2010; Westermann et al., 2005, 2006; Umbreit et al, 2014). However, despite the vast information as to how phosphorylation negatively regulates Dam1c, it is unknown whether there are specific regulatory events that promote Dam1c oligomerization to ensure accurate segregation.

Here, we used TIRF microscopy, the optical trap and genetic interaction to discover that Cdk1 phosphorylation of the Ask1 is responsible for Dam1c oligomerization. Ring formation is important to ensure that kinetochore-microtubule attachments are stabilized as its interface comes under tension during metaphase and needs to maintain processive attachments to disassembling microtubules at anaphase. Her, we also show the formation of rings must be well regulated or cells can undercome abnormal chromosome segregation.

3.3 Results

Cdk1 phosphorylation of Ask1 strengthens kinetochore-microtubule attachments

The contribution of Cdk1 phosphorylation of Dam1c's Ask1 to strengthen tip-bound kinetochore-microtubule attachments (Figure 2.1) led us to determine that phosphorylation was not enhancing the Dam1c interaction to the kinetochore. The Dam1c contains two additional functions: 1) binding to microtubules and 2) oligomerizing around the microtubule.

Cdk1 phosphorylation of Ask1 does not enhance Dam1c's microtubule binding function

The Dam1c can contact and bind microtubules as a monomer; this function may allot the complex time to then form the essential 17 individual Dam1c units to form a ring around microtubules (Jenni & Harrison, 2018). In collaboration with Jae ook Kim, we tested whether phosphorylation promotes monomeric microtubule binding using single-molecule TIRF microscopy (Ekaterina L. Grishchuk et al., 2008; Joglekar, Bouck, Molk, Bloom, & Salmon, 2006; Miranda et al., 2005; Ramey et al., 2011; Westermann et al., 2005). First, we tested whether phosphorylation promotes the initial binding of the Dam1c to the lattice of the microtubule. To do this, we monitored the binding of single monomers on individual, taxol-stabilized microtubules using a concentration of purified recombinant Dam1c (50 pM) that was low enough to prevent its oligomerization (Tien et al., 2010) (Figure 3.1A). We measured the residence times of the fluorescently labeled wild-type rDam1c$^{Ask1\text{-}GFP}$ and phosphomimetic rDam1c$^{Ask1\text{-}2D\text{-}GFP}$ monomers on the microtubules and found that they behaved identically (Figure 3.1B and 3.1C), indicating that phosphorylation does not directly affect the interaction between microtubules and Dam1c monomers.

Ask1 phosphorylation facilitates Dam1c oligomerization

Dam1c initially contacts the microtubule as a monomer and then an estimated 17 individual complexes oligomerize to form a ring around microtubules (Jenni & Harrison, 2018). Using single-molecule TIRF microscopy, we tested whether Ask1 phosphorylation alters Dam1c oligomerization. To do this we used a concentration (1 nM) that can either tilt Dam1c microtubule binding in the monomeric state or towards the formation of oligomerization around microtubule. We mixed a concentration of non-fluorescent 'dark' rDam1c (1 nM) together with a small amount of fluorescent 'tracer' rDam1c (50 pM), to enable the observation of individual monomers under these conditions (Figure 3.2A). It has previously been demonstrated that the residence times for individual, fluorescent-tagged, microtubule-binding subcomplexes are increased when they hetero-oligomerize with other, non-fluorescent microtubule-binders (Kim et al., 2017; Umbreit et al., 2014; Zelter et al., 2015). We therefore mixed wild-type 'dark' rDam1c^{Ask1} with the wild-type 'tracer' rDam1c$^{Ask1\text{-}GFP}$ and found that the mean residence time of rDam1c$^{Ask1\text{-}GFP}$ was 8 seconds (Figure 3.2B and 3.2C), which is a ~2-fold increase over the residence time of rDam1c$^{Ask1\text{-}GFP}$ alone (Figure 3.1B and 3.1C). This increase in residence time indicates the formation of oligomers that bind more stably to the microtubules than monomers (Umbreit et al., 2014). We verify that this is sensitive only as a ring begins to form. Over saturating concentrations of 'dark' rDam1c (5 nM) with fluorescent 'tracer' rDam1c (50 pM) will tip the balance to favor oligomerization. The excess rDam1c causes rDam1c$^{Ask1\text{-}2D}$ to no longer have a noticeable effect in stabilizing onto microtubules (Supplemental Figure 3.1A and 3.1B). This result emphasizes that the effect of 1 nM is at the critical concentrations that's approaching oligomerization around the microtubule and, strikingly, the residence time was more

substantially increased when phospho-mimetic rDam1c$^{Ask1\text{-}2D}$ was used instead of wild-type rDam1c^{Ask1} (Figure 3.2B and 3.2C). This observation strongly suggests that phosphorylation of Ask1 promotes oligomerization of Dam1c.

Phosphorylation enhances nDam1c microtubule coupling

It was previously shown that rDam1c can bind to microtubule tips and withstand force in the optical trap assay so as long as they can oligomerize (Franck et al., 2007; Neil et al., 2014; Charles et al., 2006). Because phosphorylated nDam1c aids kinetochore-microtubule binding and phosphorylation is important to its oligomerization, we reasoned that phosphorylated nDam1c would have an advantage of oligomerizing to microtubules and withstanding higher forces when compared to de-phosphorylated nDam1c. We approached this by using an optical trap in which we directly linked 10 nM of nDam1c to polystyrene microbeads. Following nDam1c interaction with microtubules, force was applied and then gradually increased until the interaction at the microtubule-tip ruptured (Supplemental Figure 3.2A). Phosphorylated nDam1c ruptured with a mean strength of 6.6 pN while phosphatase-treated ruptured at 4.9 pN (Supplemental Figure 3.2B and 3.2C). The preliminary data suggests phosphorylation aids Dam1c in microtubule binding.

Ask1 phospho-regulation is important for accurate chromosome segregation

We next asked whether Cdk1 phosphorylation of Dam1c affects chromosome segregation *in vivo*. Although previous work found that the double phospho-mutants of Ask1 do not exhibit major growth defects, they were never analyzed for chromosome stability (Li & Elledge, 2003). To assess chromosome segregation, we performed a quantitative chromosome transmission

fidelity assay, or sectoring assay (Duffy & Hieter, 2017; Hieter, Mann, Snyder, & Davis, 1985). This strategy utilizes a non-essential chromosome fragment III (CFIII) whose loss is a five-fold higher to WT chromosomes and can be detected and quantified by a change in the color of the yeast cells (Figure 3.3A and 3.3C, representative yeast colonies to the right). Both the *ask1-2A* and *ask1-2D* cells exhibited a significant increase in chromosome loss, with the *ask1-2A* cells exhibiting an eight-fold increase in chromosome missegregation compared to wild-type (Figure 3.3B). In looking as to which mutants had a higher frequency of chromosome loss the first cell division leading to missegregation was quantified. Preliminary data indicates Dam1c$^{Ask1\text{-}2A}$ exhibited a significant increase in chromosome loss (Figure 3.3D). Because both Ask1 mutant strains lack phosphoregulation, eventually after multiple rounds of division (Figure 3.3B) this will lead to additional chromosome loss. To maintain chromosome stability, it is important that Dam1c phosphorylation is dynamically regulated *in vivo*.

Genetic interaction of Ask1 mutants with compromised Dam1c

To further characterize the role of Ask1 phosphorylation, we asked whether defects in its regulation exhibit genetic interactions with mutations in other Dam1 complex components. We made double mutants containing the *dam1-9* allele that is mildly temperature sensitive in our strain background and assayed serial dilutions for growth at a semi-permissive temperature (Cheeseman, Enquist-Newman, Müller-Reichert, Drubin, & Barnes, 2001). While all of the single mutants were able to grow at 37 °C, the *dam1-9 ask1-2A* cells were inviable, consistent with phosphorylation promoting Dam1c function *in vivo* (Figure 3.4).

Cdk1 positive phospho-regulation of Ask1 opposes Ipl1 negative phospho-regulation on the Dam1c

The enhanced oligomerization of Dam1c *in vitro* has a role *in vivo.* While wild-type and *ask1-2A* cells had difficulty growing on plates containing microtubule drug benomyl (Supplemental Figure 2.8), *ask1-2D* cells were able to survive. These data suggest that microtubules are stabilized in *ask1-2D* cells, consistent with enhanced Dam1c oligomerization around the microtubule (Figure 3.2) (Umbreit et al., 2014). We next tested whether Ask1 phosphorylation can suppress defects in oligomerization induced by Ipl1-dependent phosphorylation of another subunit of the Dam1c, the Dam1 protein. It was previously found that Ipl1-mediated phosphorylation of Dam1-S20 inhibits Dam1c oligomerization *in vitro* (Zelter et al., 2015). Consistent with this prior discovery, *dam1-S20D* cells were benomyl sensitive but the addition of the *ask1-2D* mutation suppressed the benomyl sensitivity (Supplemental Figure 3.3A). This suppression indicates that the effect of *ask1-2D* opposes that of *dam1-S20D in vivo*, consistent with our finding that Ask1-2D promotes oligomerization *in vitro*, whereas phosphorylation of Dam1-S20 inhibits oligomerization. Taken together, these data suggest that Cdk1 phosphorylation of Ask1 promotes oligomerization of the Dam1c in a cell cycle-dependent manner to ensure accurate chromosome segregation.

Recognizing that the Cdk1 dependent Ask1-2D was able to overcome Ipl1 negative phospho-regulation on oligomerization, we decided to test as to whether Ask1-2D can rescue Ipl1 phosphorylation that leads to the downregulation of Dam1c microtubule binding (Zelter et al., 2015). Ipl1 targets three C-terminal sites on the Dam1 protein (S257, S256, S292) so we used strains with these three sites mutated to aspartic acid to mimic its phosphorylation, *dam1-3D*

cells. In a serial dilution and in the presence of benomyl, all strains were sensitive but the addition of the *ask1-2D* mutation suppressed the benomyl sensitivity (Supplemental Figure 3.3B). Despite having success in tolerating benomyl, the function of promoting oligomerization was not able to provide a full rescue to microtubule compromised cell. As suggested above, this indicates that the effect of *ask1-2D* is in a different pathway of Dam1c microtubule binding, yet the enhanced oligomerization can aid in deficient microtubule function *in vivo*.

Cdk1, Ask1 phospho-mimetic allows Dam1c to counteract the negative regulation of Ipl1. The kinase serves to weaken erroneous kinetochore-microtubule attachments, but this requires a fine-tuning mechanism (Cheeseman et al., 2002). Having either Ipl1 overexpression or its downregulation leads to mis-segregation (Barrera et al., 2014; Chan and Botstein, 1992). We decided to test mutant Ipl1, *ipl1-321,* at semi-permissive temperature (30 °C) alone or in combination with Ask1 mutants (Supplementary Figure 3.3C). In regular media, cells are growing fine, but in the presence of benomyl, WT and *ipl1-321* are sick. Yet, in the combination of a mutant kinase that can no longer downregulate the Dam1c and in combination with Ask1-2A that has decrease in oligomerization, *ipl1-321 ask1-2A* seem to almost offset each other and experience mild recue (Supplemental Figure 3.3C). This suggests that the combination has reached a balance that stabilizes benomyl treated cells. In *ask1-2D ipl1-321*, oligomerization would be enhanced by the phospho-mimetic, even overcoming any residual phosphorylation on Dam1-S20, and microtubule binding would be undisrupted due to the downregulation of Ipl1. This would suggest that Ipl1 activity in negatively regulating Dam1c function is neutralized, and therefore creating an unruly Dam1c (Supplemental Figure 3.3C). Ipl1 negatively regulates the Dam1c function, but this is a balance that can be countered with CDk1 phosphorylation

promoting stronger oligomerization. Altogether, the phosphorylation, whether the regulation is negative or positive, is required to be choreographed if not then disordered mechanism will yield an inability to distribute chromosomes.

