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Mud Binds Mitotic Kinesins Non-Claret Disjunctional and Pavarotti in D. Melanogaster

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IN *D. MELANOGASTER*

BY

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ABSTRACT

The cytoskeleton is crucial to maintaining an even distribution of chromosomes and subsequently to divide the cell during every cell cycle. During mitosis, spindle structure and movement dictates how chromosomes will be separated and act as the sole mechanism for even distribution of these chromosomes. The cytoskeleton also remains a major cellular component during cytokinesis when the cell must cleave the membrane in order to form two resulting daughter cells. Both processes require an extensive use of the immobile structures of the cytoskeleton, such as microtubules (MTs), intermediate filaments, and microfilaments, but also the mobile units, which are the motor proteins moving along them. In this study, we focus on how two kinesins, the most common class of motor protein, bind to both their cargo and to MTs. Mushroom body defect (Mud) is a *Drosophila* homolog of the human Nuclear mitotic apparatus

protein (NuMA), which are both responsible for coordinating many signaling sequences in the cell throughout mitosis. Here, we show how Mud binds to two mitotic kinesins, Non-claret disjunctional (Ncd) and Pavarotti (Pav). First, we show the binding pattern of Mud to Ncd and how this regulates a putative autoinhibition mechanism in Ncd. We believe that this Mud function allows for the activation of Ncd in order to crosslink microtubules during mitosis. Next, we determined the binding pattern between Mud and Pav, and show that phosphorylation of Pav causes an increase in binding of Mud. We hypothesize that phosphorylation of Pav is a method of activation for this interaction and that the interaction itself causes Mud to localize to the cell cortex during asymmetric cell division.

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INTRODUCTION

During mitosis, successful separation of chromosomes is imperative to proper cell function of each resulting daughter cell. In order to accomplish this, cells reorganize the cytoskeleton during prophase to create what is known as the mitotic spindle, or spindle apparatus. Microtubules (MTs), which form the fibers of the spindle, originate from spindle poles known as centrosomes and attach to chromosomes at a specific site known as the centromere. From there, the spindle pulls on the chromosome in opposing directions, which causes the sister chromatids of each chromosome to be pulled towards each centrosome on opposite ends of the cell. This chromosome bifurcation is completely dependent on spindle morphology, as it requires two spindle poles to split each chromosome in two. Many diseases have been linked to spindle morphology deformations, with some of the more severe examples of spindle malformation causing cancer, neurodevelopmental disorders, and polycystic kidney disease (Silkworth et al., 2009; Farmer et al., 2018; Zhang et al., 2010).

In unusual cases, the spindle might become deformed due to the creation of three or more centrosomes, which causes the spindle to become multipolar. Although this is usually temporary, and cells have mechanisms in place to fix this issue, unresolved multipolarity can cause aneuploidy and ultimately result in death of the resulting two daughter cells (Silkworth and Cemini, 2012). This multipolar attribute is regularly seen in cancer cells and is thought to be the result of unregulated mitotic kinases such as Aurora A (Aur-A), Aurora B (Aur-B), and Polo-like kinase 1 (Plk1) (Lens et al., 2010),

which is then exacerbated by the loss of tumor suppressor genes like p53 (Meraldi et al., 2002; Maiato and Logarinho, 2014). Specifically, the mitotic kinases Aur-A and Plk1 are involved in centrosome maturation by recruiting proteins to the pericentriolar material (PCM) in order to prime the centrosomes with γ-tubulin for MT nucleation (Crane et al., 2003; Woodruff et al., 2014; Kim and Rhee, 2014). However, it is probable that Polo-like kinase 4 (Plk4), another mitotic kinase, is directly responsible for centriole duplication, as it is among the earliest markers for centriole duplication during S-phase (Kim et al., 2013; Firat-Kiralar and Stearns, 2014). Although Plk4 is mostly known for its ability to duplicate centrioles, Aur-A and Plk-1 are also known to have other important roles in cell division, such as maintenance of the kinetochore-MT dynamics during metaphase as well as chromosome alignment, respectively (DeLuca et al., 2018; Weerdt and Medema, 2006). Both the Aurora and Plk families have orthologs in multiple lifeforms as well as a conserved protein structure between different variants of each kinase (such as Aur-A and Aur-B), suggesting that these mechanisms are evolutionarily conserved among most eukaryotes (Willems et al., 2018; Kurasawa et al., 2020).

However, spindle multipolarity is typically brief because cells have mechanisms in place to rescue multipolar phenotypes that can be caused by overexpression of Aur-A, Plk1, or Plk4. These mechanisms require the use of motor proteins such as Dynein or kinesins such as Non-claret disjunctional (Ncd) in *Drosophila* in order to cluster multiple centrosomes into two distinct poles by a process known as microtubule crosslinking (She and Yang, 2017; Simeonov et al, 2009). In this process, microtubules facing opposite ways are pulled together by minus-end-directed Dynein motor or members of

the kinesin-14 family, which includes Ncd (Goshima et al., 2005). It is thought that this process is conserved among eukaryotes, as most members of the kinesin-14 family and Dynein are conserved among multiple species as well (Wickstead and Gull, 2007; Zhang et al, 2015).

Another mitotic protein in *Drosophila* that has also been shown to rescue various abnormal spindle phenotype is Mushroom Body Defect (Mud), although the process by which it does so is not entirely known. Previous research on Mud showed that it localizes prominently at the cell cortex, the spindle poles, and the spindle MTs during mitosis (Siller et al., 2006), and that localization at both the cell cortex and the spindle pole contribute to spindle morphology. Cortical Mud localization is imperative to proper spindle positioning, as it functions through two separate mechanisms in order to orient the spindle in the direction of polarity cues located on the cell cortex (Izumi et al., 2006; Ségalen et al, 2010). In both of these mechanisms, Mud is anchored into a cortical membrane complex, and recruits Dynein to the complex in order to act as the mechanical force that pulls the closest spindle pole towards it (Johnston et al., 2013).

However, the function of Mud localized at the spindle poles is less understood. Current research shows that there is a link between Mud and the cell's ability to cluster multiple spindle poles, but the mechanism by which it does so is not known (Bosveld et al., 2017). Due to the cortical interaction between Mud and Dynein at the cortex, it is believed that Mud and Dynein also interact between spindle poles to cluster multiple poles into the correct, bipolar phenotype (Bosveld et al., 2017; Okumura et al., 2018).

Here, we define a novel Mud interaction with Ncd, and propose a mechanism by which Mud and Ncd may also be cooperating to correct multipolar spindle phenotypes.

We have also discovered that Mud interacts with another mitotic kinesin known as Pavarotti (Pav). Curiously, Pav is not related to spindle morphology until the end of mitosis, where it is known to be highly important in the formation of the contractile ring during telophase and cytokinesis (Adams et al., 1998). Mud is thought to be involved in the processes as well, although how it does so exactly remains largely unclear (Taniguchi et al., 2014). Additionally, previous research shows that Mud radiates from spindle poles in *Drosophila* neuroblasts (Siller et al., 2006), suggesting that Mud localizes to the spindle pole and subsequently moves towards the cell cortex. We suspect this movement away from the spindle pole to be the result of kinesin shuttling, as Mud itself does not have any currently known intrinsic mechanisms involving movement. Because of this, we further speculate that Mud binds to Pav to direct Mud to the cell cortex, and that once the cell moves into telophase, the pair become involved in contractile ring formation.

Chapter 1

Mud Binds the Kinesin-14 Ncd in Drosophila

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ABSTRACT

Maintenance of proper mitotic spindle structure is necessary for error-free chromosome segregation and cell division. Spindle assembly is controlled by force-generating kinesin motors that contribute to its geometry and bipolarity, and balancing motor-dependent forces between opposing kinesins is critical to the integrity of this process. Non-claret dysjunctional (Ncd), a Drosophila kinesin-14 member, crosslinks and slides microtubule minus-ends to focus spindle poles and sustain bipolarity. However, mechanisms that regulate Ncd activity during mitosis are underappreciated. Here, we identify Mushroom body defect (Mud), the fly ortholog of human NuMA, as a novel Ncd binding partner. We demonstrate this interaction involves a short coiled-coil domain within Mud (MudCC) directly binding the N-terminal, non-motor microtubule-binding domain of Ncd (NcdnMBD). We further show that the C-terminal ATPase motor domain of Ncd (NcdCTm) directly interacts with NcdnMBD as well. Mud binding competes against this self-association and also increases NcdnMBD microtubule binding in vitro. Our results describe a novel interaction between two spindle-associated proteins and suggest a potentially new mode of minus-end motor protein regulation at mitotic spindle poles.

INTRODUCTION

The kinesin superfamily of molecular motor proteins convert the chemical energy of ATP hydrolysis into microtubule (MT)-based mechanical work that enable them to perform diverse cellular tasks. These include intracellular transport, MT organization and dynamics, spindle assembly, and cytokinesis. Although detailed aspects of their structure vary, kinesins conform to a general architecture typified by a MT-binding ATPase 'head' domain, a central coiled-coil stalk region, and a cargo binding 'tail' domain (Endow et al., 2010). Motor activity must be tightly controlled to ensure proper execution of specific tasks, and kinesins have evolved several mechanisms to achieve this goal (Verhey and Hammond, 2009). Among them, autoinhibition has emerged as a means of self-regulating the function of diverse kinesin families and generally involves intra- or intermolecular interactions between motor and non-motor domains or accessory subunits that suppress MT interaction. Phosphorylation and cargo interaction represent common mechanisms for releasing these inhibited states and activating kinesin activity (Verhey and Hammond, 2009). As such, identifying specific kinesin binding partners that influence MT interaction should provide insights into the molecular mechanisms controlling their activity.

Kinesin-14 proteins, including Drosophila Non-claret dysjunctional (Ncd), represent an evolutionarily conserved subfamily that function as meiotic/mitotic-specific motors, participating in spindle assembly, spindle pole organization, and chromosome dynamics. Kinesin-14 topology is 'flipped' relative to other subfamilies, with their MT-binding

ATPase motor domain residing at the C-terminus (NcdCTm). These motors also display 'reversed' directional movement along MTs toward the minus-ends rather than plus-ends seen with most other kinesins (She and Yang, 2009). The N-terminal 'tail' domain of several kinesin-14s acts as an additional, non-motor MT-binding domain (NcdnMBD), thus allowing MT crosslinking and sliding functions essential for spindle assembly (Fink et al., 2009; Furuta and Toyoshima, 2008; Simeonov et al., 2009; Zhang and Sperry, 2004). To prevent these activities on cytoplasmic interphase MTs, an N-terminal nuclear localization sequence (NLS) signals for importin-mediated nuclear sequestration (She and Yang, 2017). Upon mitotic entry and nuclear envelope breakdown (NEBD), Ran-dependent disruption of importin binding, which occludes MT binding to the nMBD (Weaver et al., 2015), is thought to be an important step in kinesin-14 activation (Cai et al., 2009). More recently, phosphorylation of the nMBD within the Drosophila kinesin-14 Ncd was also shown to inhibit its MT binding capacity by promoting interaction with 14-3-3 (Beaven et al., 2017). Taken together, these results underscore the importance of the nMBD in both function and regulation of kinesin-14 activity, with Ncd serving as a model representative.

Here we identify the centrosomal protein Mushroom body defect (Mud; the fly ortholog of human Nuclear Mitotic Apparatus, NuMA) as a Ncd interacting protein. Mud and NuMA have established roles in spindle assembly and positioning in diverse cell types, although molecular models for these functions remain incomplete (Radulescu and Cleveland, 2010; Sun and Schatten, 2005). We delimit this interaction to a short Mud coiled-coil domain (MudCC) that directly binds with high affinity to the NcdnMBD

domain. We also find that the NcdnMBD directly interacts with the NcdCTm in trans. Mutation of MT contacting residues in the motor domain weaken this interaction, suggesting that this Ncd self-association could regulate MT binding. Finally, Mud competes against the binding of NcdCTm with NcdnMBD and also increases MT association to the isolated NcdnMBD. We suggest Mud, in addition to its other previously described roles in spindle assembly, could act as a regulator of Ncd with implications to its role in mitotic spindle function.

RESULTS

Mud directly binds Ncd

Previously, we identified a short coiled-coil domain within the C-terminal region of Mud as a substrate for Warts kinase, uncovering a phosphorylation-sensitive mode of regulating Mud localization and spindle positioning (Dewey et al., 2015). As coiled-coil domains are well-characterized protein interaction platforms (Truebestein and Leonard, 2016), we performed mass spectrometry on samples isolated from an unbiased GST pulldown of MudCC bait with Drosophila S2 whole-cell lysate prey to identify novel Mud binding partners that might convey additional functionality through this domain. One protein identified at statistically significant abundance was Ncd (99.9% Protein Threshold, 2 Peptides Minimum, 95% Peptide Threshold). Figure S1 illustrates the five unique peptides within the primary Ncd sequence that were identified in this analysis. Notably, both Ncd and Mud are known to associate with the microtubule (MT)-based

spindle apparatus during mitosis and participate in its several of its essential functions (She and Yang, 2017; Radulescu and Cleveland, 2010), suggesting the novel interaction between Mud and Ncd identified here could have implications to their function in cells.