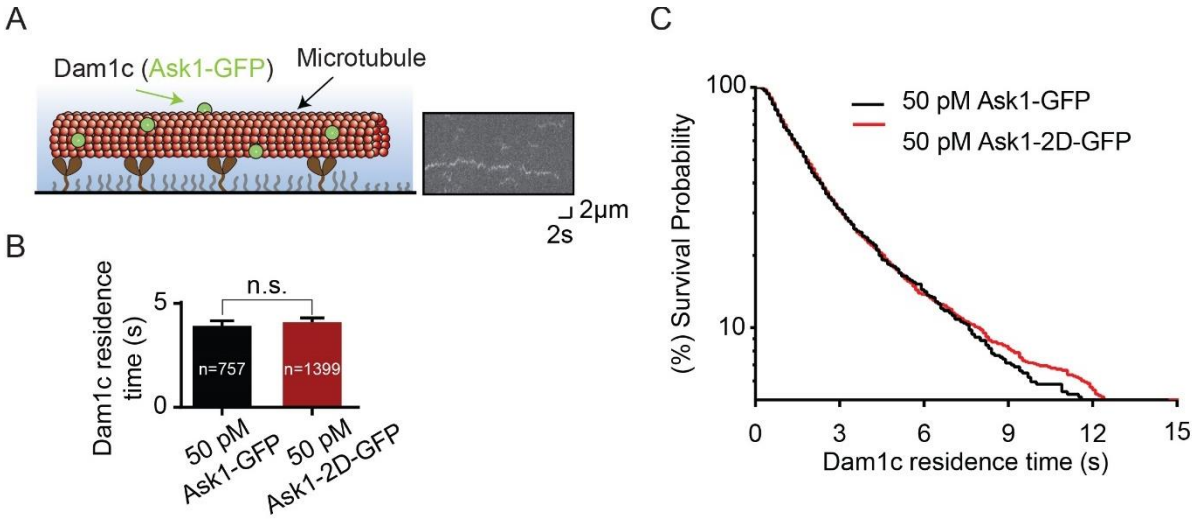
3.4 Discussion

Here, we identify a key phospho-regulatory mechanism that ensures that the Dam1c oligomerizes to preserve the attachment of kinetochores to dynamic microtubules. Although it was previously known that Cdk1 phosphorylates the Ask1 component (Li & Elledge, 2003), the underlying function of the modifications was not known. Using TIRF, the optical trap and genetic interactions, we suggest that Cdk1 phosphorylation is promoting the oligomerization of the Dam1c. In chapter 2, we determined that Cdk1-dependent Ask1 phosphorylation does not affect Dam1c's interaction to the Ndc80c or the kinetochore. In this chapter, we also ruled out another mechanism, and that is the interaction for Dam1c to bind to microtubules. Although a high-resolution structure of the Dam1c was recently solved using cryoEM (Jenni & Harrison, 2018), the C-terminal portion of Ask1 that contains the Cdk1 phosphorylation sites was not present in the structure. However, crosslinking data suggests that the C-terminal region of Ask1 can interact with a second Dam1c monomer through Spc34 (Zelter et al., 2015) bringing up the possibility that phosphorylation promotes this interaction. Future structural work will be required to elucidate the underlying contacts that are enhanced by phosphorylation to determine whether it alters intra- or inter-complex interactions.

At this time, using the information that Ask1-2D promotes oligomerization and phospho-regulation at Ask1 plays an important role in chromosome regulation, we explored the genetic

interaction between error-correction kinase. We identify a phospho-regulatory mechanism that ensures that the Dam1c is regulated in its microtubule function. In addition to Ask1-2D being able to compensate for a Dam1c oligomeric mutant, we were able to solve that Ask1-2D can only stay in its functional “lane”. Ask1-2D was not able to compensate for the lack of microtubule binding.

3.5 Figure Legend

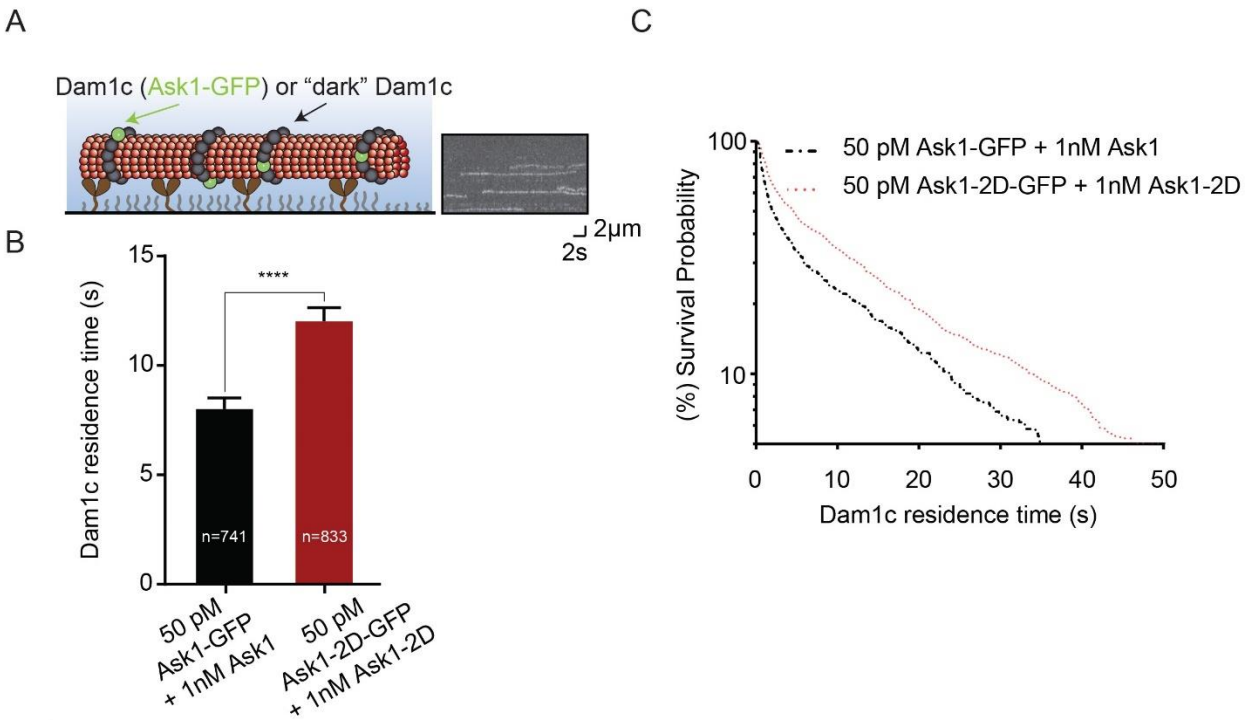


3.5.1. Figure 3.1. Ask1 phosphorylation does not enhance the Dam1c-microtubule binding.

A) Schematic of Dam1c monomer binding to microtubule TIRF assay. Dam1c binds to microtubules as a monomer at low concentrations (50 pM). To assay the effect of phosphorylation on these binding states, fluorescently labeled rDam1c (green sphere, Dad1-GFP) containing either rDam1c$^{Ask1\text{-}GFP}$ or rDam1c$^{Ask1\text{-}2D\text{-}GFP}$ at low monomeric concentrations (50 pM) was added to microtubules. The residence time of Dam1c was recorded. A representative kymograph of rDam1c$^{Ask1\text{-}GFP}$ is shown to the right.

B) Average residence time of Dam1c on the microtubule with recombinant wild-type rDam1c$^{Ask1\text{-}GFP}$ or mutant rDam1c$^{Ask1\text{-}2D\text{-}GFP}$. Bars represent average residence time ± error of the mean (n.s.= not significant).

C) Attachment survival probability versus residence time for data in B).

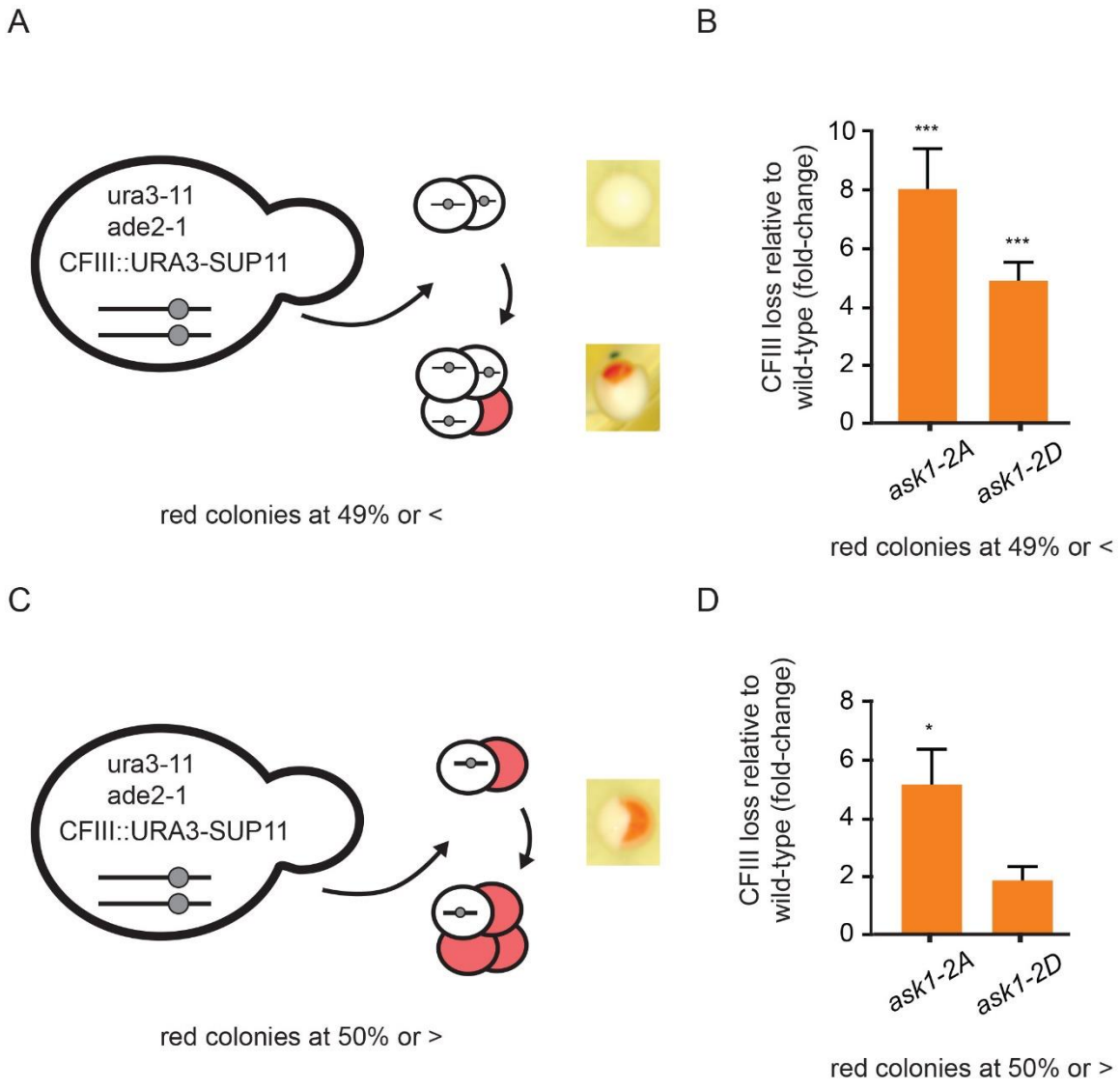


3.5.2 Figure 3.2. Ask1 phosphorylation promotes Dam1c oligomerization.

A) Schematic of Dam1c oligomerization TIRF assay. To analyze oligomerization, a higher concentration of unlabeled dark rDam1c (1 nM, grey spheres) was added to fluorescent labeled monomeric Dam1c to promote oligomerization. The residence time of Dam1c was recorded. A representative of rDam1c$^{Ask1\text{-}GFP}$ kymograph is shown.

B) Average residence time of fluorescent recombinant wild-type 50 pM, rDam1c$^{Ask1\text{-}GFP}$ or mutant 50 pM, rDam1c$^{Ask1\text{-}2D\text{-}GFP}$ with an additional 1 nM of 'dark' rDam1c^{Ask1} or rDam1c$^{Ask1\text{-}2D}$, respectively. Bars represent average residence time ± error of the mean (****= $p<0.0001$).

C) Attachment survival probability versus residence time for data in B).



3.5.3 Figure 3.3. Dynamic Ask1 phospho-regulation is important for chromosome stability *in vivo*.

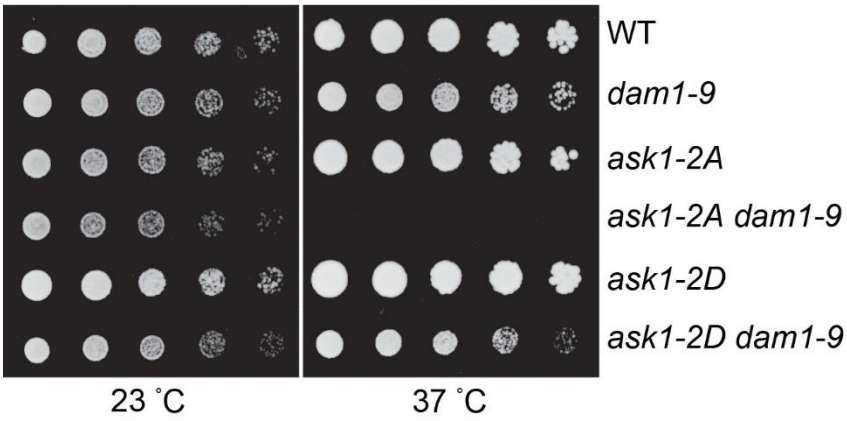
A) Schematic of a visual assay to test chromosome stability. The cell contains a non-essential chromosome III fragment (CFIII; represented as the two dark lines in the diploid cell) that carries the ochre suppressor tRNA gene SUP11. SUP11 can overcome mutant *ade2* which would normally lead to an incomplete pathway to produce a byproduct giving the cell a red pigment. Single cells with success in the first chromosomal segregation lead to a cell having more than 50%

of a white colony (top, first round of doubling from parent cell), and any sequential mis-segregations will produce a colony with less than 50% red pigment (bottom, two rounds of doubling from parent cell).

B) *Ask1-2A* (2A; SBY18724) and *ask1-2D* (2D; SBY18726) cells containing a non-essential chromosome fragment were analyzed for chromosome loss and the fold change relative to wild-type was plotted. *Ask1-2A* and *ask1-2D* both had significant loss relative to wild-type but not to each other. Error bars indicate the standard error of the mean (SEM; n = 5). Approximately 10,000 colonies were analyzed for each replicate for a total of five replicates. The statistical significance was determined using student's t-test (***= $p<0.0005$).

C) Schematic of a visual assay to test chromosome stability assay used to calculate the frequency of chromosome loss per cell division. Single cells with an unsuccessful first round of chromosomal segregation lead to first round of cells having exactly 50% of a red colony (top, first round of doubling from parent cell), and any sequential mis-segregations will produce colony with less than 50% of white pigment (bottom, two rounds of doubling from parent cell).

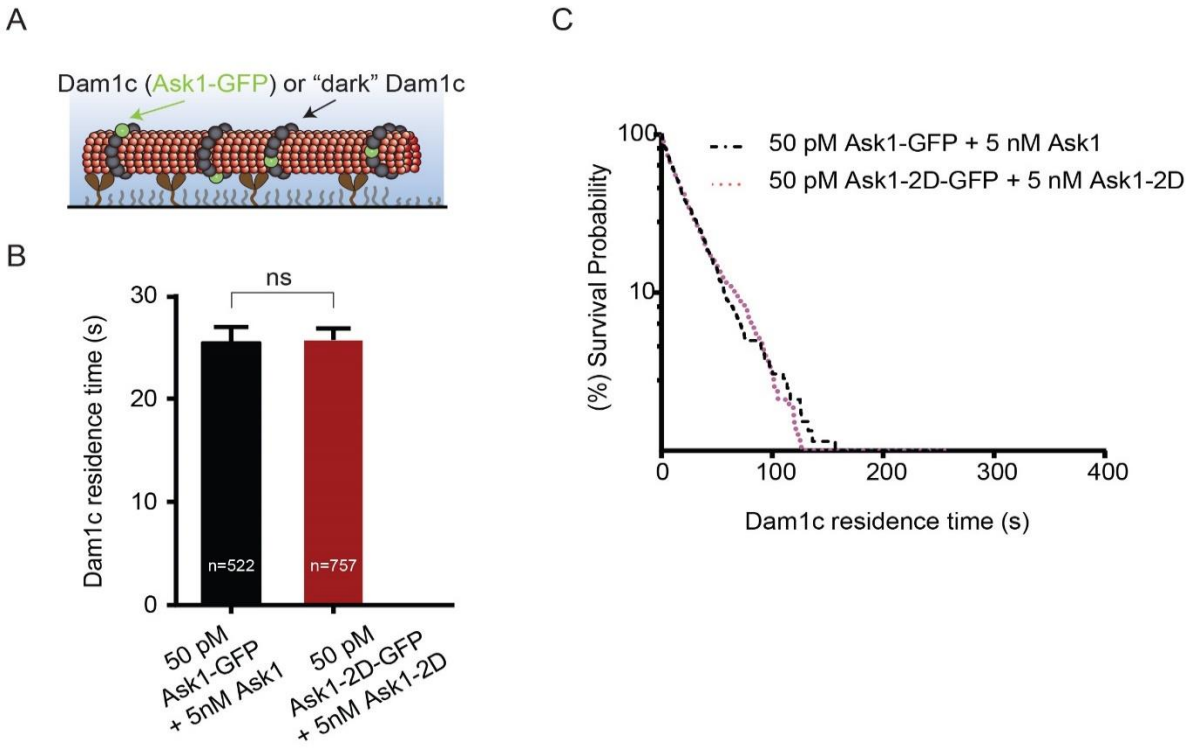
D) Same samples as B) but only the cells that lost the non-essential chromosome fragment in the first round of cell division are included in this analysis. Data plotted is the fold change relative to wild-type cells. *Ask1-2A* had significant loss relative to wild-type. Error bars indicate the standard error of the mean (SEM; n = 5). Approximately 10,000 colonies were analyzed for each replicate for a total of five replicates. The statistical significance was determined using student's t-test (*= $p<0.05$).



3.5.4 Figure 3.4. Genetic interaction of Ask1 mutants with compromised Dam1c

Five-fold serial dilutions of WT (SBY18086), *dam1-9* (SBY19132), *ask1-2A* (SBY18088), *ask1-2A dam1-9* (SBY19136), *ask1-2D* (SBY18089) and *ask1-2D dam1-9* (SBY19138) cells were plated onto yeast media. Samples were grown at 23 °C (left) or 37 °C (right).

3.6 Supplemental Figure Legend

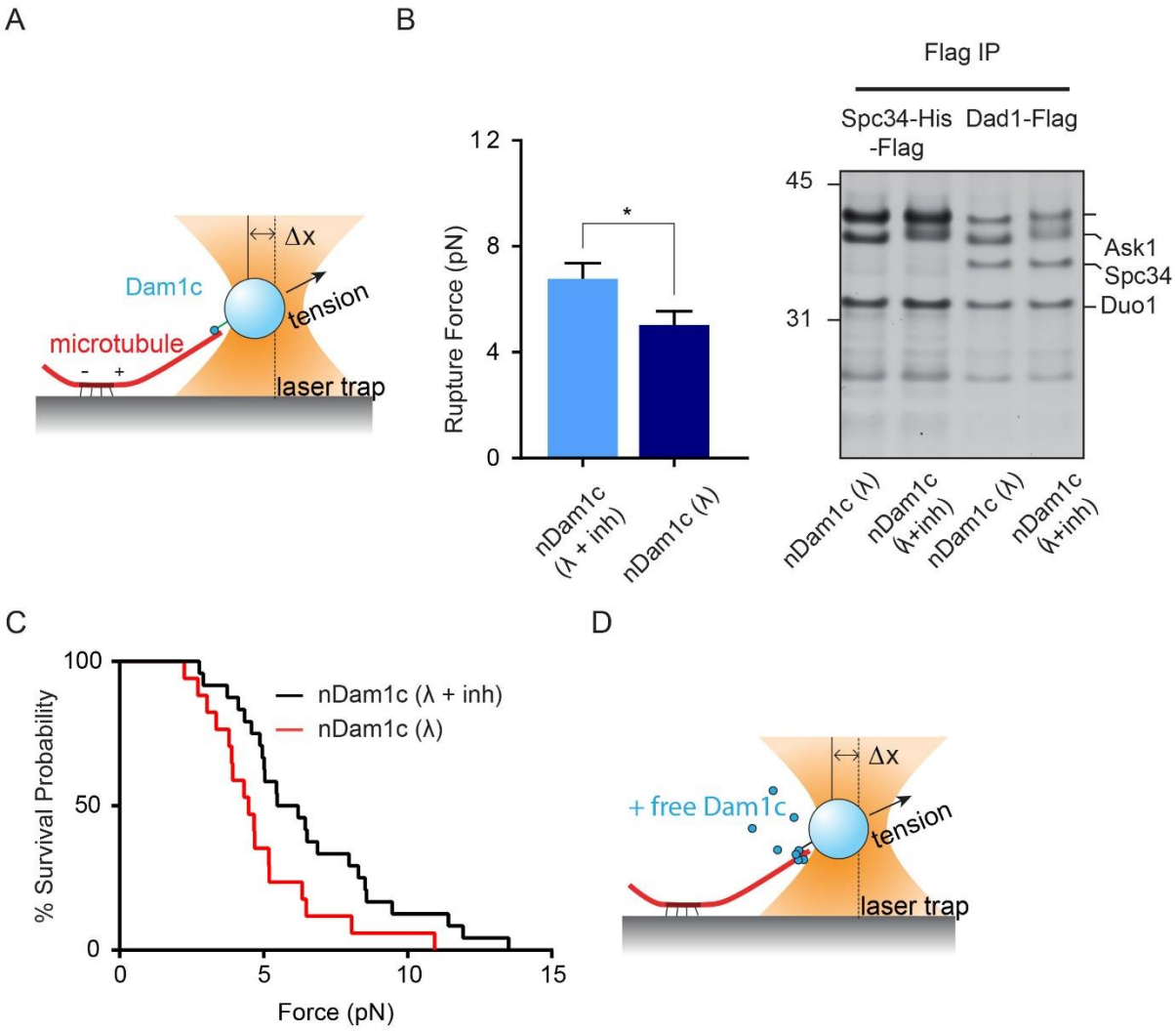


3.6.1 Supplemental Figure 3.1. Ask1 phosphorylation does not influence abundant Dam1c in its intrinsic microtubule oligomerization.

A) Schematic of Dam1c oligomerization TIRF assay. At a high concentration of unlabeled dark rDam1c (5 nM, grey spheres), that ensure ring formation, fluorescent labeled monomeric Dam1c was added. The residence time of Dam1c was recorded.

B) Average residence time of fluorescent recombinant wild-type 50 pM, rDam1c$^{Ask1\text{-}GFP}$ or mutant 50 pM, rDam1c$^{Ask1\text{-}2D\text{-}GFP}$ with an additional 5 nM of dark rDam1c^{Ask1} or rDam1c$^{Ask1\text{-}2D}$, respectively. Bars represent average residence time ± error of the mean (n.s.= not significant).

C) Attachment survival probability versus residence time for data in B).



3.6.2 Supplemental Figure 3.2. phosphorylated nDam1c has an increased microtubule coupling

A) Schematic of laser trap assay as in Figure 2.1A modified to exclude kinetochores and instead linking purified nDam1c (solid blue sphere) to the optically trapped bead.

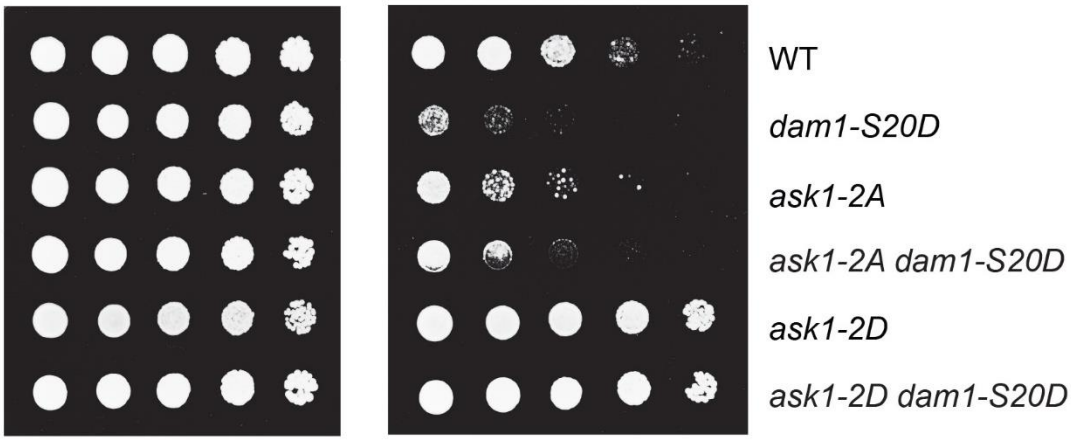
B) Mean rupture forces of Dam1c (SBY13745; Spc34-His-FLAG). The Dam1 complex was either treated with phosphatase (λ) or phosphatase with inhibitors (λ + inh) and analyzed by silver-

stained SDS-PAGE (right panels). Samples are to the left of nDam1c with untagged Spc34 protein to demonstrate Spc34 is tagged and no longer at the 34 kDa marker. Molecular weight markers (kDa) are indicated on the left of the gel. Error bars represent standard error of the mean (SEM; n = 17-24 events). P-values were determined using a two-tailed unpaired t test (*= $p<0.05$).