We next sought to confirm the Mud/Ncd interaction, determine if it is direct, and map its structure-function relationship using equilibrium binding experiments with recombinantly purified components. Unlike conventional kinesin motors, Ncd motility is directed toward the MT minus-end (Endow, 1999), and its molecular topology is reversed relative to plus-end kinesins, with its ATPase motor domain residing at its C-terminus. The Ncd N-terminus contains a second, non-motor MT-binding domain (Beaven et al., 2017; Karabay and Walker, 1999), with a central coiled-coil separating these dual MT-interacting regions (Fig. 1A). We cloned and recombinantly purified full-length Ncd, along with each of these three domains individually, as Maltose-binding protein (MBP) fusions from E. coli. Attempts were made to isolate high purity proteins, although each MBP:Ncd product remained prone to some C-terminal degradation or incomplete bacterial translation, with NcdnMBD being most susceptible likely due to its lack of significant globular structure (Wendt et al., 2003). These MBP fusion baits were then immobilized on solid amylose resin and used in in vitro pulldown experiments with purified MudCC as soluble prey. Binding was quantified across a range of MudCC concentrations and equilibrium dissociation binding constants (KD) were calculated for each MBP:Ncd protein tested. MudCC binding to MBP:NcdFL was modest and had a calculated affinity in the micromolar range (Fig. 1B). Binding to MBP:NcdCTm was similar to full-length, also showing a relatively weak dissociation constant (Fig. 1E). In contrast,

a high affinity, nanomolar interaction was measured with the isolated MBP:NcdnMBD domain (Fig. 1C). No binding was detected to MBP:NcdCC at any Mud concentration tested (Fig. 1D). Lastly, we examined binding to MBP:NcdCTm containing mutation of three key MT-contacting amino acids (see below). This mutant displayed a significant impairment for MudCC binding, suggesting the low-affinity binding to NcdCTm occurs at a site that overlaps with its MT binding. Overall, these results demonstrate that (1) Mud binds Ncd directly in vitro, (2) Mud binding to Ncd is primarily mediated through high-affinity association with the NcdnMBD, and (3) Mud binding the NcdnMBD appears to be restrained within the context of the NcdFL protein.

The NcdnMBD domain was recently shown to contain two tandem phosphorylation sites that regulate its direct interaction with 14-3-3, ultimately leading to altered affinity for MTs (Beaven et al., 2017). To determine if such modifications affect Mud binding, we tested phosphomimetic (serine-to-aspartate) NcdnMBD mutants and found that neither single mutant nor a double mutant significantly affected affinity for MudCC binding (Fig. S2). Although it remains possible that naturally phosphorylated Ncd in cells may display altered binding, we conclude that MudCC directly binds the NcdnMBD domain in a manner that is likely independent of its phosphorylation status.

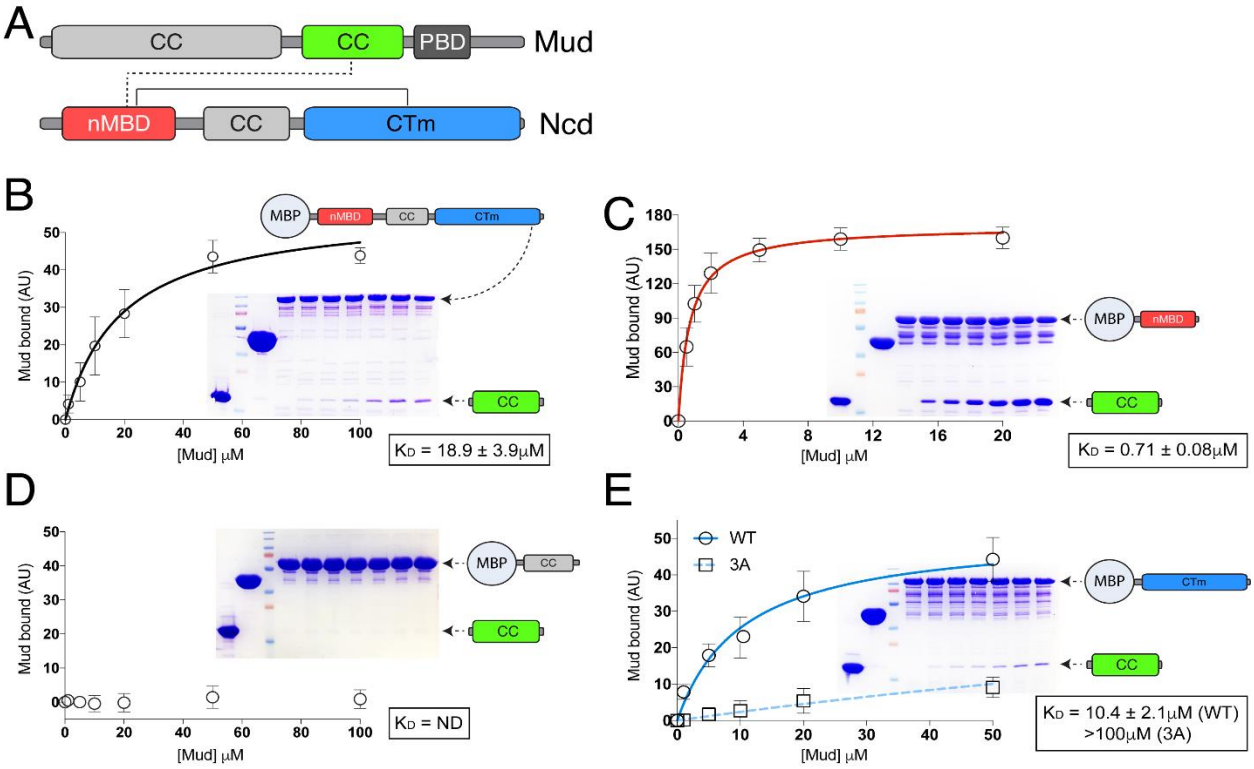

Fig. 1. Mud directly binds Ncd in vitro.

(A) Domain architectures of Mud (*top*) and Ncd (*bottom*). Mud is a large coiled-coil (CC) domain protein, with the C-terminal of these CC domains (*green*) representing the focus of this study. This Mud^{CC} domain precedes the Pins-binding domain (PBD). Ncd is a 'reversed topology' kinesin protein with its ATPase MT motor domain (CTm; *blue*) at its C-terminus. Ncd has a second, non-motor MT binding domain (nMBD; *red*) at its N-terminus, which is followed by a central CC domain (*grey*). Note these color schemes are retained throughout the remaining figures. (B) MBP alone (*third column on shown gel*) or as a fusion to Ncd^{FL} was immobilized on amylose resin and incubated without or with increasing concentrations of Mud^{CC} (1–100 μM). Gel shown is representative of 4 independent experiments, and the graph depicts the average ± standard deviation values for Mud^{CC} bound at indicated concentrations in arbitrary intensity units (AU) normalized to respective MBP:Ncd^{FL} bands. The equilibrium dissociation constant binding affinity is shown in the solid box. The first lane shows the purified Mud^{CC} used. (C) MBP alone (*third column on shown gel*) or as a fusion to Ncd^{nMBD} was immobilized on amylose resin and incubated without or with at increasing concentrations of Mud^{CC} (0.5–10 μM). Gel shown is representative of 4 independent experiments, and the graph depicts the average ± standard deviation values for Mud^{CC} bound at indicated concentrations in arbitrary intensity units (AU) normalized to respective MBP:Ncd^{nMBD} bands. The equilibrium dissociation constant binding affinity is shown in the solid box. The first lane shows the purified Mud^{CC} used. (D) MBP alone (*second column on shown gel*) or as a fusion to Ncd^{CC} was immobilized on amylose resin and incubated without or with at increasing concentrations of Mud^{CC} (1–100 μM). Gel shown is representative of 4 independent experiments, and the graph depicts the average ± standard deviation values for Mud^{CC} bound at indicated concentrations in arbitrary intensity units (AU) normalized to respective MBP:Ncd^{CC} bands. As no measurable binding could be detected, the dissociation constant was not determined (ND). The first lane shows the purified Mud^{CC} used. (E) MBP alone (*second column on shown gel*) or as a fusion to Ncd^{CTm} was immobilized on amylose resin and incubated without or with at increasing concentrations of Mud^{CC} (1–50 μM). Gel shown is representative of 4 independent experiments. The graph depicts the average ± standard deviation values for Mud^{CC} bound at indicated concentrations in arbitrary intensity units (AU) normalized to respective MBP:Ncd^{CTm} bands. The equilibrium dissociation constant binding affinity is shown in the solid box. For all experiments shown, the amount of MBP:Ncd added was kept constant across conditions and attempts were made to equalize total bait proteins loaded for analysis. All values for Mud^{CC} bound were normalized to MBP bait proteins for each respective gel lane (see Materials and methods). In all cases, the MBP input shown was incubated with the highest concentration of Mud^{CC} used in each respective binding curve. Molecular weight standards in each gel are labeled in kilodaltons (kD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

NcdnMBD and NcdCTm self-associate in vitro

The ability of MudCC to bind directly to both N- and C-terminal regions of Ncd in isolation, albeit with a much stronger preference for the N-terminal domain, inspired us to consider the possibility that these two MT-binding domains may interact to regulate Mud binding to the full-length Ncd protein, potentially explaining its significantly reduced binding affinity compared with the isolated NcdnMBD (Fig. 1B,C). Such self-associations between distinct kinesin domains have been described for several other subfamilies and often substantiate mechanisms of regulation (Verhey and Hammond, 2009). To test this hypothesis, we first examined whether the NcdnMBD and NcdCTm could directly interact in trans as isolated recombinant proteins. Indeed, soluble NcdnMBD bound to MBP:NcdCTm in a dose-dependent manner with a low-micromolar affinity (Fig. 2B). Similar results were obtained when inverting the interaction order and instead examining soluble NcdCTm binding to MBP:NcdnMBD (Fig. 2C), further validating this novel interaction. We then examined the interaction of these isolated domains with immobilized MBP:NcdFL and found that each had significantly reduced binding affinity when compared to binding to their respective isolated counterpart (Fig. S3). These results are consistent with interactions between NcdnMBD and NcdCTm regions within full-length proteins bound to the resin competing against binding to isolated domains in solution.

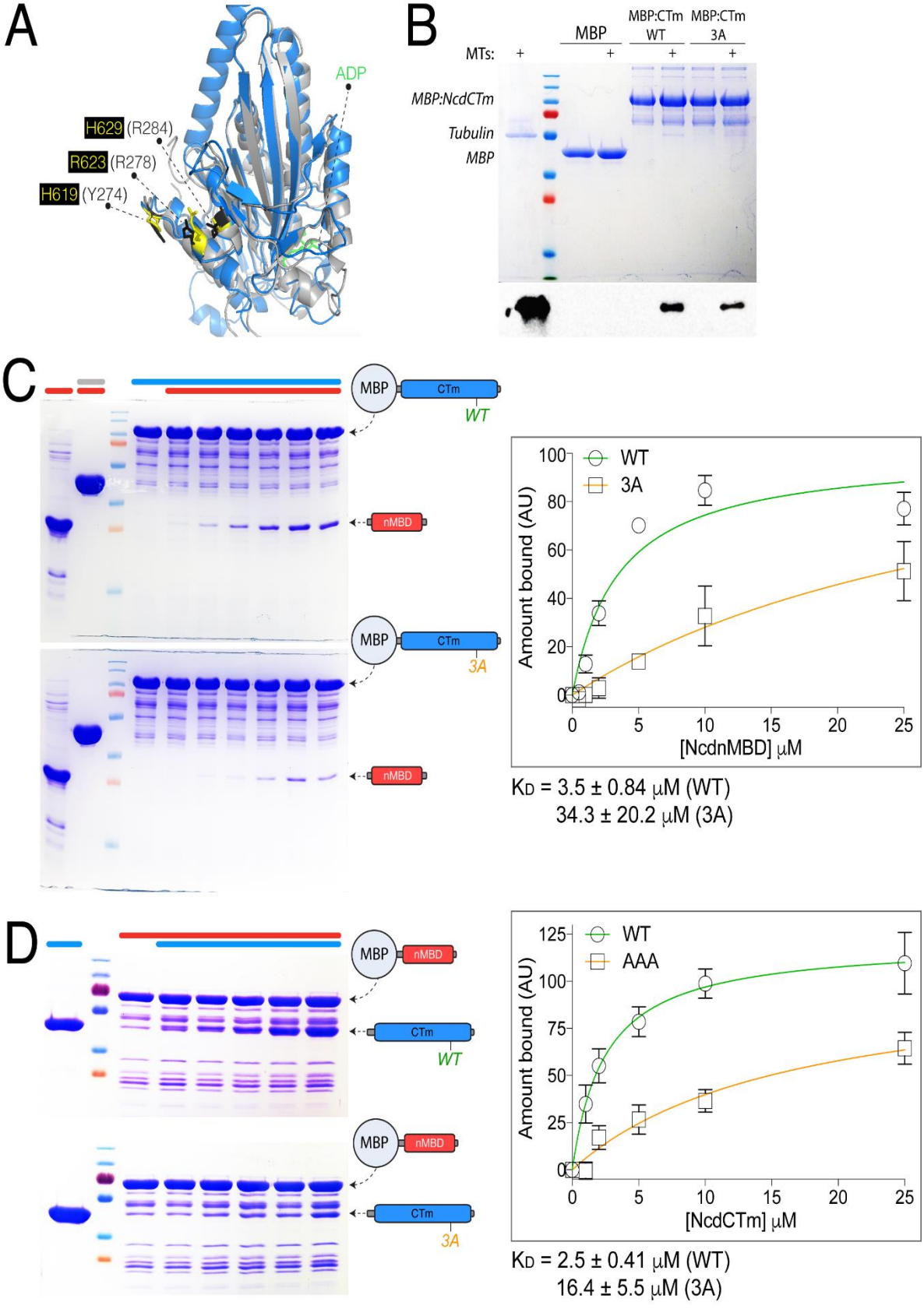
A
ADP
H629 (R284)
R623 (R278)
H619 (Y274)
B
MBP
MBP:CTm WT
MBP:CTm 3A
MTs:
MBP:NcdCTm
Tubulin
MBP
C
MBP
CTm
WT
nMBD
MBP
CTm
3A
nMBD
WT
3A
Amount bound (AU)
[NcdnMBD] μM
KD = 3.5 ± 0.84 μM (WT)
34.3 ± 20.2 μM (3A)
D
MBP
nMBD
CTm
WT
MBP
nMBD
CTm
3A
WT
AAA
Amount bound (AU)
[NcdCTm] μM
KD = 2.5 ± 0.41 μM (WT)
16.4 ± 5.5 μM (3A)