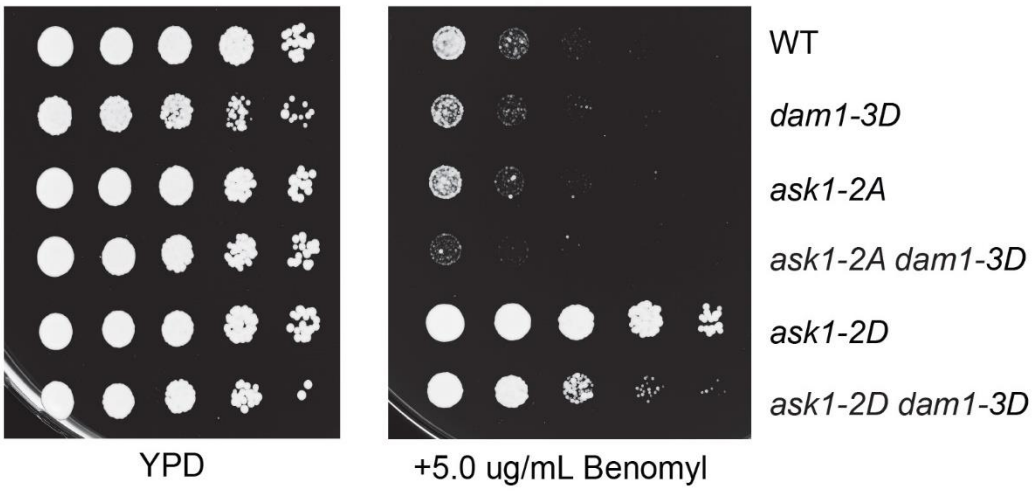
C) Attachment survival probability versus force for data in B).

D) Schematic of laser trap assay with bead bound Dam1c and an add back of free Dam1c.

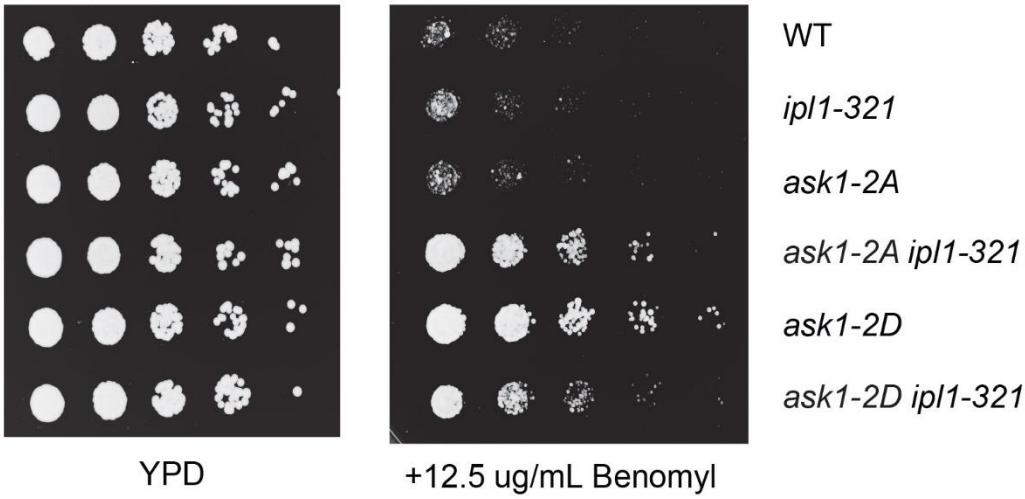
A


23 ˚C
WT
dam1-S20D
ask1-2A
ask1-2A dam1-S20D
ask1-2D
ask1-2D dam1-S20D
YPD
+7.5 ug/mL Benomyl

B


23 ˚C
WT
dam1-3D
ask1-2A
ask1-2A dam1-3D
ask1-2D
ask1-2D dam1-3D
YPD
+5.0 ug/mL Benomyl

C


30 ˚C
WT
ipl1-321
ask1-2A
ask1-2A ipl1-321
ask1-2D
ask1-2D ipl1-321
YPD
+12.5 ug/mL Benomyl

3.6.3 Supplemental Figure 3.3. Ask1 phosphorylation and lack of Ipl1 phosphorylation lead to a decrease in cell viability.

A) Five-fold serial dilutions of WT (SBY19361), *dam1-S20D* (SBY18466), *ask1-2A* (SBY19314), *ask1-2A dam1-S20D* (SBY18468), *ask1-2D* (SBY19319), or *ask1-2D dam1-S20D* (SBY18469) cells were plated on YEP media or with the addition of benomyl (7.5 µg/mL)

B) Five-fold serial dilutions of WT (SBY18086), *dam1-3D* (SBY18103), *ask1-2A* (SBY18088), *ask1-2A dam1-3D* (SBY18105), *ask1-2D* (SBY18089), or *ask1-2D dam1-3D* (SBY18107) cells were plated on YEP media or with the addition of benomyl (5 µg/mL).

C) Five-fold serial dilutions of WT (SBY18086), *ipl1-321* (SBY18599), *ask1-2A* (SBY18088), *ask1-2A ipl1-321* (SBY18601), *ask1-2D* (SBY18089), or *ask1-2D ipl1-321* (SBY18603) cells were plated on media at semi-restrictive temperature 30 °C containing YEP or with the addition of benomyl (12.5 µg/mL) at semi-restrictive temperature 30 °C

***Benomyl plates are labeled based on the intended concentrations, but benomyl can crash in solution so the concentration on the plate may be imprecise. Regardless, Benomyl plates are reported as such and cells will be compared within the same plate.

3.7 Material and Methods

Strain construction: Yeast Strains and Plasmids

Saccharomyces cerevisiae strains used in this study are derived from or backcrossed to SBY3 to be isogenic with the W303 background and are listed in Supplementary Table 1. Dam1-9 is gifted from Barnes lab and backcrossed to SBY3 or SBY4. Dam1-3D (S257D, S265D, S292D) is gifted from Barnes lab and backcrossed to SBY3 or SBY4. All strains containing the following epitope-tagged genes are generated by standard PCR-based integration techniques at the endogenous loci as described in (Longtine et al., 1998) and are fully functional. Primer sequences are listed in Supplementary Table 2. Spc34-His-Flag was made with primer SB4440 and SB4441 and template plasmid pSB1590.

TIRF microscopy

Flow chambers were constructed using glass slides and functionalized coverslips as reported before (Gestaut et al., 2008; Tien et al., 2010). Coverslips were 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. 50 pM GFP-tagged Dam1c ^{Ask1} or Dam1c$^{Ask1\text{-}2D}$ was used to test the direct binding of the Dam1c to microtubules. For testing oligomerization on microtubules, an additional 1 nM of the untagged Dam1 complex was incubated. For testing the sensitivity of Dam1c phosphorylation was only at 1 nM concentration, an addition of 5 nM of the untagged Dam1 complex was incubated with 50 pM GFP-tagged

Dam1c ^{Ask1} or Dam1c$^{Ask1\text{-}2D}$. Experiments using GFP-tagged Dam1 complex had an additional 0.8 mg/mL κ-casein and 50mM KCl.

Single particle tracking and analysis was carried out with custom software (available on request and developed in LabVIEW (National Instruments) and Igor Pro (Wavemetrics) (Gestaut, Cooper, Asbury, Davis, & Wordeman, 2010; Gestaut et al., 2008; Tien et al., 2010; Umbreit et al., 2014). Mean residence times were carried out through bootstrapping analysis (Kim et al., 2017; Umbreit et al., 2014). Each residence time data set was randomly resampled with replacement. All the data sets presented formed normal distributions; mean and standard deviation of the bootstrapped dataset are reported.

Spotting assay and chromosome loss assay

For the spotting assay, the desired strains were grown overnight in YPD medium. The following day, cells were serially diluted 5-fold and spotted on YPD or YPD+ Benomyl (5.0 - 12.5 µg/mL). Plates were incubated at specified temperature, 23 °C for 3 days, 30 or 37 °C for 1 or 2 days. The chromosome loss assay techniques are described in (Duffy & Hieter, 2017). Briefly, strains were grown on -URA plates to ensure selection for the chromosome fragment. The following day, the strains were diluted in water and spread onto YPD plates containing a fifth fold dilution of adenine (Adenine 0.001%). Cells were diluted to obtain about 200 colonies/plate and then grown at 23 °C for 7-12 days before placing them at 4 °C for 2-3 days. Total colonies and colonies with red sectoring were counted Figure 3.3A or colonies with at least 50%, half the colony, that was red.

Optical trap assays

Optical trap-based bead motility assays were performed as in (Akiyoshi et al., 2010; Miller et al., 2019). Streptavidin-coated 0.56-µm polystyrene beads (Spherotech) were functionalized with biotinylated anti-penta-His antibody (Qiagen) and stored in BRB80 containing 8 mg/ml BSA and 1 mM DTT at 4 °C with continuous rotation. Beads were decorated with purified nDam1c (via Spc34-6His-3Flag) in a total volume of 60 µl incubation buffer (BRB80 containing 8.0 mg/mL κ-casein and BSA 1 mM DTT). Dam1c particles were diluted such that the concentration of Duo1 was ~10 nM and incubated with 6 pM beads for 1 h at 4 °C. Any unbound Dam1 complex was removed by pelleting the beads down (13,200 RPM for 10 minutes at 4 °C) and following up with multiple washes of incubation buffer and the resuspending at the original volume. The concentration of nDam1c was obtained by comparing the intensity of Spc34 to dilutions of BSA standards on silver-stained SDS-PAGE using a standard curve. The BSA standards and its derived equation were run on the same gel for the calculated Dam1c concentrations. nDam1c was incubated in the microtubule growth buffer (see below). Dynamic microtubule extensions were grown from coverslip-anchored GMPCPP-stabilized microtubule seeds in a microtubule growth buffer consisting of BRB80, 1 mM GTP, 250 µg/ml glucose oxidase, 25 mM glucose, 30 µg/mL catalase, 1 mM DTT, 1.4-1.5 mg/mL purified bovine brain tubulin and 1 mg/mL κ-casein. Assays were performed at 23 °C. Rupture force experiments were performed as in (Akiyoshi et al., 2010; Miller et al., 2019) and as described above in Chapter 2 Material and Methods. Briefly, an optical trap was used to apply a force of ~1-3 pN in the direction of microtubule assembly. Once beads were observed to track with microtubule growth for a distance of ~100-300 nm (to ensure end-on attachment), the applied force was increased at a constant rate of 0.25 pN/s until bead detachment. Records of bead position over time were generated and analyzed using custom

software (Labview and Igor Pro, respectively) and used to determine the rupture force, which was marked as the maximum force sustained by the attachment during each event. Note, the concentration of Dam1c is half the concentration that has been previously been used (Umbreit et al., 2014).

Immunoblot and silver stain analysis

As described above.

Protein biochemistry

As described above.

3.8 Acknowledgement of contribution of collaborators and funding

We are grateful to the Sue Biggins (S.B.), Trisha N Davis (T.N.D), and Charles L Asbury (C.L.A.) lab for their contributions to this chapter. A special thanks to Jae ook Kim that contributed to TIRF experiments.

I received support by a Ford Foundation predoctoral fellowship. This work was supported by R35GM130293 (to T.N.D.), R35GM134842 (to C.L.A.) and R01GM064386 (to S.B.). This work was also supported by the Genomics, Proteomics and Scientific Imaging Shared Resources of the Fred Hutch/University of Washington Cancer Consortium (P30 CA015704).

4 Chapter 4: Motor protein Cin8 and Kar3 contribute to strengthening kinetochore-microtubule attachment

4.1 Summary

Motor proteins have been shown to influence microtubule spindles and chromosome movement, but studies looking at their roles at the kinetochore are incomplete (Wordeman, 2010). Because motor proteins are known to interact with kinetochores, it would be imperative to investigate as to whether they function in fastening kinetochores to microtubules (Gupta et al., 2018). Here, we used biochemistry, genetics and a reconstitution system to discover that yeast motor proteins, Cin8 and Kar3, interact directly with kinetochores and are playing a role in kinetochore-microtubule coupling. In the absence of Cin8, kinetochores have weakened microtubule binding. Preliminary data suggests Kar3 motor protein may have a role in the retention or stability of the Dam1c at the kinetochore. I propose that motor proteins support kinetochore-microtubule coupling based on literature describing Cin8 targeting phosphatase to Ipl1 phosphorylation sites and Kar3 facilitating the retention of Dam1c. Both functions will ensure kinetochore-microtubule attachments are steadied as kinetochores come under tension during the disassembling mechanical force imposed by microtubules.

4.2 Introduction

Regulation of kinetochore–microtubule attachment via motor proteins.

Kinetochores perform an essential function in chromosome segregation by bridging it to microtubules. Our understanding of the components that are involved in directly coupling the microtubule to the kinetochore is gradually increasing. However, there are additional important proteins, such as motor proteins, whose role in promoting kinetochore-microtubule attachment is incomplete. The goal is to understand how nuclear yeast motor proteins (Kip1, Cin8, Kip3, Kar3) regulate these attachments.

Motor proteins are excellent candidates to bridge kinetochore-microtubule interactions, but they are not essential, so it has been difficult to understand their contribution to the process. In *S. cerevisiae*, data has shown that motor proteins play a role in spindle assembly and orientation, chromosome positioning and segregation (Hoyt et al., 1992; Hildebrandt & Hoyt, 2000). In addition, motor proteins control kinetochore microtubule length and, when mutated this results in a decrease of kinetochore-microtubule tension required for proper chromatid segregation (Gardner et al., 2008). Through imaging, motor proteins have been shown to localize to kinetochores and via chromatin immunoprecipitation they are found to interact with the centromere (Tytell & Sorger, 2006). Motor proteins localize kinetochore components to the tips of the microtubules (Biggins, 2013). This leads to the suggestion that motor proteins have a role as a direct or intermediate interactor to influence the control between kinetochore and microtubule attachment. Here, we used genetics and a reconstitution system to discover that yeast motor proteins, Cin8 and Kar3, interact directly with kinetochores and Kar3 may influence

the retention or stability of the Dam1c at the kinetochore that ultimately leads to the strength of kinetochore-microtubule attachment.

4.3 Results

4.3.1 Cin8 contributes to strengthening the kinetochore–microtubule attachment under high tension

Cin8 is found at interpolar microtubules, and as a formed tetramer it captures anti-parallel microtubules. Following a clustering of the kinesin at the microtubule's minus end, it can switch from a minus-end to its accepted plus-end direction to help separate and stabilize the spindle apparatus (Hoyt et al., 1992; Saunders and Hoyt, 1992; Shapira et al., 2017). These functions are occurring on microtubules but their impact reaches mechanisms in chromosome stability (Hoyt et al., 1992). Because motor proteins contribute to the stability of kinetochore-microtubule interaction, it would be conceivable that motor proteins have a role at the kinetochore. To approach how kinesins can influence kinetochore microtubule attachment in yeast, we first wanted to determine which motor proteins co-purify with kinetochores. Purifying kinetochores was achieved by pulling down on its Mtw1 complex subunit, the Dsn1 protein, and detecting additional proteins that co-purify. To determine if Cin8 purifies with kinetochores, we fused Cin8 with a SNAP tag to fluorescently mark the protein. The IP of Dsn1 reveals that Cin8 co-purifies with kinetochores (Figure 4.1A). To understand Cin8 function at the kinetochore, we decided to purify kinetochores with depleted Cin8 protein and test kinetochore-microtubule coupling *in vitro*. Purifications of kinetochores were done from some cells containing the motor protein

fused with a plant specific auxin inducible degron (AID) system at its C-terminus, *cin8-V5-AID*. Kinetochores with C-terminally tagged constructs on Cin8 -V5 or -V5-AID look like untagged Cin8 except for the migration shift due to the addition of the fusion protein (Figure 4.1B, silver-stained SDS-PAGE, top; and immunoblot, bottom). Cells with *cin8-AID* alleles were treated with auxin hormone IAA (Indole 3 acetic Acid) before harvesting cells. Cells with Tir1 that promote the E3 ligase will interact and ubiquitinate the protein of interest for degradation (Nishimura et al., 2009). Kinetochores were purified with Cin8 and degraded Cin8 (data not shown). Using the optical trap, we reconstituted kinetochore-microtubule coupling and assayed the strength of attachment. Kinetochores carrying Cin8 ruptured at an average of 8.7 pN while the degradation of Cin8 weakens attachments as the rupture force drops to 6.5 pN (Figure 4.1C, top and bottom). This suggests that kinesin Cin8 has an important role in kinetochore-microtubule attachment.

4.3.2 Kar3 facilitates Dam1c at the kinetochore to strengthen the kinetochore–microtubule attachment

In discovering that Cin8 directly had an impact in kinetochore-microtubule attachment, we decided to evaluate if there were additional kinetochore bound motor proteins that are found in the nucleus: Kip1, Kip3 and Kar3. First, to determine which additional motor proteins co-purify with the kinetochore, a pull-down assay was carried out. We purified kinetochores from cells with motors containing GFP tags and analyzed their presence by immunoblotting. Proteins Kip1 and Kar3 were readily present at the kinetochores, but Kip3 was not (Figure 4.2A). I decided to focus on Kar3 because Kar3 is implicated in aiding the initial attachment of the kinetochore to the microtubule, and is influential in establishing biorientation (Liu et al., 2011)

Kar3 is a minus-end directed motor that heterodimerizes with Cik1 or Vik1, two non-motor proteins that regulate Kar3 function (Manning et al., 1999). The Kar3-Cik1 heterodimer is particularly interesting since this interaction is required to localize Kar3 to the plus end tips of microtubules (Liu et al., 2010). Kar3-Cik1 has also been found to directly bind to the kinetochore protein Ndc80 of the Ndc80c and to be dependent on the Spc105 protein for its kinetochore localization (Mieck et al., 2015; Pagliuca et al., 2009). These observations suggest that Kar3 may play a role in regulating kinetochore-microtubule attachments. However, its role has not been investigated because it has been difficult to separate Kar3's multiple functions in the cell. In moving forward, Kar3 motor protein was excluded from the kinetochore and then investigated as to whether it carried a function in kinetochore-microtubule coupling.

To exclude Kar3 from the cell, Kar3 protein was fused with an AID system to conditionally degrade the protein, which did not impact cell growth (Supplemental Figure 4.1A). Cells containing *kar3-AID; OsTir1* allele and treated with auxin downregulated the Kar3 protein within 30 minutes (Figure 4.2B). Without the plant based E3 ligase system, auxin did not affect cell growth or Kar3 protein (Figure 4.2B and Supplemental Figure 4.1A). Induced Kar3 degradation caused the cells to be sick (Supplemental Figure 4.2A). Using the optical trap, kinetochores purified from cells with Kar3 rupture at WT forces, 9.4 pN, while depleted Kar3 kinetochores rupture at 6.7 pN. The minus end-directed motor protein, Kar3 has a role in kinetochore-microtubule interaction.

4.4 Discussion

kinetochores are required to tightly couple microtubules as they approach chromosomal segregation. The Ndc80c and Dam1c have major roles in their binding, but other key players have not been investigated. Motor proteins are difficult to study within the cell because they carry an assortment of functions. Cin8 for instance can cross-link microtubules, promote spindle pole body separation, and help microtubules slide to separate spindle pole bodies during Anaphase B (Hildebrandt & Hoyt, 2000; Straight, Sedat, & Murray, 1998). This ultimately makes Cin8 function is important to reduce the chance of chromosome loss (Hoyt, He, Loo, & Saunders, 1992). Therefore, we wanted to directly look at kinetochores in the absence of additional function that Cin8 carries within the mitotic cell. Using purified kinetochores that lack Cin8 motor protein we were able to begin to understand its impact directly at the kinetochore-microtubule interface.

Preliminary data on the depletion of Cin8 led us to identify kinetochores with a reduced function in microtubule binding (Figure 4.1). This work was interesting and was then pursued by a postdoctoral trainee in the lab, Amitabha Gupta and collaborators. The drop of rupture force was attributed to Cin8's role in recruiting Protein Phosphatase 1 (PP1) to the kinetochore. PP1 can then remove phosphorylation from the Ndc80c, specifically the unstructured N-tail that when phosphorylated it leads to an active inhibitory affect towards microtubule coupling (Suzuki et al., 2018; Umbreit et al., 2012).

In addition to finding Cin8 at the kinetochore, we discovered Kip1 and Kar3. Kip1 is considered to be redundant to Cin8, but Kip1's role in maintaining proper chromosomes segregation is weaker (Hoyt et al., 1992). Therefore, we decided to investigate Kar3.

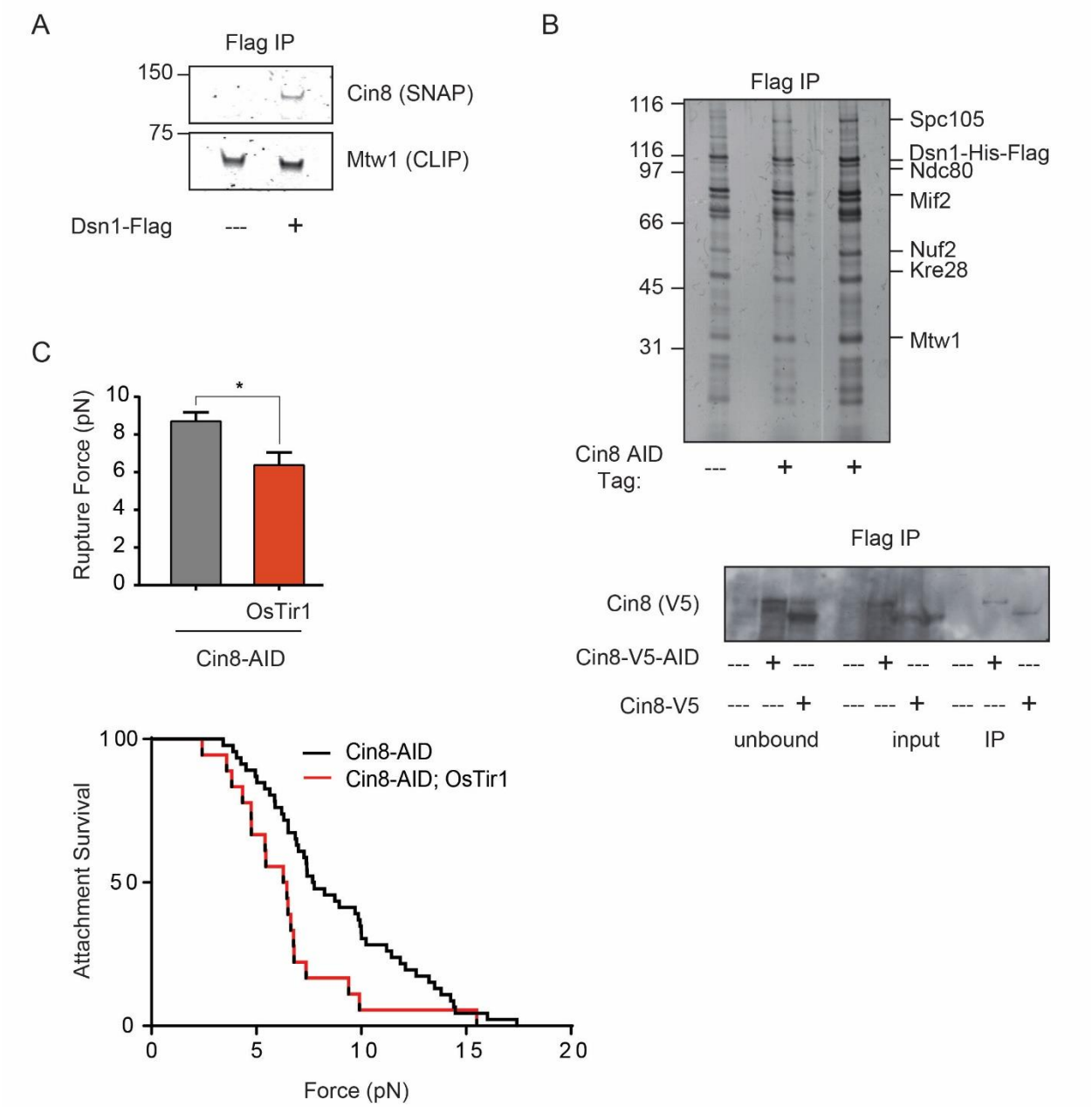
Kar3 has important roles and is known to crosslink microtubules, maintain the spindle apparatus, antagonize the outward forces of plus-end directed motor proteins Kip1 and Cin8, and is also found to interact with kinetochore Ndc80c (Gardner et al., 2008; Hoyt, He, Totis, & Saunders, 1993; Mieck et al., 2015). This was intriguing and at the same time it was important to know that the role it has at the microtubule-kinetochore interface remained unsolved. This led us to investigate Kar3 at the kinetochore. In using our reconstitution system, the optical trap, degradation of Kar3 led to a decrease in kinetochore-microtubule rupture force. Despite the finding that Kar3 drives the kinetochore to the minus end in attempt to create an end-on attachment, this would not explain the decrease in rupture force (Tanaka et al., 2005). Kar3 may play additional roles than the transportation of whole kinetochores. Perhaps it has a role in driving kinetochore binding proteins to kinetochores, but that experiment has not been done and would be fitting since without Kar3, kinetochore-microtubule binding is weakened.