Fig. 2. Ncd MT-binding domains self-associate in vitro.
(A) Structural image of the triple alanine "3A" mutation within the Ncd L12 loop. Image depicts a superposition of the Ncd (RCSB 2NCD, blue) with that of the prototypical human kinesin-1 motor domain (RCSB 1BG2, grey). L12 loop amino acids that were mutated to alanine are indicated in yellow and grey for Ncd and kinesin-1, respectively. A bound ADP molecule (green) is shown for reference. (B) MBP alone or fused to the NcdCTm domain (wild-type, WT or 3A mutant) was immobilized on amylose resin and incubated with taxol-stabilized polymerized MTs. Samples were resolved on SDS-PAGE gels and stained with coomassie blue (top) or transferred to nitrocellulose membranes and probed with an α-Tubulin antibody (bottom). The 3A mutation reduced MT binding, consistent with previous findings (Woehlke et al., 1997; Asenjo and Sosa, 2009; Alonso et al., 2009). Gel shown is representative of 3 independent experiments. (C) MBP alone (grey bar, second lane) or as a fusion to NcdCTm (blue bar; WT, top or 3A, bottom) was immobilized on amylose resin and incubated in the absence or presence of increasing concentrations of NcdnMBD (red bar; 0.5–25 μM; MBP input shown was incubated with 25 μM). Left: gels shown are representative of 4 independent experiments. Right: saturation binding curves show average ± standard deviation values for NcdnMBD bound at indicated concentrations for WT (green) and 3A (orange). The 3A mutant results in an ~10-fold reduction in binding affinity. (D) MBP fused to NcdnMBD (red bar) was immobilized on amylose resin and incubated in the absence or presence of increasing concentrations of NcdCTm (blue bar; 1–25 μM). Left: gels shown are representative of 4 independent experiments. Right: saturation binding curves show average ± standard deviation values for NcdCTm bound at indicated concentrations for WT (green) and 3A (orange). The 3A mutant results in an ~7-fold reduction in binding affinity. In all conditions, the amount bound was performed similarly to that described in Fig. 1 and the Materials and methods. In the case of NcdCTm binding to MBP:NcdnMBD (D), particular attention was carefully given to background subtraction of the obscuring band that runs at a similar molecular weight as NcdCTm. Molecular weight standards in each gel are labeled in kilodaltons (kD). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To map a putative interaction sight within NcdCTm, we introduced a triplet of alanine mutations in the L12 loop of the Ncd motor domain (NcdCTm3A, H619A/R623A/H629A; Fig. 2A). These residues lie within the predicted MT-binding site (Sablin et al., 1998; Sosa et al., 1997; Woehlke et al., 1997), with related mutations in kinesin-1 having also been shown to reduce MT interaction (Asenjo and Sosa, 2009). Although not a complete loss-of-function, this NcdCTm3A mutant showed reduced binding to taxol-stablized MTs in vitro (Fig. 2A), consistent with Ncd L12 loop involvement in MT binding (Alonso et al., 1998). We next tested the ability of NcdCTm3A to bind to NcdnMBD in trans to determine if the L12 loop is also important for the Ncd self-association. Similar to the effects of this mutant on MudCC binding (Fig. 1E), the 3A mutant caused an ~10-fold

reduction in binding affinity to NcdnMBD when tested as either the soluble or MBP-immobilized fraction (Fig. 2C,D). Taken together, these results suggest that the site of both MudCC binding and NcdnMBD self-association overlap with the MT binding surface of the NcdCTm domain and suggest a network of interactions that could play an important role in regulating Ncd function.

Mud competes against Ncd self-association and enhances MT interaction with NcdnMBD

Having established that NcdnMBD directly binds both MudCC and NcdCTm, and that Mud and NcdnMBD each had reduced binding affinity to the NcdCTm3A mutant (Figures 1C and 2), we next examined whether these interactions are mutually exclusive. To do this, we immobilized MBP:NcdCTm on amylose resin and examined how its interaction with a single concentration of NcdnMBD (at the ~KD of 2 μM) is affected by addition of increasing concentrations of MudCC. As shown in Figure 3, binding of MudCC resulted in a concentration-dependent reduction in the interaction between NcdCTm and NcdnMBD, demonstrating that MudCC directly competes against NcdnMBD/NcdCTm binding. This result suggests that Mud binding could act as a mechanism to disengage the Ncd self-association to regulate its function.

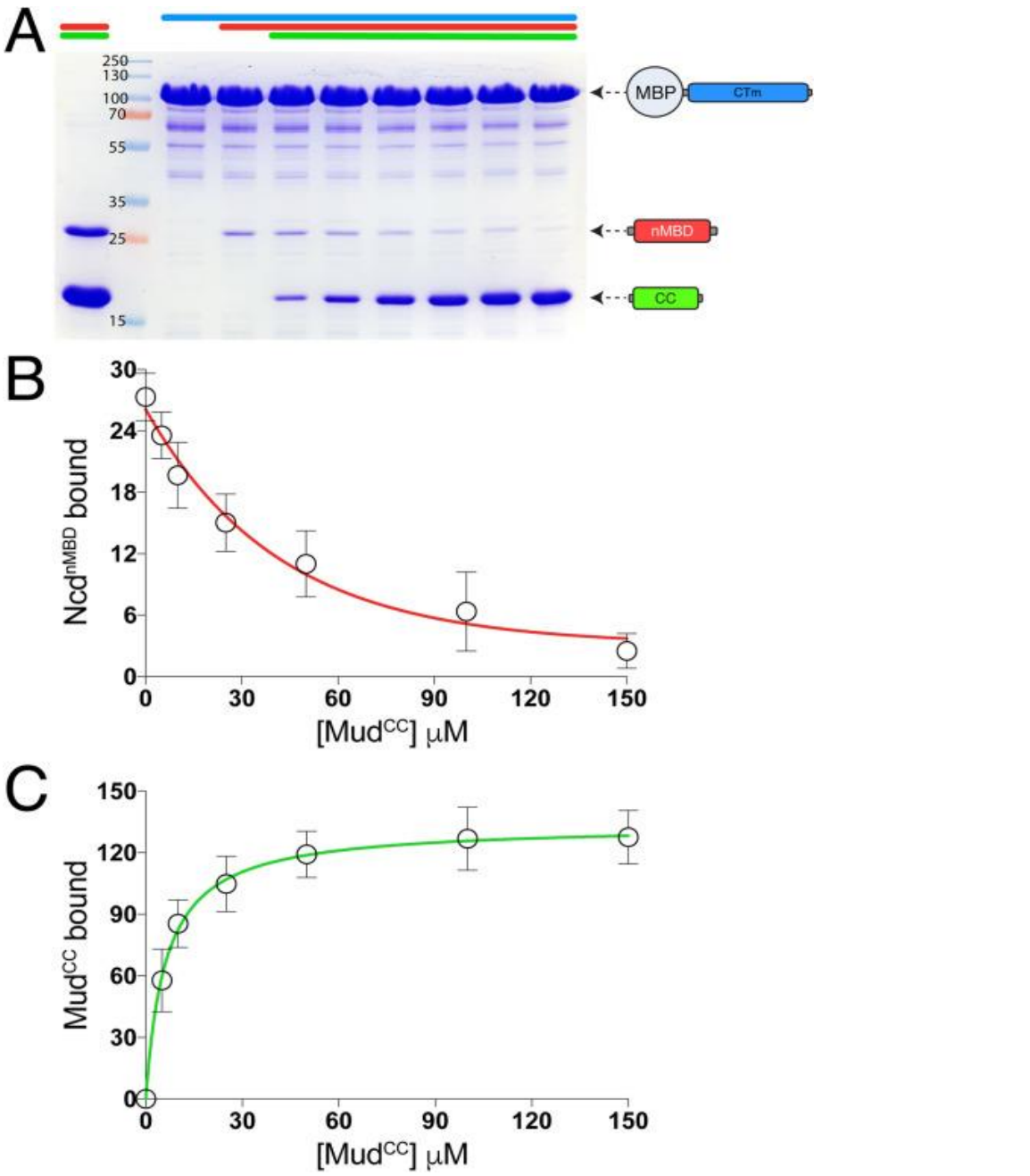

Fig. 3. Mud directly competes against Ncd self-association.
(A) MBP-fused NcdCTm (blue) was immobilized on amylose resin and subsequently incubated without or with 2 μM NcdnMBD (red) and increasing MudCC (5 μM–150 μM; green). Gel shown is representative of 5 independent experiments. Molecular weight standards in each gel are labeled in kilodaltons (kD). (B) Effects of MudCC on the interaction between MBP:NcdCTm and NcdnMBD. Curve plots the average ± standard deviations for the amount of NcdnMBD bound to MBP:NcdCTm (as a function of MudCC concentration for 5 independent experiments. (C) Curve plots the average ± standard deviations for the amount of MudCC bound to MBP:NcdCTm as a function of MudCC concentration in the presence of 2 μM NcdnMBD for 5 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

To explore how Mud might control Ncd function, we next examined how MudCC binding influences the interaction between NcdnMBD and taxol-stabilized MTs. Surprisingly, association with MudCC increased MT binding to MBP:NcdnMBD (Fig. 4A). Examining this effect across a range of MT concentrations revealed that Mud binding acts primarily to increase the affinity of MT binding to NcdnMBD without significantly increasing its maximal capacity for MT binding at higher concentration (Fig. 4B). A precise mechanism for this effect remains unclear at this time. The NcdnMBD has been shown to contain two MT-contacting sites (Karabay and Walker, 1999); it is possible that Mud binding affects the relative conformations of these regions to influence their association with MTs. Nonetheless, these results demonstrate that a MudCC/NcdnMBD/MT trimeric complex is not only possible but may exist as a high-affinity complex. We conclude that MudCC reduces self-association between Ncd domains while also enhancing its MT binding through direct interaction with the nMBD.

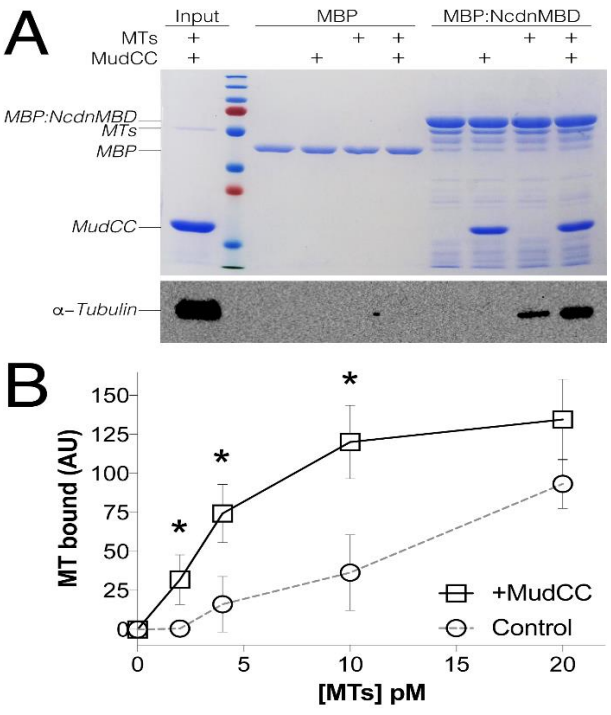

Fig. 4. Mud enhances MT binding to NcdnMBD in vitro.
(A) MBP alone or as a fusion to NcdnMBD was immobilized on amylose resin. Reactions were then incubated in the absence or presence of taxol-stabilized MTs, MudCC, or both as indicated. Coomassie stained gel (top) shows near equal levels of bait proteins as well as the MudCC interaction with MBP:NcdnMBD. Anti-αTubulin western blot (bottom) depicts an increased amount of MTs bound in the presence of MudCC. Molecular weight standards in the gel are labeled in kilodaltons (kD). (B) Curves representing the effect of MudCC (10 μM) on concentration-dependent MT binding to MBP:NcdnMBD. Binding was determined from pixel intensity measurements of tubulin bands on western blot images and are plotted as arbitrary intensity units (AU). Mud significantly increases the amount of bound MTs at lower concentrations without altering the total binding capacity at saturation. *, $p < 0.05$ relative to Control, Student's t-test for respective MT concentrations.