In investigating as to whether there is a decrease of outer kinetochore protein, by purifying kinetochore with degraded Kar3, it was noticeable that Dam1 protein of the Dam1c was missing (Supplementary Figure 4.1B and C). Unfortunately, immunoblot V5 tag was not exposed long enough to detect the Kar3 IP; Despite needing to repeat this experiment, past results suggest it is present (Figure 4.2). To check that the kinetochore association of Kar3 was not dependent on microtubules but directly interacting with kinetochores, benomyl was used. In using benomyl, it looks as though Dam1c was pulled down with kinetochores at similar levels when compared to Kar3 depleted kinetochores (Supplementary Figure 4.1D). At this point it would be important to verify if Kar3 remains present at kinetochores in the presence or absence of benomyl. Additionally, it would be important to test if Kar3 can rebind to benomyl treated Kar3-depleted

kinetochores. If Kar3 can bind back, then these kinetochores can be tested on the optical-trap to investigate as to whether Kar3 is serving the kinetochore-microtubule interface.

Motor proteins can bind both microtubules and kinetochore subunits. It would be interesting to find a yeast motor protein serving a function like Dam1c or the Ndc80c. In this case, both Cin8 and Kar3 facilitate the kinetochore-microtubule interface by reducing weak attachments and potentially, with the aid of Kar3, increase or retain Dam1c at the kinetochores.

4.5 Figure Legend



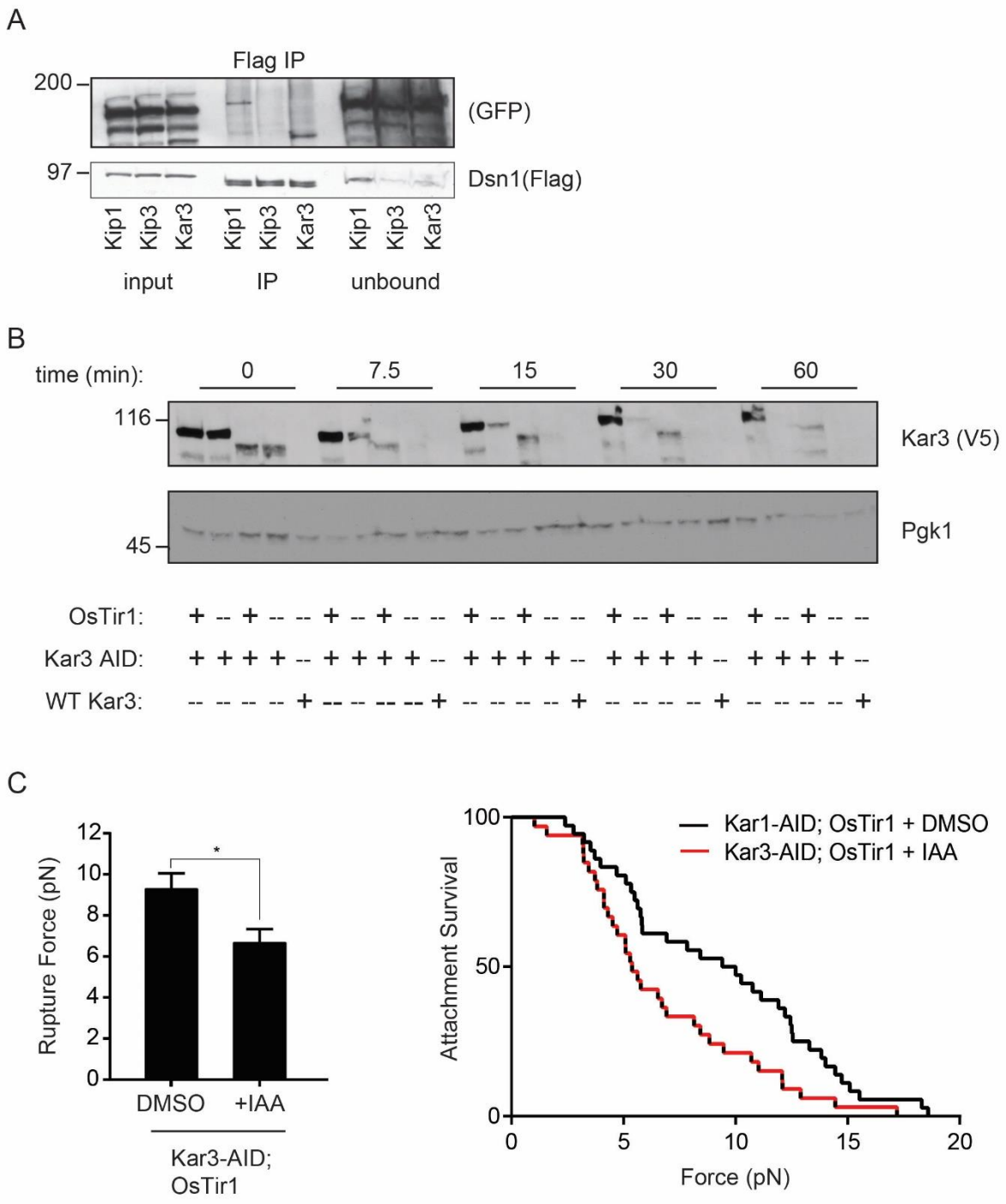
4.5.1 Figure 4.1. Yeast kinesin Cin8 localization to kinetochores to strengthens kinetochore-microtubule attachment.

A) Using α-flag beads, Kinetochores were purified via Dsn1-Flag immunoprecipitation from cell lysates. Both strains, SBY10327 and SBY15778 contain the Mtw1 protein CLIP-tagged, but only SBY15778 has the Cin8 protein SNAP-tagged. Kinetochores retained on beads are dyed, washed

and eluted using α-flag peptide. The presence of kinetochore bound proteins were analyzed by SDS-PAGE and identified by fluorescent imaging of Mtw1-CLIP and Cin8-SNAP.

B) Using α-flag beads, Kinetochores of WT Cin8 (SBY13592), Cin8-V5-tagged (SBY14049) and Cin8-V5-AID construct (SBY14050) were purified via Dsn1-Flag immunoprecipitation from cell lysates, washed and eluted using α-flag peptide. Kinetochores were visualized by silver stained SDS-PAGE (top) and by immunoblot with α-V5 antibodies (bottom). Molecular weight markers (kDa) are indicated on the left.

C) Mean rupture forces for kinetochores purified from strains containing *cin8-AID* (SBY14379) or *cin8-AID*; *OsTir1* (SBY14380). Error bars for standard error of the mean (SEM; n = 18-48 events). P-values were determined using a two-tailed unpaired t test (*= $p<0.05$).


A
Flag IP
200
(GFP)
97
Dsn1(Flag)
Kip1
Kip3
Kar3
Kip1
Kip3
Kar3
Kip1
Kip3
Kar3
input
IP
unbound
B
time (min):
0
7.5
15
30
60
116
Kar3 (V5)
45
Pgk1
OsTir1:
Kar3 AID:
WT Kar3:
C
Rupture Force (pN)
12
10
8
6
4
2
0
*
DMSO
+IAA
Kar3-AID;
OsTir1
Attachment Survival
100
50
0
Kar1-AID; OsTir1 + DMSO
Kar3-AID; OsTir1 + IAA
0
5
10
15
20
Force (pN)

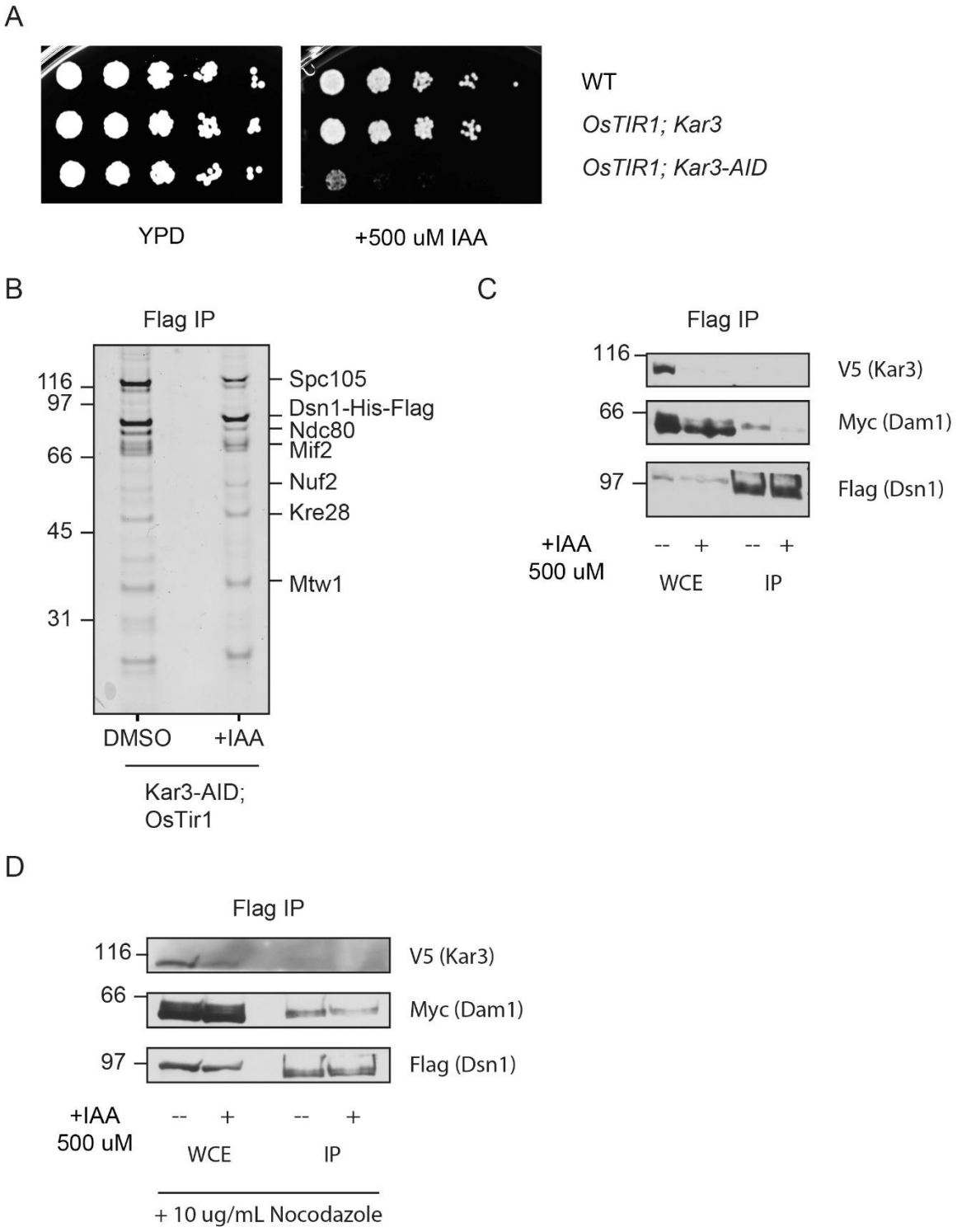
4.5.2 Figure 4.2. Kar3 motor plays an important role in strengthening kinetochore-microtubule attachment.

A) Kinetochores from cell lysates were purified via Dsn1-Flag immunoprecipitation. Strains with V5 tags on motor proteins Kip1(SBY15712) Kip3 (SBY15714) and Kar3 (SBY15716) were retained on beads, washed and eluted using α-flag peptide. The presence of kinetochore and bound motor proteins were analyzed by SDS-PAGE and identified by immunoblot with α-Flag and α-V5 antibodies, respectively. Molecular weight markers (k Da) are indicated on the left.

B) Kar3 protein is monitored from cell lysates of strains; *kar3-AID* (SBY15934); *kar3-AID*, *OsTIR1* (SBY15932); *kar3-AID* (SBY15959); *kar3-AID, OsTIR1* (SBY15957); and untagged WT cells (SBY3). Cells were treated with 500 µM of auxin and then harvested at select time points (in minutes) as noted in the figure. The presence of Kar3 protein was analyzed by SDS-PAGE immunoblot with α-V5 antibodies (top). Protein Pgk1 was used as a loading control (bottom). Molecular weight markers (kDa) are indicated on the left.

C) Mean rupture forces for kinetochores purified from strains containing *kar3-AID; OsTir1* (SBY15932) treated with DMSO or 500 µM of auxin 30 minutes before harvesting. Error bars for standard error of the mean (SEM; n = 33-36 events). P-values were determined using a two-tailed unpaired t test (*= $p<0.05$). Surivival plot (right).

4.6 Supplementary Figure Legend



4.6.1 Supplemental Figure 4.1. Kar3 localization helps retain Dam1c at kinetochores independent of microtubules.

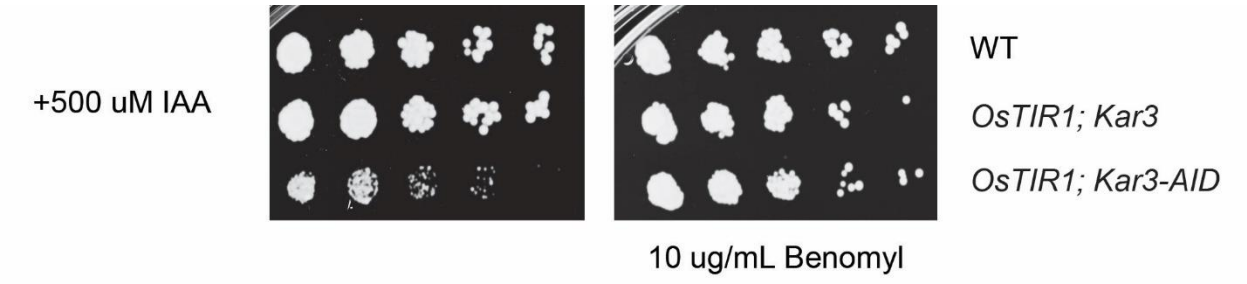
A) Five-fold serial dilutions of *WT* (SBY3), *kar3-3V5* (Kar3; SBY15938), and *kar-3V5-AID* (Kar3-AID; SBY15932). Cells were plated onto yeast extract peptone containing

DMSO or top-plated 500 µM auxin.

B) *Kar3-AID; OsTir1* (SBY15932) treated with DMSO or 500 µM of auxin 30 minutes before harvesting. Kinetochores were visualized by silver stained SDS-PAGE (right) and used in the optical trap Figure 4.2.

C) Kinetochores from cell lysates were purified via Dsn1-Flag immunoprecipitation. Strain *kar3-AID; OsTir1; dam1-myc* (SBY16176) is treated with DMSO or 500 µM of auxin 30 minutes before harvesting.

D) same as C) with the addition of 100 µg/mL Nocodazole 30 minutes before harvesting.



4.6.2 Supplemental Figure 4.2. Degradation of depolymerase Kar3 rescues cells in benomyl.

Five-fold serial dilutions of *WT* (SBY3), *kar3-3V5* (Kar3; SBY15938), and *kar-3V5-AID* (Kar3-AID; SBY15932). Cells were plated onto yeast extract peptone containing DMSO or top-plated 500 µM auxin.

***Benomyl plates are labeled based on the intended concentrations, but benomyl can crash in solution so the concentration on the plate may be imprecise. Benomyl plates are reported as such.

4.7 Materials and Methods

Strain construction: Yeast Strains and Plasmids

All strains are derivatives of W303 (SBY3). MTW1-CLIP was made with primers SB3109 and SB3111 and template plasmid pSB1824. Cin8-SNAP-3V5 was made with primers SB4524 and SB4525 and template plasmid pSB2402. Cin8-V5 was made with primers SB4524 and SB4525 and template plasmid pSB2068. Cin8-3V5-IAA17 was made with primers SB4524 and SB4525 and template plasmid pSB2067. Kar3-V5 was made with primers SB4904 and SB4905 and template plasmid pSB2068. Kar3-3V5-IAA7 was made with primers SB4904 and SB4905 and template plasmid pSB2066. Kar3-3V5-IAA17 was made with primers SB4904 and SB4905 and template plasmid pSB2067. pGPD1-OsTIR1 from pSB2273 was SwaI digested and pGPD1-OsTIR1 was integrated at HIS3 (single integration plasmid) into SBY3. Dam1-9MYC was gifted from the Winey lab.

In vitro SNAP and CLIP labeling of Cin8 and Mtw1, respectively.

To examine the binding of Cin8 to WT kinetochores, Kinetochores and Cin8 were fluorescently labeled following a three-hour α-flag bead incubation. Dsn1-Flag kinetochores were pulled down as described above and then incubated in 100uL buffer containing SNAP-Surface 647 dye and CLIP-surface 547 dye (10 µM in BH 0.15 M KCl and phosphatase and protease inhibitors) for 30 min with continuous gentile agitation while immobilized on beads. The excess dye was washed away with three washes (first wash was in 4 °C for five minutes) of BH 0.15 M KCl and protease inhibitors and then the kinetochore was eluted as described above.

Conditional auxin inducible degron

Protein depletion came by using an Auxin Inducible Degron (AID) system as described in (Suzuki et al., 2018 and Nishimura et al., 2009). Cells expressed fusions of the protein of interest to an auxin responsive protein (IAA7 or IAA17) at the endogenous locus. Cells also expressed Tir1, which is necessary for induced degradation. 500 mM IAA (indole-3-acetic acid) was added to media for 30 minutes to 120 minutes to induce degradation of the AID-tagged protein prior to harvesting. AID treated serial dilutions plates were prepared to contain 500 uM IAA. Compound was diluted into DMSO and auxin hormone IAA was spread with acid-washed glass beads and allowed to dry before use. A plate contains about 20 mL, so IAA was added in calculation to this final volume.

Optical trap assays

Optical trap-based bead motility assays were performed as in (Akiyoshi et al., 2010; Miller et al., 2019). Streptavidin-coated 0.44-µm polystyrene beads (Spherotech) were functionalized with biotinylated anti-penta-His antibody (Qiagen) and stored in BRB80 containing 8 mg/ml BSA and 1 mM DTT

Nocodazole treated cells

Thirty minutes prior to harvesting cells, 100 µg/mL of Nocodazole was added and cells were then spun and washed to be immunoprecipitated as described above.

Immunoblot and silver stain analysis.

As described above.

Protein biochemistry.

As described above

4.8 Acknowledgements

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5 Chapter 5: Conclusion

Accurate chromosome segregation requires that every pair of sister kinetochores biorient and attach to microtubules from opposite poles (Tanaka, 2005). However, early in mitosis, kinetochores initially make random attachments to microtubules, which results in some pairs of sister kinetochores that are attached to the same pole instead of opposite poles. These incorrect kinetochore-microtubule attachments must be destabilized and provide a second opportunity for bi-orientation to be made, and previous work showed that phosphorylation of the Dam1c by the Ipl1 protein kinase facilitates this process (Cheeseman et al., 2002; Gestaut et al., 2008; Kalantzaki et al., 2015; Kang et al., 2001; Keating et al., 2009; Lampert et al., 2010; Lampson & Grishchuk, 2017; Sarangapani et al., 2013; Tien et al., 2010; Zelter et al., 2015; Jin & Wang, 2013). As mitosis proceeds and kinetochores make proper attachments to microtubules, the attachments must be stabilized to resist the tension that is generated by biorientation. While it has been known that the kinetochore-bound Dam1 complex oligomerizes around microtubules to stabilize stable kinetochore-microtubule attachments, it hasn't been established whether Dam1c oligomerization is positively regulated. The work that I present reveals that Cdk1 phosphorylation of Dam1c subunit, Ask1, leads to efficient oligomerization around the microtubule to effectively aid the kinetochore to withstand the force exerted by microtubules.

Phosphorylation of Dam1c has a positive role in Dam1c mechanism

Initial identification of the Dam1c was found to interact with microtubules and have an important role in mitosis (Hofmann et al., 1998). Post translational modification of Dam1c were found on various subunits of the Dam1c, with a major emphasis on phosphorylation (Kang,

Cheeseman, & Kallstrom, 2001; Cheeseman et al., 2002; Legal, Zou, Sochaj, Rappsilber, & Welburn, 2016; Li, 2002; Li & Elledge, 2003; Shah et al., 2019). Phosphorylation has served as a negative regulator to Dam1c as it compromises kinetochore coupling to microtubules (Sarangapani, Akiyoshi, Duggan, Biggins, & Asbury, 2013). Dam1c is negatively phospho-regulated by targeting its function in 1) interacting to its kinetochore receptor, the Ndc80c 2) making contact to microtubules and/or 3) the oligomerization around the microtubule (Kalantzaki et al., 2015; Lampert, Hornung, & Westermann, 2010; Tien et al., 2010; Gestaut et al., 2008; Ramey et al., 2010; Zelter et al., 2015; H.-W. Wang et al., 2007; Zelter et al., 2015). Once bi-orientation occurs it is important that contacts are not lost. Despite majority of the data indicating that phosphorylation disrupts Dam1c function, Mps1 phosphorylation was shown to have a role in an alternate attachment of kinetochores to microtubule (Shimogawa et al., 2006) and Cdk1 phosphorylation functions in maintaining the health of the rest of the complex (Li & Elledge, 2003a). In our findings, we show that Cdk1 phosphorylation of Ask1 positively regulates its function in oligomerization.

Cdk1 phosphorylation of Dam1c rescues attachment strength of kinetochores lacking Dam1c

Temperature sensitive Dad1-1 kinetochores are depleted of Dam1c and can only withstand low forces when they are pulled on the microtubule tip (Akiyoshi et al., 2010). To reconstitute complete kinetochores, add-back of rDam1c to Dad1-1 kinetochores was done, but it was an incomplete rescue as kinetochores could not hold WT forces. Remarkably, the add-back of phosphorylated nDam1c was able to hold as well as WT. In discovering that phosphorylation of Dam1c was involved in kinetochore attachment strength, the kinase was sought after. Cdk1 phosphorylation of its consensus sites, Ask1-S216, S250, proved to be necessary for complete

rescue of Dad1-1 kinetochores. In changing the Ask1 Cdk1-sites to alanine (Ask1-2A), to prevent phosphorylation, testing Dam1c in an add-back to Dad1-kinetochores we observed that the rupture force dropped to 7 pN. Despite the implication that there were additional sites to test, the add-back of phosphorylated Ask1, via phospho-mimetics or Cdk1 phosphorylation, was able to rescue Dad1-1 kinetochores to WT levels. We were able to determine that Cdk1 activity was enough to increase Dam1c function. Next, we investigated the mechanism as to how Cdk1 phosphorylation of Ask1 produced a strong kinetochore-microtubule attachment.

Dam1c ring formation was negatively regulated by targeting Ipl1-site, Dam1-S20. The phospho-mimetic version, Dam1-S20D was found to inefficiently oligomerize around microtubules and when the kinetochore-microtubule interface was under tension it produced weak attachments (Sarangapani et al., 2013; Zelter et al., 2015). In investigating the mechanism of Dam1c, we were able to find that Cdk1-dependent Ask1 phosphorylation (Ask1-2D) could better oligomerize. In understanding that oligomerization can overcome microtubule depolymerization we decided to test Ask1-2D in benomyl, a microtubule depolymerizing drug (Umbreit et al., 2014). With cells carrying both Dam1-S20D and Ask1-S2D in plating the cells onto benomyl, which make WT and Dam1-S20 cells poor growing, Ask1-2D function was able to compensate Dam1-S20D and survive. This indicates that the negative regulation of Ipl1 is countered with Cdk1 activity. At this point, experimentally it would have been great to test the combination of Dam1-S20D Ask1-2A on the optical trap as another way to show that Ask1-2D is serving to oligomerize and aid in kinetochore-microtubule interface.

Knowing that phosphorylation of Ask1 promotes oligomerization, it would be reasonable to state that Dam1c would therefore undergo new conformational changes and make different

contacts either within (-intra) the complex or with its neighboring (-inter) Dam1c (Legal et al., 2016; Zelter et al., 2015). It is not possible to determine contact points of Ask1 Cdk1-sites since electron microscopy of Dam1c structure was produced with that region of Ask1 being truncated. It has been suggested that Ask1 C-terminal ends contact the Ndc80c, and crosslinking mass spectrometry bolsters this claim. (Jenni & Harrison, 2018; Kim et al., 2017). Yet, we find that this was not the case when we tested single molecule binding of rDam1c to rNdc80c or in testing phosphorylated nDam1c to Dad1-1 kinetochores.