The Mud coiled-coil domain localizes to spindle poles in an Ncd-dependent manner

Having delineated the Mud/Ncd interaction in vitro, we next sought to investigate its relevance within a cellular context, particularly as it pertains to key aspects of mitotic spindle activity. To do so we used Drosophila S2 cells, a well-established model system for studying spindle assembly and function (Goshima, 2010; Moutinho-Pereira et a., 2010). We first examined the localization of transiently-transfected GFP-tagged MudCC. In addition to cytoplasmic localization, GFP:MudCC showed prominent localization to spindle poles in most cells. Quantification of the pole:cytoplasm intensity revealed ~2-fold accumulation at spindle poles (Fig. 5). Treatment of cells with interfering dsRNA (RNAi) against Ncd significantly reduced pole localization. In most cells, NcdRNAi caused spindle assembly abnormalities (see below), yet GFP:MudCC mislocalization was notable even in treated cells with relatively intact spindles. In contrast, NcdRNAi did not affect pole localization of endogenous, full-length Mud (Fig. 5). In separate experiments, we expressed a full-length GFP:Ncd and found that MudRNAi treatment did not significantly affects its spindle pole localization (Fig. S2), suggesting Mud is not required for Ncd localization. Knockdown of Abnormal spindles (Asp) did significantly reduce endogenous Mud at spindle poles, as expected (Bosveld et al., 2017), but did not affect that of the isolated GFP:MudCC. Ample evidence supports a role for Mud and Dynein cooperation at spindle poles (Radulescu and Cleveland, 2010); however, treatment of cells with RNAi targeting the Dynein heavy chain (Dhc64CRNAi) also did not affect GFP:MudCC localization. Together, these results suggest that the MudCC domain is sufficient for pole localization and occurs specifically through an Ncd-dependent mechanism. It is likely

that other Mud domains, namely its N-terminal coiled-coils, are responsible for interactions with Dynein and Asp and that these interactions provide a more substantive contribution to Mud localization.

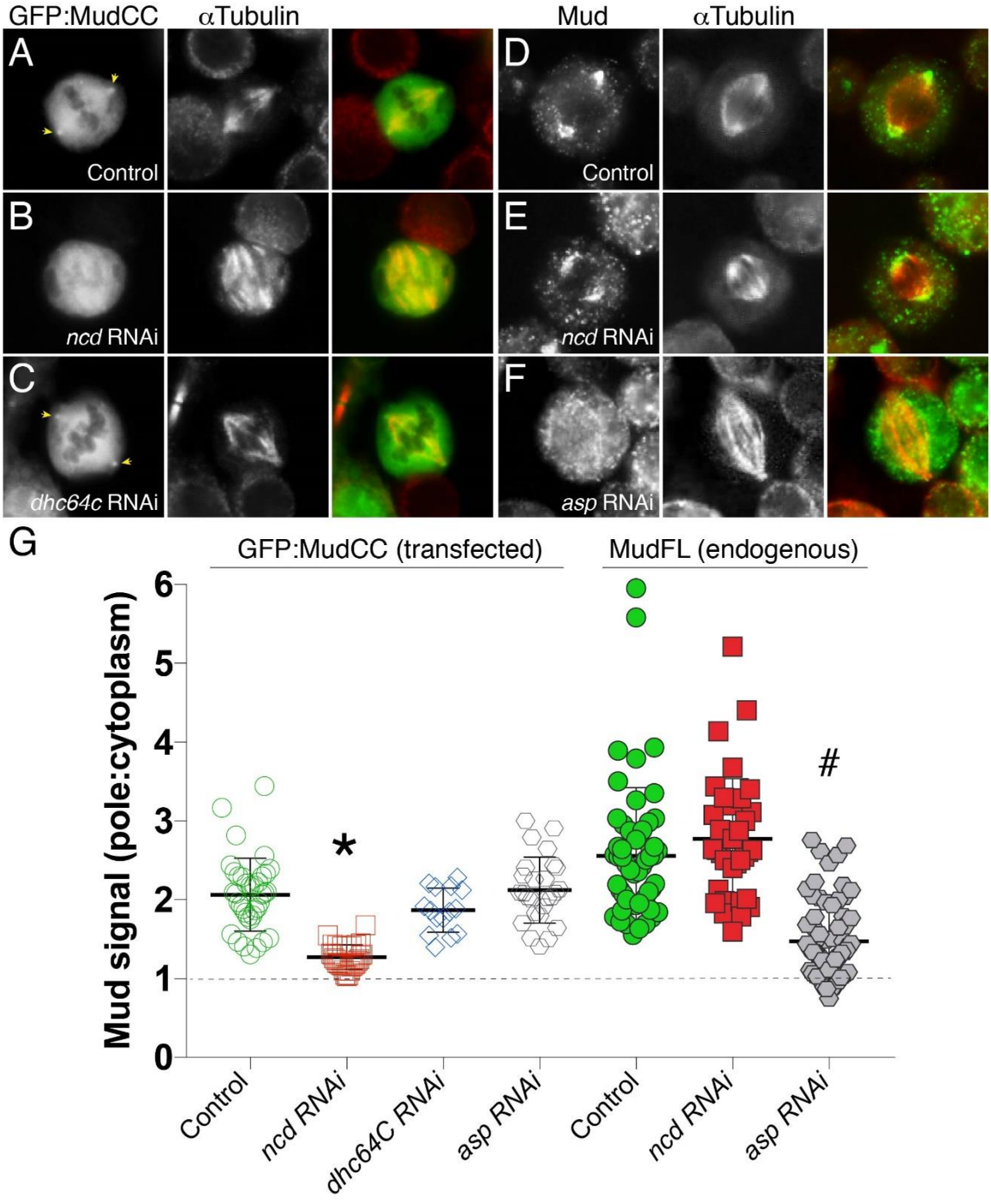

Fig. 5. MudCC localizes to mitotic spindle poles in an Ncd-dependent manner
(A-C) S2 cells were transiently transfected with GFP:MudCC and treated without (A) or with RNAi against *ncd* (B) or *dhc64c* (C) for 5 days. Cells were fixed and stained with an antibody against α-Tubulin (red).
(D-F) S2 cells were treated without (D) or with RNAi against *ncd* (E) or *asp* (F) for 5 days. Cells were fixed and stained with antibodies against Mud (green) and α-Tubulin (red).
(G) Quantification of spindle pole relative to cytoplasm intensities for GFP ('transfected', left) or Mud ('endogenous', right) under each condition indicated. *, $p<0.05$ relative to GFP:MudCC control; #, $p<0.05$ relative to Mud control, ANOVA with Tukey's post-hoc test.

Mud and Ncd knockdowns share phenotypic traits in spindle assembly

The Ncd-dependent MudCC pole localization suggests that the Mud/Ncd interaction could be relevant to spindle assembly and function. Ncd has been shown to play roles in spindle pole focusing, centrosome clustering, and, in some systems, spindle length and geometry (Petry, 2016). We found that both NcdRNAi and MudRNAi resulted in unfocused spindle poles as quantified by significant increases in the width of MT minus ends averaged across both poles (Fig. 6A,B). The exact role of kinesin-14 motors in controlling spindle morphology is somewhat uncertain, as previous studies have reported inconsistent results (Cai et al., 2009; Sharp et al., 2000; Sharp et al., 1999). We found that MudRNAi and NcdRNAi both resulted in a shortening of spindles (measured as pole-to-pole distance) together with a widening of spindle fibers at the metaphase plate (Fig. 6A,C). The primary phenotype observed following Dhc64CRNAi treatment was detached centrosomes, consistent with previous studies (Goshima et al., 2005), indicating its inability to infringe on GFP:MudCC localization is unlikely due to ineffective knockdown (Fig. S5A). Overexpression of GFP:MudCC was sufficient to induce an increase in spindle length:width ratio; however, it did not significantly affect pole width. Notably, this MudCC-induced spindle lengthening was suppressed by NcdRNAi treatment (Fig. 6C). A similar phenotype was observed with GFP:NcdFL, overexpression

of which resulted in an increased spindle length:width ratio that was suppressed by MudRNAi treatment (Fig. 6C). We conclude that both Mud and Ncd are necessary for maintenance of spindle pole focusing and that they cooperate to control proper spindle geometry.

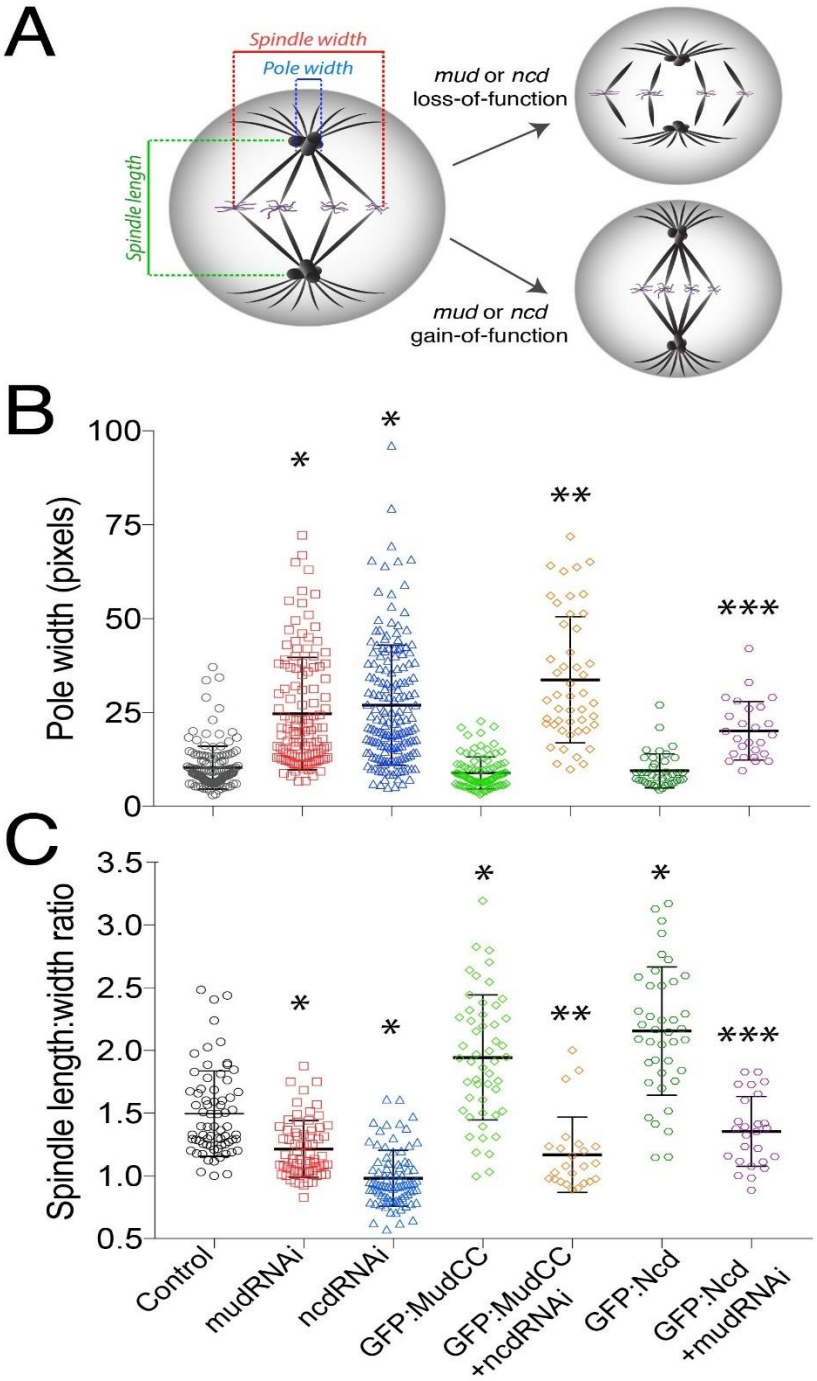

Fig. 6. Mud and Ncd cooperate in mitotic spindle assembly
(A) Schematic representation of how spindle morphology measurements were conducted (left). Pole focusing was determined using pole widths (blue), which were measured as the distance span of MT minus-ends at both spindle poles averaged. Spindle geometry was assessed as a ratio between the length (green; distance between poles) and the width (red; distance across spindle equator). Generally, reducing Mud or Ncd function leads to short, wide spindles (with unfocused poles; right/top), whereas overexpression of MudCC or Ncd results in long, narrow spindles (right/bottom).
(B) Quantification of spindle pole widths for the indicated conditions. Knockdown of mud or ncd results in broad spindle poles. Expression of GFP:MudCC or GFP:Ncd had slightly narrower poles, although these did not reach statistical significance relative to control. The mud and ncd knockdowns remained significant even in the presence of these GFP fusion expressions. *, $p<0.05$ relative to Control; **, $p<0.05$ relative to GFP:MudCC; ***, $p<0.05$ relative to GFP:Ncd, ANOVA with Tukey's post-hoc test.
(C) Quantification of spindle morphology measurements for indicated conditions. RNAi against mud or ncd caused shorter, wider spindles, whereas expression of GFP:MudCC or GFP:Ncd resulted in longer, narrower spindles. Treatment with NcdRNAi suppresses the effects of GFP:MudCC, and treatment with MudRNAi suppresses the effects of GFP:Ncd. *, $p<0.05$ relative to Control; **, $p<0.05$ relative to GFP:MudCC; ***, $p<0.05$ relative to GFP:Ncd, ANOVA with Tukey's post-hoc test.