Cdk1 phosphorylation of Ask1 establishes efficient oligomerization when the kinetochore is under high tension

Cdk1 is a cell cycle control kinase whose function was well characterized in *S cerevisiae* from studies of its homolog Cdc28 (Hartwell et al., 1974). Cdk1 also serves to phosphorylate Ask1 of the Dam1c as observed when inhibiting kinase function (Bishop et al., 2000; Li and Elledge). It phosphorylates Ask1 in a cell cycle dependent manner (Li & Elledge, 2003a; Peter & Herskowitz, 1994). Cdk1 most likely interacts with different cyclins that phosphorylate Ask1 as the cell proceeds through the cell-cycle and switch to a mitotic cyclin, such as Clb2, at the end. This may be a form of regulating its specificity in phosphorylating Ask1 and ensuring it is fully phosphorylated by the time mitosis is in play to withstand the microtubule's poleward force of ~12 pN (Loog & Morgan, 2005; Nathalie Grandin and Steven Reed, 1993; Ubersax, Woodbury, Quang, & Paraz, 2003; Asbury, 2017). Cdk1 phosphorylation ensures that the kinetochore can therefore have its oligomeric rings to serve and holdfast onto dynamic microtubules. Despite our kinetochores holding forces at 9 pN, we may be excluding additional factors that would help cells overcome the 12 pN, or perhaps within the cell all protofilaments peel away only as the cell

transitions to anaphase. In this case, the major role for Ndc80c and Dam1c is to continuously track the microtubule since the only opposing force is viscous drag from within the cell (Nicklas, 1983; Asbury, 2017).

Dam1c, Ask1-2D can be used to investigate whether its binding to the Ndc80c is better facilitated when Ndc80c scaffolds from the Cnn1 or Mtw1 arm

In this thesis we also explored the two arms, Cnn1 and Mtw1, of the Ndc80c that may have specificity as to which phosphorylated state of the Dam1c it will interact with. The two arms may be responsible for recruiting the increases in copy numbers of Ndc80c and therefore enhance its attachments to the Dam1c and the microtubule (Malvezzi et al., 2013; Lang et al., 2018; Altunkaya et al., 2016). An alternative is that the Cnn1 pathway, which comes in late mitosis, may outcompete the Mtw1 pathway to bind Ndc80c and alter its conformation to hold the Dam1c in an orientation that no longer permits Ipl1 phosphorylation, but does allow for continuous Cdk1-dependent phosphorylation. Inspiration to this wild idea comes from the fact that the interacting components of the MIND/Mtw1 complex can reverse the Ndc80c inhibited state to enhance microtubule binding (Scarborough, Davis, & Asbury, 2019). If Cnn1 can alter the conformation of the Ndc80c, then perhaps it can also alter how it interacts with the Dam1c. Crosslinking mass-spectrometry data on Dam1c and Ndc80c identifies that there are three sites of interaction. This may suggest that the Ndc80c adjusts its interaction with Dam1c and makes the tight connection with an already prominent Cdk1-phosphorylated Dam1c ring to form a stronger microtubule attachment. This is speculative, but something fun to ponder about.

Kar3 and its role in retaining Dam1c at the kinetochore

In addition to looking at Dam1c, I was also exploring motor proteins. Both Cin8 and Kar3 were found at the kinetochores. Cin8 was found to have a role in kinetochore-microtubule attachment. Using the optical trap system, we found that the rupture strength was weakened when Cin8 was absent. It was found by Zusuki and collaborators that Cin8 had an important role in leading PP1 to phosphatase Ipl1-phosphorylated sites to strengthen microtubule attachments. Kar3 was also responsible for enhancing kinetochore-microtubule attachment, but its function was not explored to completion. Findings outside of our lab were made to show that Kar3 interacted with the Ndc80c, but this is not enough to explain weakened kinetochore-microtubule attachments (Mieck et al., 2015). We found that in the absence of Kar3 protein, there was less Dam1c retained at the kinetochore. This is a big finding, but it requires additional assays and an order of operations (Kar3 deposition vs retention of Dam1c) to build a case of confidence and a beautiful story.

6 Supplementary Tables

6.1 Supplementary Table 1. Strains

All strains are derivatives of W303 (SBY3)

Strain	**Relevant Genotype**
SBY3 (W303)	*MATa ura3-1 leu2-3,112 his3-11 trp1-1 can1-100 ade2-1 bar1-1*
SBY8253	*DSN1-6His-3Flag:URA3*
SBY8944	*DSN1-6His-3Flag dad1-1:KanMx*
SBY12464	*DAD1-3Flag:TRP1*
SBY13486	*Dsn1-His-FLAG:URA3 his3-11:Ask1-12myc:HIS3 dad1-1:KanMX*
SBY13507	*DAD1-3Flag:TRP1 cdc28-13*
SBY13509	*DAD1-3Flag:TRP1 cdc28-as1*
SBY13538	*DAD1-3V5:HIS*
SBY13592 (diploid)	*his3-11/his3-11:OSTIR1-myc:HIS Dam1-myc9:TRP/DAM1 DSN1-HIS-FLAG:URA3+/DSN1*
SBY14049 (diploid)	*his3-11/his3-11:OSTIR1-myc:HIS Dam1-myc9:TRP/DAM1 DSN1-HIS-FLAG:URA3+/DSN1 CIN8-3V5:KanMX6/+*
SBY14050 (diploid)	*his3-11/his3-11:OSTIR1-myc:HIS Dam1-myc9:TRP/DAM1 DSN1-HIS-FLAG:URA3+/DSN1 CIN8-3V5-IAA17:KanMX6/+*
SBY14379	*DSN1-HIS-FLAG:URA3+ CIN8-3V5-IAA17:KanMX6*
SBY14380	*DSN1-HIS-FLAG:URA3+ CIN8-3V5-IAA17:KanMX6 his3-11:OSTIR1-myc:HIS*
SBY15712	*DSN1-HIS-FLAG:URA3 Kip1-GFP*
SBY15714	*DSN1-HIS-FLAG:URA3 Kip3-GFP*
SBY15716	*DSN1-HIS-FLAG:URA3 Kar3-GFP*
SBY15932	*Dsn1-HIS-FLAG:URA3 Kar3-3V5-IAA17:KanMX6 his3::pGPD1-OsTIR1:HIS3*
SBY15934	*Dsn1-HIS-FLAG:URA3 Kar3-3V5-IAA17:KanMX6 his3::pGPD1-OsTIR1:HIS3*
SBY15938	*his3::pGPD1-OsTIR1:HIS3 Dsn1-HIS-FLAG:URA3 Kar3-3V5:KanMX6.*
SBY15957	*Dsn1-HIS-FLAG:URA3 Kar3-3V5-IAA7(1-125):KanMx6 his3::pGPD1-OsTIR1:HIS3*
SBY15959	*Dsn1-HIS-FLAG:URA3 Kar3-3V5-IAA7(1-125):KanMx6*
SBY16176	*DSN1-HIS-FLAG:URA3+ his3::pGPD1-OsTIR1:HIS3 Dam1-myc9:TRP Kar3-3V5-IAA17:KanMX6*
SBY16766	*DAD1-SNAP-3V5:KanMx*
SBY16826	*MTW1-CLIP:KanMx dad1-1:KanMx*
SBY16828	*DSN1-His-Flag:URA3 MTW1-CLIP:KanMx dad1-1:KanMx*
SBY17831	*DAD1-3Flag:TRP1 ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*
SBY17833	*DAD1-3Flag:TRP1 ask1::KanMx leu2-3,112::ask1-S216D, S250D:LEU2*
SBY18086	*ask1::KanMx leu2-3,112::ASK1:LEU2*
SBY18088	*ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*

SBY18103	*dam1-3D:KANMX ask1::KanMx6*
SBY18105	*dam1-3D:KANMX ask1::KanMx6 leu2-3,112::Ask1-S216A, S250A:Leu2*
SBY18107	*dam1-3D:KANMX ask1::KanMx6 leu2-3,112::Ask1-S216D, S250D:Leu2*
SBY18466	*DSN1-6His-3Flag:URA3 dam1(S20D):KanMx ask1::KanMx leu2-3,112::ASK1:LEU2*
SBY18468	*DSN1-6His-3Flag:URA3 dam1(S20D):KanNMx ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*
SBY18469	*DSN1-6His-3Flag:URA3 dam1(S20D):KanMx ask1::KanMx leu2-3,112::ask1-S216D, S250D:LEU2*
SBY18606	*CDC28-3V5:KanMx*
SBY18722	CFIII (CEN3.L.YPH278)*URA3-SUP11 ask1::KanMx leu2-3,112::ASK1:LEU2*
SBY18724	CFIII (CEN3.L.YPH278)*URA3-SUP11 ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*
SBY18726	CFIII (CEN3.L.YPH278)*URA3-SUP11 ask1::KanMx leu2-3,112::ask1-S216D, S250D:LEU2*
SBY19132	*dam1-9:kanMx ask1::KanMx leu2-3,112::ASK1:LEU2*
SBY19136	*dam1-9:kanMx ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*
SBY19314	*DSN1-6His-3Flag: URA3 ask1::KanMx leu2-3,112::ask1-S216A, S250A:LEU2*
SBY19319	*DSN1-6His-3Flag: URA3 ask1::KanMx leu2-3,112::ask1-S216D, S250D:LEU2*
SBY19361	*DSN1-6His-3Flag:URA3 ask1::KanMx leu2-3,112::ASK1:LEU2*

6.2 Supplementary Table 2. Primers

Primers (all listed 5' to 3')

Primers	**Sequence**
SB3109	GTTAGTATAGATATTGAAGAGCCTCAATTGGATTTACTTGATGATGTGTTAGGTTCTGGTGGTTCTGGTATGGACAAAGACTGCGAAATGAAGCGCAC
SB3111	CATTCGTGAATACATACATCATATCATAGCACATACTTTTTCCCACTTTATATTACCGCGGCCGCATAGGCCACTAGTGGATCTG
SB4440	AATTAGGCGGCTAGAAATGGAGATAGCCAACTTGCAAGAGGAATTCAAGCTTGGGTTAATTAAC
SB4441	AGGACAAAGGAAATATCTATTCTTGAGGAGAAGTGCTCCCGATTCGGTAATCTCCGAACAG
SB4214	CATCGACGAGCAACCTACTTTATCTCAATCGAAAACGAAGAGGGAACAAAAGCTGGAGCT
SB4215	ATTTAGGATAATATTAGGAGAGACAGAGGGAACCGCAACTCTATAGGGCGAATTGGGT
SB4448	CATCGACGAGCAACCTACTTTATCTCAATCGAAAACGAAGGCGGCCGCTCTAGAACTAGTGG
SB4449	ATTTAGGATAATATTAGGAGAGACAGAGGGAACCGCAACTCCCCCTCGAGGTCGACGGTATCG

SB4524	GAAAATGTGGACAATGAGGGCTCGAGAAAAATGTTAAAGATTGAACGGATCCCCGGGTTAATTAA
SB4525	CCACTAGTTTGAATATATATTCGACTGAAAGGCAATATCAAGAATTCGAGCTCGTTTAAAC
SB4904	ATTTGCGTCTAAAGTGAATTCTACCAGATTGGTTAGTAGAAAACGGATCCCCGGGTTAATTAA
SB4905	GTCAAAGGAGTGAAAAAGACCAGAAAAGGCCTTGACCGAATTCGAGCTCGTTTAAAC
SB5091	CATCGACGAGCAACCTACTTTATCTCAATCGAAAACGAAGCGGATCCCCGGGTTAATTAA
SB5092	ATTTAGGATAATATTAGGAGAGACAGAGGGAACCGCAACTGAATTCGAGCTCGTTTAAAC
SB5468	GATTACACTCGAGGTGTCTTATTATTTAGGTTT
SB5469	TGTAATCCGGCCGCTATCTATTCGTAGAAAAAT
SB5602	GCAGCAGTACGGGTCTAGTTCCAGCATGGTACCGGCTCCGATTGTGCCCAATAAAATGCG
SB5603	GCAGCAGTACGGGTCTAGTTCCAGCATGGTACCGGATCCGATTGTGCCCAATAAAATGCG
SB5604	CGGGTGGTGGTGCCCCTGCTTCAGAGGCGCGCTCTCTATATTATTGCTGTTCTCATCG
SB5605	CGGGTGGTGGTGCCCCTGCTTCAGAGGATCGCTCTCTATATTATTGCTGTTCTCATCG
SB5892	CGCCAGAAGAGCAGCCATCCACCCCTACTTCCAAGAATCACGGATCCCCGGGTTAATTAA
SB5893	AGTAGCATTTGTAATATAATAGCGAAATAGATTATAATGCGAATTCGAGCTCGTTTAAAC

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