Mud and Ncd are necessary for chromosome alignment

Ncd mutants have previously been shown to cause scattered chromosomes in both mitotic and meiotic spindles (Hatsumi and Endow, 1992). This, together with their similar effects on both pole integrity and spindle architecture, prompted us to next examine how Mud and Ncd knockdown each affect chromosome congression and alignment. For this analysis, we used an S2 cell line stably expressing GFP fused to the Centromere identifier (CID) gene, the Drosophila ortholog of the CENP-A centromere protein that marks centromeres (Dewey and Johnston, 2017). We found that compared to control cells, NcdRNAi and MudRNAi treatments each significantly increased the frequency of cells with non-congressed and improperly aligned chromosomes (Fig. 7). We conclude that Mud- and Ncd-mediated spindle assembly is a prerequisite for proper chromosome alignment.

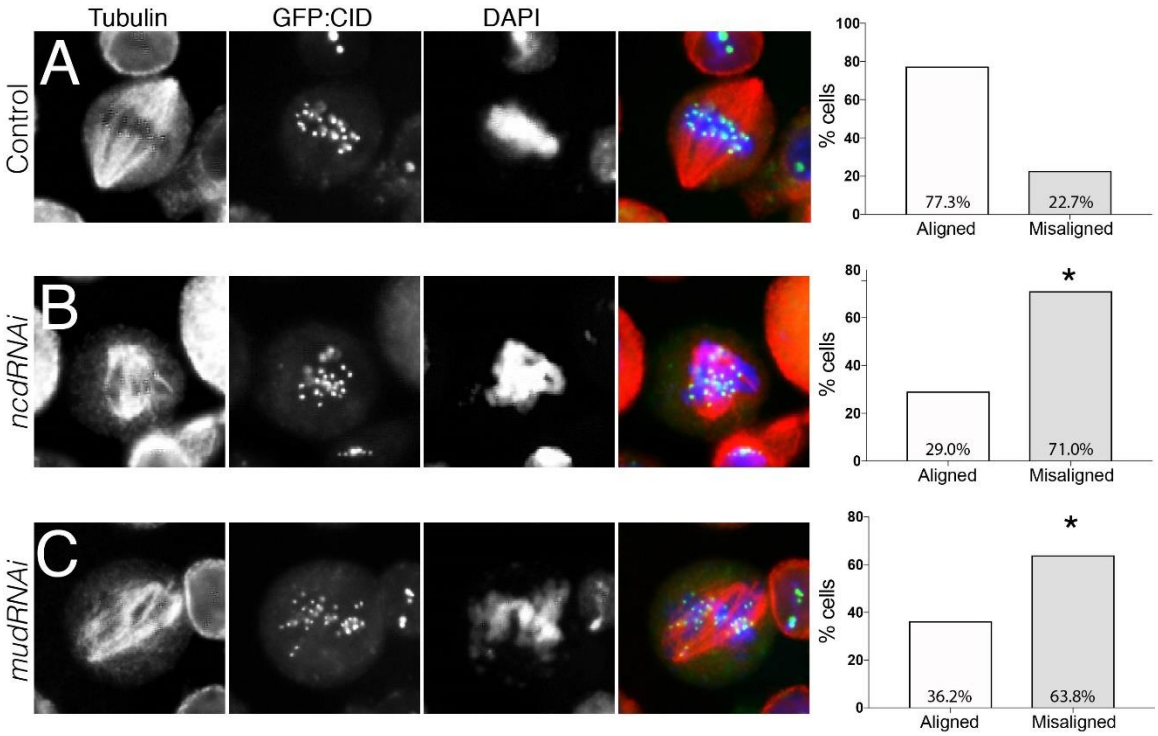

Figure 7 – Mud and Ncd knockdown impairs chromosome alignment
S2 cells stably expressing GFP:CID (centromere identifier) were treated without (A) Control or with RNAi targeted against (B) ncd or (C) mud for 5 days. Cells were formaldehyde fixed and stained with an antibody against Tubulin (red) as well as DAPI (blue). Both ncdRNAi and mudRNAi lead to a significant percentage of cells with misaligned chromosomes. *, $p<0.05$ relative to Control, Fisher's exact test.

Mud and Ncd are necessary to suppress multipolar divisions

Abnormal chromosome alignment can lead to segregation defects during anaphase. One underlying mechanism associated with abnormal chromosome alignment and segregation is multipolar divisions that occur as a consequence of centrosome amplification (Marthiens et al., 2012). Such aberrant mitoses have been implicated in the generation of chromosome instability and aneuploidy often witnessed in cancer cells (Vitre and Cleveland, 2012). To avoid such events, cells have evolved mechanisms that cluster centrosomes into a pseudo-bipolar spindle to suppress multipolar mitoses. To ascertain how Mud and Ncd loss affects bipolar division, we performed live-cell imaging of S2 cells stably co-expressing GFP:CID and mCherry:α-Tubulin (Dewey et al.,

2019). Although S2 cells are naturally multipolar at an observable frequency, we used RNAi against Regulator of cyclin A1 (Rca1) in all conditions tested to increase the frequency of supernumerary centrosomes (Ricolo et al., 2016). Cells with >2 (typically 3 or 4) visible centrosomes just prior to nuclear envelope breakdown were selected for imaging (Dewey et al., 2019), which was carried through telophase or cytokinesis. In control cells (Rca1RNAi alone), extra centrosomes were dynamically clustered into bipolar spindles in 71% of cells, leading to otherwise normal bipolar divisions (Fig. 8A). Treatment with NcdRNAi lead to a significant increase in the frequency of cells with multipolar spindles (77%), which subsequently lead to tripolar divisions (Fig. 8C). These results are consistent with previous reports implicating Ncd as a core component of centrosome clustering (Basto et al., 2008). We next examined MudRNAi-treated cells and found a similar frequency (68%) of cells undergoing tripolar mitoses (Fig. 8D). Treatment with Dhc64CRNAi did not affect the efficiency of clustering, with 77% of cells showing a normal bipolar division (Fig. 8B). The lack of Dhc64CRNAi effects seen here, as well as with MudCC localization above, were not due to a lack of RNAi efficiency as western blot analysis revealed a reduction in Dhc expression in these cells (Fig. S5C). Furthermore, Dhc64CRNAi resulted in an increased frequency of detached centrosomes from the mass of spindle K-fibers as previously described (Goshima et al., 2005) (Fig. S5A,B). These results demonstrate a role for Mud in suppressing multipolar spindles and suggest it operates through Ncd rather than Dynein to achieve this effect in S2 cells.

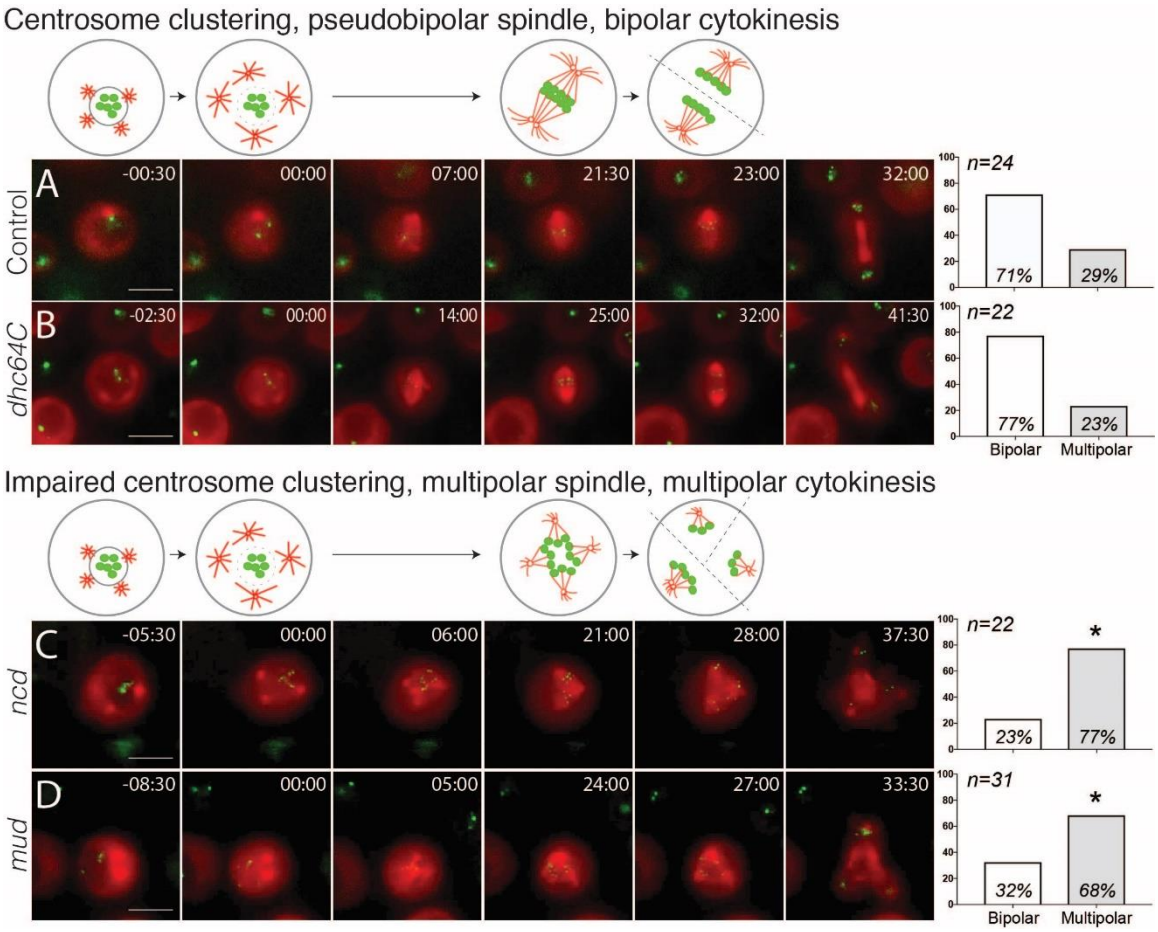

Fig. 8. Mud and Ncd are required for centrosome clustering and suppression of multipolar divisions
S2 cells stably expressing GFP:CID and mCherry:α-Tubulin were treated with RNAi targeting rca1 alone (A) Control or together with RNAi targeted against (B) dhc64C (C) ncd, or (D) mud for 5 days. Live imaging of cell divisions with >2 noticeable centrosomes was conducted from just prior to NEBD to late telophase or cytokinesis. In the majority of cases, control and dhc64CRNAi-treated cells were capable of clustering excess centrosomes into pseudo-bipolar spindles that suppressed multipolar divisions. Treatment with ncdRNAi or mudRNAi, however, causes a significant reduction in this clustering function, which leads to increased frequency of multipolar divisions. *, $p<0.05$ relative to control, Fisher's exact test.

DISCUSSION

Maintenance of proper spindle structure throughout mitosis is essential for correct and efficient segregation replicated chromosomes into daughter cells. Although our understanding of the molecular mechanisms that control this complex process has

become increasingly clear in recent years, many details remain to be elucidated (Petry, 2016). Herein, we have detailed a novel interaction between two prominent spindle-associated proteins, the structural protein Mud and the kinesin-14 motor Ncd. Mud directly binds the non-motor MT-binding domain at the Ncd N-terminus with a sub-micromolar affinity, which is ~25-fold greater compared that measured with the full-length Ncd protein. Furthermore, we show a direct self-association between the NcdnMBD domain and the C-terminal ATPase motor domain and demonstrate that Mud binding competitively uncouples this Ncd self-association. Both MudCC and NcdnMBD binding to the NcdCTm are significantly impaired by mutations to the MT-contacting L12 loop. Altogether, these results suggest that interactions between Ncd MT-binding domains could act as an important regulatory mechanism, with Mud binding acting to reverse such control. The ability of Mud to facilitate MT binding to the NcdnMBD further implies a positive impact on its function. Our results support a model in which Mud may act as a key regulator of not only Dynein but also Ncd, two essential MT motors functioning at MT minus ends (Akhmanova and Steinmetz, 2019).

By competing against the self-association between the two distinct MT-binding domains of Ncd, Mud could act to enhance motor domain association with MTs and thus the motility of Ncd, similar to that seen with cargo-stimulated kinesin-1 motility (Blasius et al., 2007). Alternatively, low-affinity Mud binding to NcdCTm could directly impact its MT binding or catalytic function at high concentrations, although we are unaware of a precedence for such interactions at kinesin motor domains. It is also possible that Mud binding has no impact, direct or indirect, on motor domain activity, but instead exerts its

function solely through non-catalytic MT interactions with the NcdnMBD. The existence of an apparent MudCC/NcdnMBD/MT complex yielding increased MT binding (Fig. 4) is consistent with this model, and is similar to regulation of the human kinesin-14, HSET (Chavali et al., 2016). Parsing such hypotheses will require future experiments, the blueprints for which are outlined by the detailed interaction studies presented in this study.

Several diverse members of the kinesin superfamily participate in distinct processes of mitotic spindle assembly and function. In many cases, multiple kinesins cooperate by exerting opposing forces that demand precise spatial and temporal regulation to ensure balanced, productive coordination (Petry, 2016; Yount et al., 2015). Kinesin-14s such as Ncd are no exception to this rule, with excessive activity leading to altered spindle geometry and chromosome dynamics (Cai et al., 2009). In contrast, reduced kinesin-14 function leaves the opposite force generating kinesin-5 unopposed, leading to outward forces that unfocus spindle poles (Hentrich and Surrey, 2010; Yukawa et al., 2015). These finding make it clear that proper control of kinesin-14 activity is essential for spindle function. Current models of kinesin-14 regulation are primarily linked to nuclear dynamics. Prior to mitosis, kinesin-14 is bound to importin and sequestered in the nucleus to prevent excessive bundling of interphase MTs. Upon nuclear envelope breakdown, the chromatin-derived Ran gradient is thought to dissociate importin to liberate the kinesin-14 to exert is mitotic function (Weaver et al., 2015; Ems-McClung et al., 2003; Goshima and Vale, 2005). It is not clear whether Mud/NuMA might impact this process, but, interestingly, NuMA itself also undergoes a Ran-dependent activation

process to remove importin binding to a region near the analogous MudCC domain examined in this study (Chang et al., 2017; Nachury et al., 2001). As the active Ran gradient dissipates toward spindle poles (Caudron et al., 2005; Kalab et al., 2002), the primary site of Ncd action, it is possible that Ran and Mud could serve as spatially independent regulators of kinesin-14 function. More recently, phosphorylation of the NcdnMBD was shown to preclude its MT association by promoting a mutually-exclusive interaction with 14-3-3, a mechanism required for proper meiotic spindle assembly (Beaven et al., 2017). The Ncd/Mud interaction was not sensitive to phosphomimetic mutations at these sites (Fig. S2), however, suggesting this phosphorylation likely acts primarily as an inhibitory mechanism. Cargo binding serves as an important regulatory mechanism for many kinesin families, including kinesins-1, -2, -3, -7, and -13. In these cases, cargo interactions typically relieve inhibited conformations maintained through self-associations between distinct kinesin domains or with regulatory binding proteins (Yount et al., 2015; Hirokawa et al., 2009). The interaction between the NcdnMBD and NcdCTm domains (Fig. 2) suggests a possible role in regulating Ncd function, although this will certainly require further investigation. It should be noted that most kinesin-14s, owing to their extended coiled-coil stalk domains, exist as rigid homodimers with limited structural flexibility (Wang et al., 2018). Thus, interactions between motor and non-motor domains, whatever their functional consequence, would likely occur intermolecularly in trans. While kinesin-14s do not have cargo per se, the interactions of Mud or 14-3-3 with the N-terminal nMBD could act in an analogous fashion to finely regulate MT crosslinking function during mitosis.

Recently, another large centrosomal protein, CEP215, was discovered as an HSET-interacting protein (HSET being the human kinesin-14 ortholog of Ncd) (Chavali et al., 2016). CEP215, also known as CDK5RAP2, is the human ortholog of Drosophila centrosomin (Cnn), a large coiled-coil protein critical for centrosome assembly and maintenance (Megraw et al., 1999). Interestingly, this interaction was delimited to a coiled-coil domain-containing region within the N-terminus of CEP215 directly binding to the nMBD of HSET (Chavali et al., 2016), a result that closely resembles how Mud binds Ncd (Fig. 1A,C). CEP215 binding serves as a MT minus end recruitment signal for HSET, perturbation of which leads to centrosome-spindle detachment and reduced clustering efficiency (Chavali et al., 2016). It is not currently known whether a conserved function for Cnn exists in controlling Ncd localization, however. If so, determining whether Cnn and Mud can bind the NcdnMBD simultaneously, or if their interactions are mutually exclusive, could offer additional insight into the specific role for each in Ncd function. Whether the MT-binding domains of HSET self-associate, as with Ncd, and how CEP215 binding may impact such interaction will also require future studies to resolve. Nevertheless, these results, taken together with those reported here, highlight an apparent role for coiled-coiled domain containing centrosomal proteins in regulating localization and activity of the kinesin-14 proteins. Resolving the precise molecular mechanisms involved as well as their evolutionary conservation will be critical next steps.

What functional roles might the Mud/Ncd interaction contribute to inside the cell? Kinesin-14 members are well known to play prominent roles in the focusing of mitotic

spindle poles and in the clustering of excess centrosomes into bipolar spindles (She and Yang, 2017; Simeonov et al., 2009; Petry, 2016). Loss of these functions can lead to multipolar divisions, chromosome segregation errors, and aneuploidy that affect cell viability (Marthiens et al., 2012). In fact, inhibiting clustering in cancer cells with centrosome amplification has been suggested as a potentially novel therapeutic avenue (Marthiens et al., 2012; Mariappan et al., 2018). RNAi-based screening studies in both human and fly cells have identified numerous other genes necessary for this process, notably Ncd/HSET (Goshima et al., 2007; Leber et al., 2010), although a role of Mud/NuMA has not been clearly defined. Recent studies in Drosophila wing disc epithelial cells demonstrated that Mud mutants exhibited unfocused spindle poles and supernumerary centrosomes, although cells divided in a pseudo-bipolar manner following centrosome clustering (Bosveld et al., 2017). Studies suggesting a role for NuMA have largely extended from its known interaction with the Dynein complex, with NuMA providing a localization cue for Dynein-dependent coalescence of MT minus ends (Hueschen et al., 2017). A role for Dynein itself in centrosome clustering per se has also been subject of conflicting evidence, however (Marthiens et al., 2012; Goshima et al., 2005, Kwon et al., 2008; Quintyne et al., 2005). Recent studies have shown that Mud and Dynein function to couple centrosomes to spindle fibers prior to mitosis. Loss of this function causes centrosome displacement from spindles leading to incorrect inheritance of both centrosomes into one of the two daughters (Bosveld et al., 2017). Thus, Mud (or Dynein) loss can be a contributor to the development of supernumerary centrosomes as well. Finally, multiple models have been proposed to explain the cooperative functions

of NuMA, HSET, and Dynein in spindle assembly and chromosome dynamics (Gordon et al., 2001); the interaction between Mud and Ncd (and potentially conserved in NuMA and HSET) described here posits an additional mode by which these essential regulators may be linked. Thus, Mud may serve a vital role in several aspects of spindle pole integrity, with specific functions being carried out through its interaction with distinct minus-end motors, Dynein and Ncd. The molecular basis for such regulation will require further exploration, but our results here suggest that Mud could regulate Ncd function through facilitation of MT binding. Future studies of these potential functions in the diverse model tissues that Drosophila offer would be of substantial merit.

Proper spatial and temporal control of motor protein function is essential for diverse cellular processes, particularly for those that must tightly coordinate force generation to ensure a faithfully executed cell division. Kinesin dysfunction has been linked to errors in spindle assembly, spindle orientation, and chromosome segregation, all of which can lead to deleterious consequences for tissue homeostasis. Our discovery of a direct Mud/Ncd interaction provides new insight into the regulation of this essential mitotic kinesin. Future questions that will be important to resolve include: What is the interplay between Mud and 14-3-3 in Ncd regulation? Does Mud binding control the motor activity of Ncd and its processivity? What is the role of the Mud/Ncd interaction in vivo and is it ubiquitous or tissue specific? Are the NcdnMBD/NcdCTm and MudCC/NcdnMBD interactions evolutionarily conserved mechanisms for kinesin-14 regulation? Is the Mud/Ncd interaction regulated by other cellular components?

MATERIALS AND METHODS

Antibodies

Primary antibodies used include mouse anti-α-Tubulin (DM1A, Sigma, 1:1000), rat anti-α-Tubulin (Sigma, 1:500), rabbit PH3 (Abcam, 1:5000), mouse Dynein heavy chain (DSHB, 1:1000), and a rabbit Mud antibody generated in this study (YenZym, 1:1000). All secondary antibodies used in S2 cell immunofluorescence (cross absorbed to prevent cross reactivity) experiments were purchased for Jackson ImmunoResearch (1:250). HRP-linked secondary antibodies used in western blot experiments were purchased from Abcam and used at 1:2000 dilution.

Cloning and plasmid construction

Cloning was performed using PCR amplified fragments obtained from an S2 cell cDNA library template. The Mud coiled-coil domain (amino acids 1760–1906) was cloned into the bacterial expression pBH plasmid using 5'-BamHI and 3'-SalI restriction sites, generating a TEV cleavable 6×His fusion. Full-length Ncd (amino acids 1-700) or individual domains (nMBD: amino acids 1-199; coiled-coil: amino acids 200-332; CTm: amino acids 333-700) were cloned as 6×His, GST, or MBP fusions by cloning into pBH, pGEX, or pMAL plasmids, respectively, using 5'-KpnI and 3'-SalI or 5'-NdeI and 3'-SalI restriction site combinations. Site-directed mutagenesis was carried out with a standard PCR protocol using KOD-XL DNA polymerase.

Protein purification

All proteins were expressed in BL21(DE3) E. coli under induction of isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown in standard Luria–Bertani broth supplemented with 100 μg/ml ampicillin. Transformed cells were grown at 37°C to an OD600 ∼0.6 and induced with 0.2 mM IPTG overnight at 18°C. Cells were harvested by centrifugation (5000 × g for 10 min), and bacterial pellets were resuspended in lysis buffer and flash-frozen in liquid nitrogen. Cells were lysed using a Branson digital sonifier and clarified by centrifugation (12,000 × g for 30 min).

For 6×His-tagged proteins, cells were lysed in N1 buffer (50 mM Tris pH8, 300 mM NaCl, 10 mM imidazole) and coupled to Ni-NTA resin for 3 h at 4°C. Following extensive washing, proteins were eluted with N2 buffer (50 mM Tris pH8, 300 mM NaCl, 300 mM imidazole). The 6×His tag was removed using TEV protease during overnight dialysis into N1 buffer. Cleaved products were reverse affinity purified by a second incubation with Ni-NTA resin and collection of the unbound fraction. Final purification was carried out using an S200-sephadex size exclusion column equilibrated in storage buffer (20 mM Tris pH8, 200 mM NaCl, 2 mM DTT).

For MBP-tagged proteins, cells were lysed in lysis buffer (50 mM Tris pH 8, 300 mM NaCl, 2 mM DTT) and coupled to amylose resin for 3 h at 4°C. Following extensive washing, proteins were eluted with elution buffer (50 mM Tris pH8, 300 mM NaCl, 2 mM DTT, 50 mM maltose). Final purification was carried out using an S200-sephadex size exclusion column equilibrated in storage buffer (20 mM Tris pH8, 200 mM NaCl, 2

mM DTT). For all NcdCTm-containing proteins (ATPase motor domain), final storage buffers included 2mM MgCl2 and 100 µM ATP.

Pulldowns assays and microtubule interaction studies

Equivalent amounts of GST- or MBP-fused Ncd bait constructs were absorbed to glutathione or amylose agarose, respectively, for 30 min at room temperature and washed three times to remove unbound protein. These bait proteins represent the constant component in the binding experiments, and were kept at low concentrations (200-500 nM) relative to the variable component and dissociation constant. Subsequently, soluble untagged prey proteins were added at varying concentrations for 2 h at 4°C with constant rocking in wash buffer (20 mM Tris, pH 8, 100 mM NaCl, 0.2% Triton-X100; supplemented with 5 mM MgCl2 and 100 □M ATP for reactions involving the NcdCTm domain). Incubation for different times (e.g. 1 or 3 h at 4°C, or 1 h at RT) produced similar results, indicating that this experimental framework had established equilibrium binding conditions. Reactions were then washed four times in wash buffer, and resolved samples were analyzed by coomassie blue staining of SDS-PAGE gels. All gels shown in figures are representative of at least 4 independent experiments.

For MT pulldowns, taxol-stabilized MTs were generated from a □□□-tubulin dimer stock per manufacturer protocol (Cytoskeleton, Inc.). Polymerized MTs were maintained at room temperature, and all pulldown reactions involving MTs were conducted for 1 h at

room temperature to avoid cold-induced MT depolymerization. Proteins were transferred to nitrocellulose blots and analyzed with a BioRAD ChemiDoc imager.

All interactions were quantified using ImageJ software. Briefly, gel or blot images were converted to greyscale and individual band intensities were measured using the boxed 'Measure' analysis tool. The size of measurement box was kept the same across all concentrations and was initially determined by the size of the largest bound band, typically at the highest concentration tested. To ensure accurate measurements of bound proteins, the intensities of bands for bound prey were normalized to that of the corresponding band for bait protein under each respective condition. For example, when calculating the affinity of Mud for Ncd, the intensity of the bound MudCC band at a given concentration was normalized to the MBP:Ncd band in the same gel lane. Binding curves shown in figures plot these normalized intensities (expressed as arbitrary units, 'AU') as a function of prey protein concentration. Dissociation binding constants were calculated in GraphPad Prism using a one-site binding isotherm regression analysis. All plots and statistics were also performed in Prism.

S2 cell maintenance, transient transfection, and RNAi treatment

Schneider S2 cells (Invitrogen) were grown in Schneider's insect media (Sigma) supplemented with 10% heat-inactivated fetal bovine serum (Gibco; SIM). Stocks were passaged every 3–4 d and maintained at 28°C in the absence of CO_2. For transient transfection of pMT:GFP:Mud^{CC} and pMT:GFP:Ncd^{FL}, ~6×10^6 cells were placed in individual wells of six-well culture dishes for 30 min

in 4 mL of SIM. Cells were then transfected with 1 µg total DNA using the Effectene reagent system according to manufacturer protocols (Qiagen). Following 48 h incubation, transgene expression was induced by the addition of $CuSO_4$ (500 µM) for and additional 24 h.

Primers used for RNAi construction were designed using the SnapDragon Web-based service (www.flyrnai.org/snapdragon), and all primer synthesis was carried out by Invitrogen. Primer sets that amplify segments of ~200–600 base pairs within the coding sequence of desired targets were optimized for efficiency and specificity and synthesized with T7 promoter sequence recognition tags. Targeted sequences were designed to recognize all isoforms of desired transcript. PCR-amplified sequences were transcribed to yield double-stranded RNA using the Megascript T7 kit (Ambion) following the recommended protocol. For RNAi treatment, S2 cells were seeded in six-well dishes at 6×10^6 cells per well in 1 ml of serum-free Schneider growth media and incubated with 10 µg of desired RNAi. After 1 h, 3 ml of SIM was added, and cells were incubated for an additional 5 d before subsequent assays.

S2 cell immunofluorescence

S2 cells were mixed with an equal volume of fresh SIM in 24-well dishes containing 12-mm-diameter round glass coverslips coated with poly-L-lysine. Cells were incubated for 2–3 h to allow for adherence to coverslips and to increase the percentage of mitotic cells. Cells were then fixed using a treatment of 4% paraformaldehyde in PBS for 15 min at room temperature. Fixed cells were washed three times (5 min each) with wash buffer (0.1% Triton X-100 in PBS), followed by a 1-h incubation with block buffer (0.1% Triton X-100 and 1% bovine serum albumin [BSA] in PBS). Primary antibodies diluted in block buffer were then incubated with slides overnight at 4°C. Following primary antibody incubation, slides were washed three times with block buffer. Secondary antibodies were then added and incubated at room temperature for

2 h. Antibodies were removed, and slides were washed four times with wash buffer. Finally, coverslips were inverted and mounted using EverBrite Hardset reagent (VWR), with or without DAPI, and stored at 4°C before imaging. Imaging was performed using an Olympus IX83 inverted fluorescence microscopes and collected under oil immersion at 60× magnification.

Live-cell imaging

Live-cell movies were acquired using S2 cells stably expressing inducible GFP:CID and mCherry-α-tubulin transgenes[33]. Cells (300 μL) were seeded at a density of 2×10^6 cells/mL into Nunc Lab-Tek II 4-chambered coverglass chambers precoated with poly-L-lysine. After 1 h, chambers were placed onto an Olympus IX-83 inverted epifluorescence microscope, and appropriate cells (those with >2 centrosomes in G2 phase of the cell cycle[33]) were located and imaged at either 30-s or 1-min intervals using a Hamamatsu Orca-Flash 4.0LT camera, with three z-stacks taken at each interval. Movies were converted to AVI or MOV files and analyzed using ImageJ.

SUPPLEMENTAL FIGURES

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  1 MESRLPKPSG LKKPQMPIKT VLPTDRIRAG LGGGAAGAGA FNVNANQTYC GNLLPPLSRD
 61 LNNLPQVLER RGGGARAASP EPMKLGHRAK LRRSRSACDI NELRGNKRTA AAPSLPSIPS
121 KVSRLGGALT VSSQRLVRPA APSSITATAV KRPPVTRPAP RAAGGAAAKK PAGTGAAASS
181 GAAAAAPKRI APYDFKARFH DLLEKHKVLK TKYEKQTEDM GELESMPQQL EETQNKLIET
241 ESSLKNTQSD NECLQRQVKQ HTAKIETITS TLGRTKEELS ELQAIHEKVK TEHAALSTEV
301 VHLRQRTEEL LRCNEQQAAE LETCKEQLFQ SNMERKELHN TVMDLRGNIR VFCRIRPPLE
361 SEENRMCCTW TYHDESTVEL QSIDAQAKSK MGQQIFSFDQ VFHPLSSQSD IFEMVSPLIQ
421 SALDGYNICI FAYGQTGSGK TYTMDGVPES VGVIPRTVDL LFDSIRGYRN LGWEYEIKAT
481 FLEIYNEVLY DLLSNEQKDM EIRMAKNNKN DIYVSNITEE TVLDPNHLRH LMHTAKMNRA
541 TASTAGNERS SRSHAVTKLE LIGRHAEKQE ISVGSINLVD LAGSESPKTS TRMTETKNIN
601 RSLSELTNVI LALLQKQDHI PYRNSKLTHL LMPSLGGNSK TLMFINVSPF QDCFQESVKS
661 LRFAASVNSC KMTKAKRNRY LNNSVANSST QSNNSGSFDK
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Fig. S1. Ncd peptides identified by mass spectrometry in GST:MudCC pulldown.
Shown is the primary sequence of Ncd in 10 amino acid spacing depicted as single-letter abbreviations. Highlighted in distinct colors are five unique peptides identified in mass spectrometry of samples isolated from pulldowns using a GST:MudCC bait and Drosophila S2 cell lysate prey.

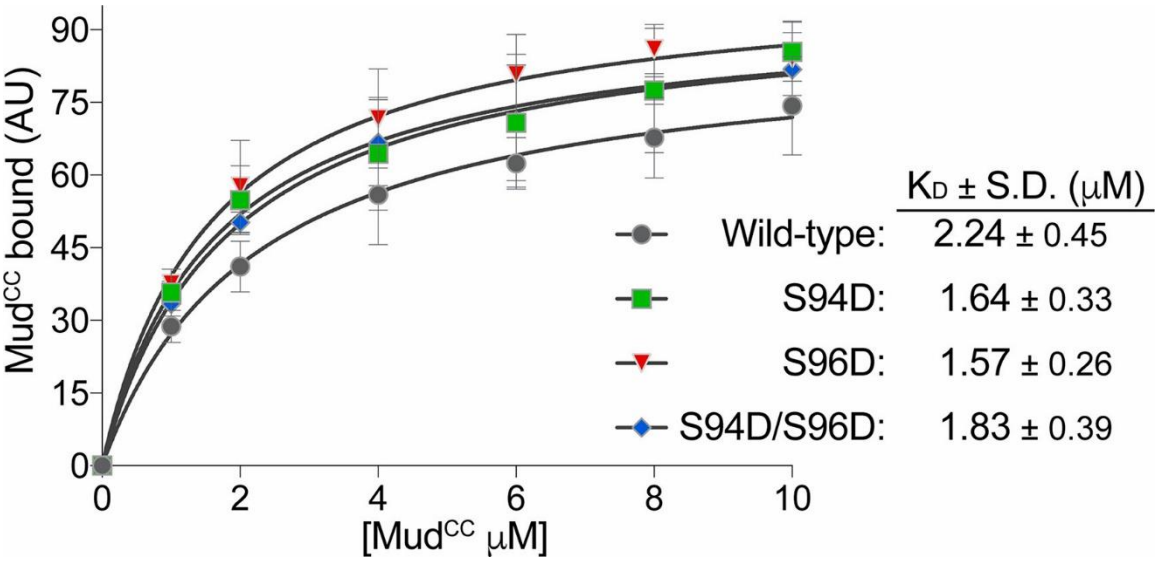

Fig. S2. Phosphomimetic NcdnMBD mutations do not affect MudCC binding.
GST-fused NcdnMBD bait as wild-type (grey circle), S94D single mutant (green square), S96D single mutant (red inverted triangle), or S94D/S96 double mutant (blue diamond) were immobilized on glutathione agarose and subsequently incubated with the indicated concentrations of soluble MudCC prey. Plots show the normalized intensities of bound Mud as a function of its concentration. The equilibrium binding constants for each are listed, which were determined from 4 independent experiments.

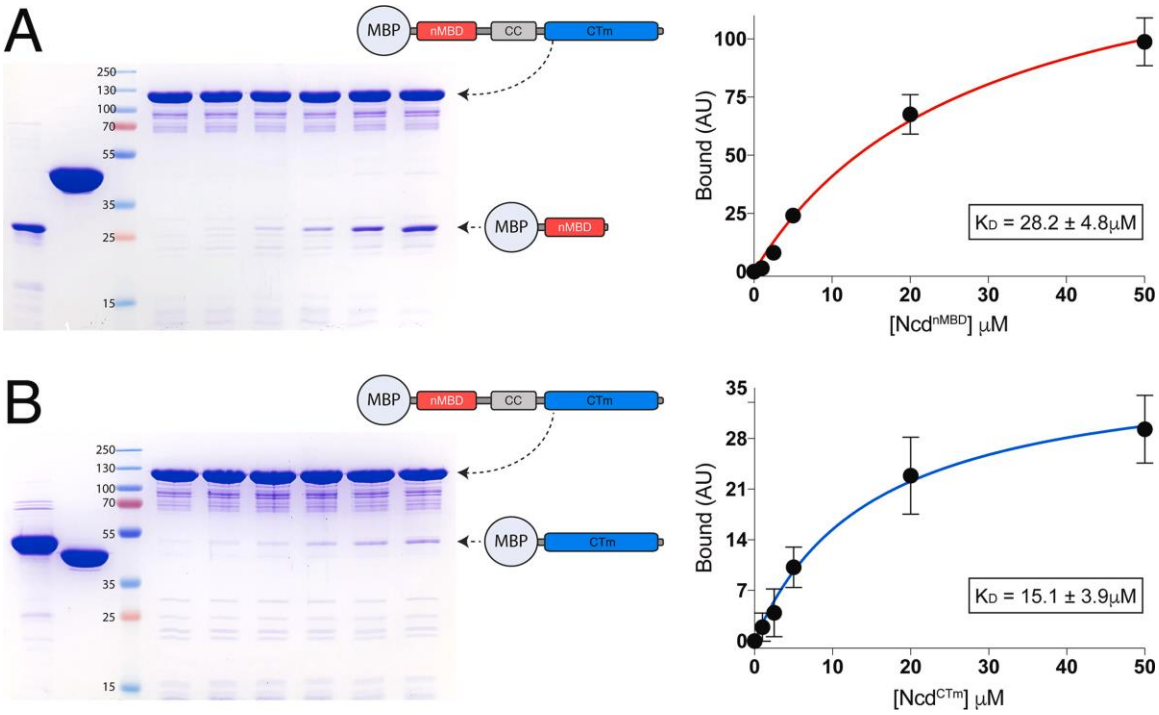

Fig. S3. MT-binding regions of Ncd have reduced affinity for NcdFL.
(A) Left: MBP-fused NcdFL (or MBP alone; second lane) was coupled to amylose resin and incubated in the absence or presence of increasing concentrations of soluble, isolated NcdnMBD. Gel shown is representative of 4 independent experiments. The first lane shows the purified NcdnMBD used. Right: Saturation binding curves demonstrate that the binding affinity was significantly reduced compared to that measured for the isolated MBP:NcdCTm domain (see Fig. 2C).
(B) Left: MBP-fused NcdFL (or MBP alone; second lane) was coupled to amylose resin and incubated in the absence or presence of increasing concentrations of soluble, isolated NcdCTm. Gel shown is representative of 4 independent experiments. The first lane shows the purified NcdCTm used. Right: Saturation binding curves demonstrate that the binding affinity was significantly reduced compared to that measured for the isolated MBP:NcdnMBD domain (see Fig. 2D).

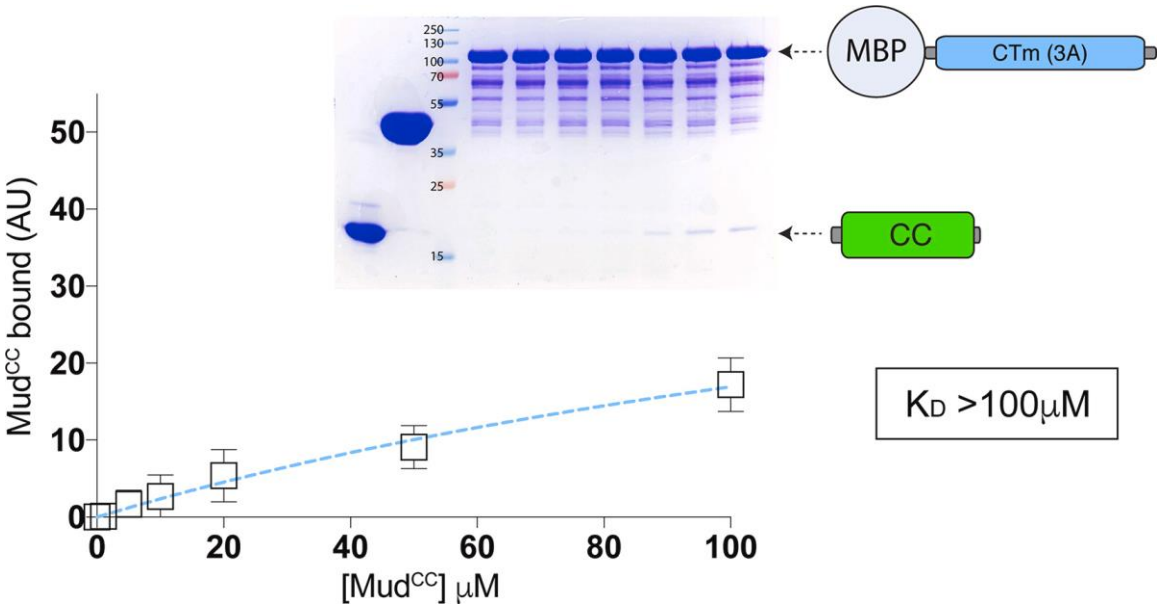

Fig. S4. The 3A mutation in NcdCTm has reduced MudCC binding.
MBP alone (second column on shown gel) or as a fusion to NcdCTm3A was immobilized on amylose resin and incubated without or with at increasing concentrations of MudCC (1–100 μM). Gel shown is representative of 4 independent experiments. The graph depicts the average ± standard deviation values for MudCC bound at indicated concentrations in arbitrary intensity units (AU) normalized to respective MBP:NcdCTm3A bands. The equilibrium dissociation constant binding affinity, which could not be quantitatively calculated due to limited binding, is shown as a >100 μM estimation in the solid box. The y-axis is scaled identically to that in Fig. 1E for comparison with the wild-type MBP:NcdCTm.

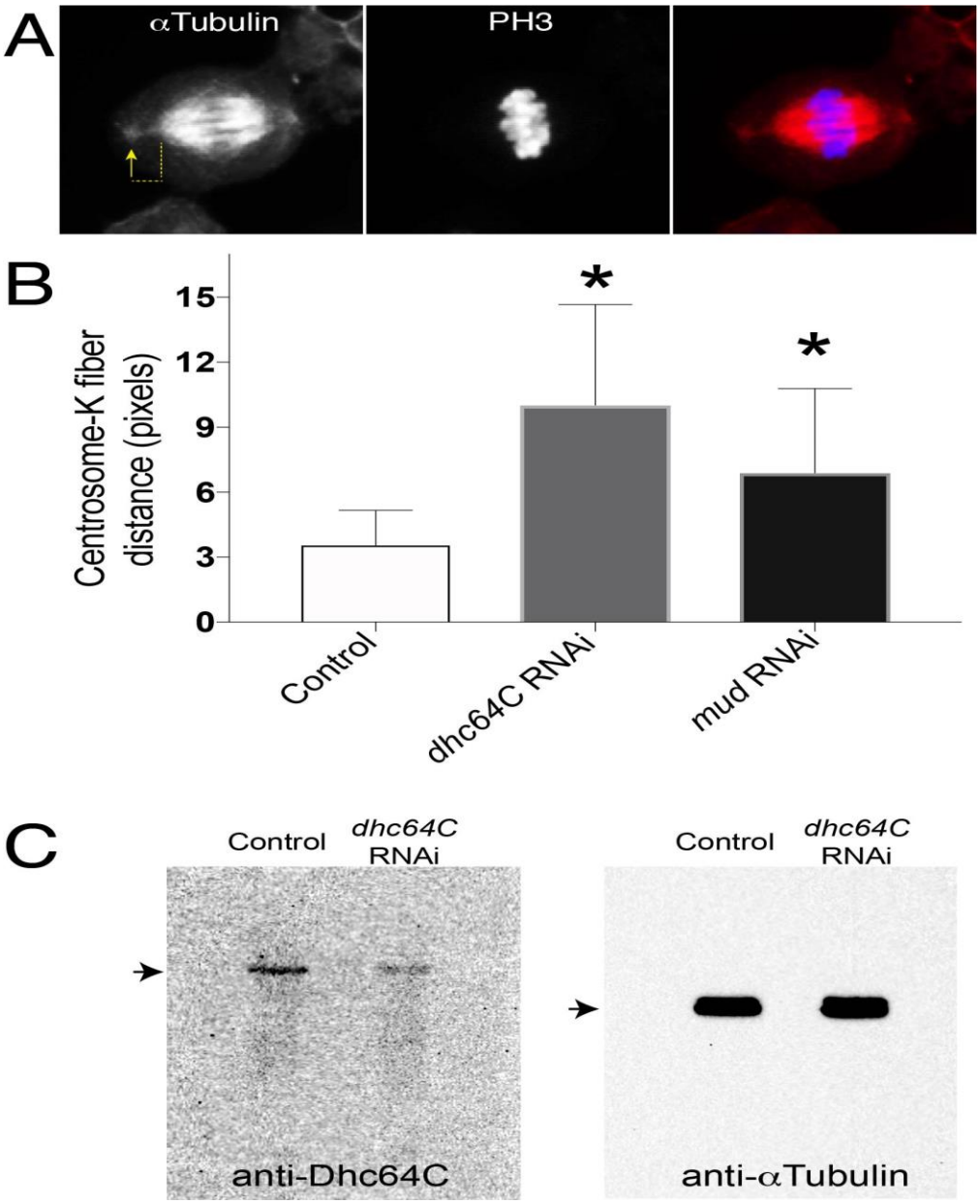

Fig. S5. Dhc64CRNAi treatment efficiently reduces DHC expression and leads to detached centrosomes
(A) S2 cells were treated with RNAi targeting the Dynein heavy chain (dhc64C) for 5 days, formaldehyde fixed, and stained with antibodies against Tubulin (red) and PH3 (blue). To assess centrosome detachment, a previously known Dynein phenotype28, the distance between visible centrosomes (yellow arrow) and the base of spindle K-fibers (dashed yellow line) were measured.
(B) Quantification of centrosome detachment distances for the indicated genotypes (n=30). Treatment with dhc64CRNAi leads to a significant increase in distance, as does mudRNAi. *, $p<0.05$ compared to Control. ANOVA with Tukey's post-hoc test.
(C) Western blot analysis of Control and dhc64CRNAi-treated cells shows that treatment leads to a significant reduction in Dynein expression. αTubulin was used as a loading control.

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V.C. contributed to experimental design, performed experiments, and assisted with manuscript development. C.A.J. designed the research project, performed experiments, analyzed data, prepared figures, and wrote the manuscript.

Chapter 2

Mud Binds the Kinesin-6 Pavarotti in *Drosophila*

Vincent Cutillas and Christopher A. Johnston

ABSTRACT

Mushroom Body Defect (Mud), and its human ortholog NuMA, both have multiple functions during cell division. Mud has been observed in *Drosophila* cells to be crucial to maintaining spindle bipolarity, as well as aligning the spindle with cortical cues so the cell can divide in accordance with nearby tissue organization. In order to regulate the process of spindle orientation, Mud initially localizes to the spindle poles, and is then thought to move towards the cell cortex to recruit Dynein and pull the spindle towards the cortical cues, although the mechanism by which it transits remains unclear. Our research suggests that a kinesin-6 family member, Pavarotti (Pav), may be responsible for this shift in localization, after identifying a novel interaction between Pav and Mud, which seems to be mediated by phosphorylation of Pav. We also show preliminary data suggesting colocalization between Pav and Mud, further promoting the idea that Pav is a crucial component in cortical Mud localization and its resultant role in spindle capture.

INTRODUCTION

Cortical membrane structures of the cell define an axis for the cell to divide during mitosis. This defined axis is essential for the cell to divide while conforming to the nearby tissue structure in multicellular organisms. However, in order for the spindle to align itself with the cortical cues, a motor mechanism must be in place in order to generate a force that pulls a misaligned spindle back into the correct axis of division. In *Drosophila*, the mechanism by which cells do so requires two major proteins: Dynein, a minus-end directed motor protein, and Mushroom Body Defect (Mud), which acts as a direct link between the cortical membrane complex and Dynein.

When cells must divide in a way that requires a specific directionality, such as asymmetric cell division or divisions within structured tissues, the cell establishes an intrinsic polarity in order to align the spindle parallel to the directional cues. To begin, cell polarity in many cell types is created by a system of three major membrane proteins, Par3 (Bazooka in *Drosophila*) and a heterodimer consisting of atypical protein kinase C (aPKC) and Par6, which together are known as the Par complex (Chen and Zhang, 2013). This Par complex in turn regulates the localization of other polarity components to establish overall cell polarity domains (Lang and Munro, 2017). Following the creation of these distinct regions, membrane complexes involved in the capture and alignment of the spindle are then recruited to the cortex, which include Inscuteable (Insc), Partner of Inscuteable (Pins), and Discs large (Dlg) (Bellaïche et al., 2001). This becomes one of the two pathways that recruits Mud to the membrane

cortex, thereby allowing for the cortical anchoring of Dynein, which then creates a mechanism by which astral microtubules (MTs) can be pulled towards this membrane complex to align the spindle (Mauser and Prehoda, 2012).

Mud's recruitment to the cortex has been shown in previous studies to be linked to phosphorylation of Mud by the mitotic kinase, Warts (Wts). Wts knockdown reduces cortical Mud localization, although Mud remains localized at spindle poles. Reduced cortical recruitment is thought to be due to the Pins binding domain of Mud (Mud^{PDB}) remaining bound to a C-terminal coiled-coil domain of Mud, Mud^{CC}, rather than cortically located Pins (Dewey et al., 2015). These results lead us to speculate that Mud originates at the spindle pole early in mitosis and is then shuttled to the cortex via an unknown transporter protein. Having identified the binding partners of Mud that were part of the motor protein class, we suggest – using preliminary data – that Pav is possibly responsible for the cortical localization of Mud by acting a transporter from the spindle pole to the cell cortex in a phosphorylation-dependent manner.

RESULTS

Mud binds to Pav at the cargo domain in a phosphorylation-sensitive manner

Previous research on Mud showed that Mud^{CC} is responsible for inhibiting Mud^{PBD} association with Pins (Dewey et al., 2015). In order to determine possible binding partners and regulatory proteins of the Mud^{CC} domain, we performed mass

spectrometry on samples of purified Mud^{CC} from pulldown experiments using whole-cell extract from *Drosophila* S2 cells. A kinesin from the kinesin-6 family known as Pav was among the statistically significant abundant proteins bound to the Mud^{CC} domain. This interaction was notable, as a direct association between Pav and Mud had not been identified yet, and the only clear link between the two was that Pav and Mud are localized at the cell cortex before the onset of metaphase (Minestrini et al., 2003; Dewey et al., 2015; Siller et al., 2006).

Because kinesins contain a conserved domain topology, we first wanted to determine whether Mud^{CC} was binding to the motor (Pav^{NT}), stalk (Pav^{CC}), or cargo domains of Pav (Pav^{CT}). We used a single concentration of soluble, purified Mud^{CC} in an *in vitro* pulldown experiment against *E. coli* lysates containing Pav^{NT}, Pav^{CC}, and Pav^{CT} domains, each tagged with a glutathione-s-transferase (GST) protein, and immobilized on glutathione-sepharose beads. A first attempt showed that at our chosen concentration of Mud^{CC}, it had bound strongly to Pav^{CT}, and also had some, albeit lower, binding to Pav^{CC} (Figure 1A). These findings not only confirmed that Mud bound to Pav, but that Mud seemed to be a cargo of Pav, since it bound to its cargo domain.

Once this was determined, we next inquired as to whether there were any similarities in sequences between Pav^{CT} and another kinesin that was recently discovered to bind to Mud, known as Non-claret Disjunctional (Ncd) (Cutillas and Johnston, 2021). Although most kinesins have conserved motor domains, they differ in the length of their stalk domains and sequence of their cargo domain. Surprisingly though, Pav and Ncd did share a highly specific sequence of amino acids in both their

cargo domains, which was ^{741}RRSRSAGD^{748} in Pav and ^{92}RRSRSACD^{99} in Ncd. Although phosphomimetic mutations at S94 and S96 caused no change in the affinity of Mud for Ncd in previous studies (Cutillas and Johnston, 2021), the phosphomimetic mutation at S745 seemed to drastically increase the binding of Mud for Pav in a preliminary round of results (Figure 1B). Interestingly, phosphomimetic mutations at both S743 and S745 seemed to revert Mud binding back to that of wild-type Pav.

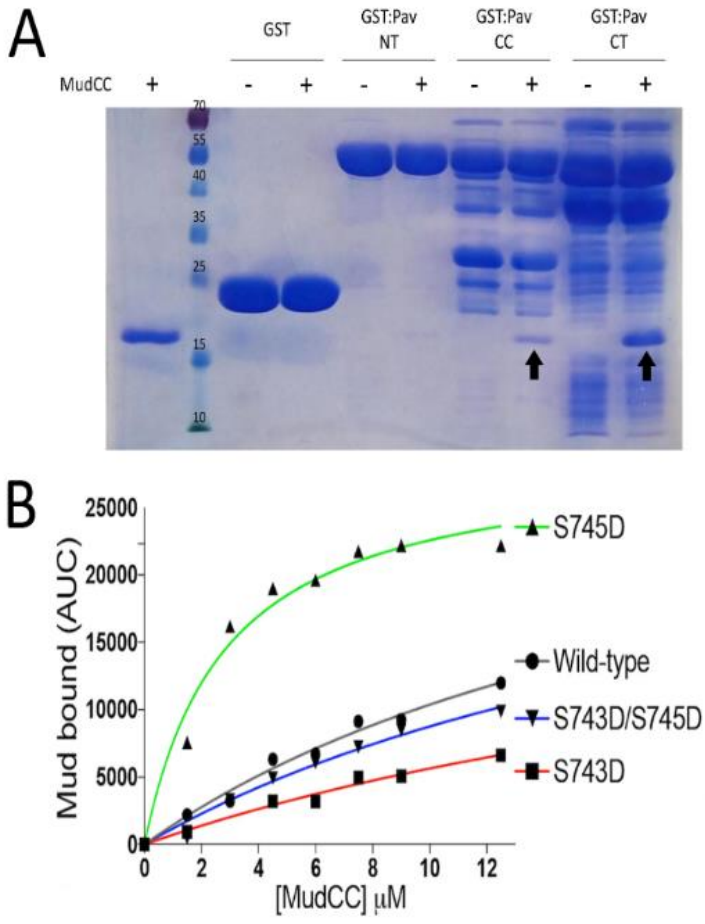

Fig. 1. Mud binds kinesin-6 Pavarotti
(A) Mud^{CC} (black arrow) binds the stalk and cargo domains of Pav, labelled as PavCC and PavCT, respectively. (B) Phosphomimetic mutation at S745 (green) in the cargo domain of Pav seems to cause an increase in affinity of Mud for Pav. Single mutations at S743 (red) and a double phosphomimetic mutation at both S743 and S745 (blue) seems to have relatively no effect on Mud affinity for the Pav cargo domain compared to wild-type (grey).

DISCUSSION

Spindle capture is an essential part of mitosis in multicellular organisms, as this mechanism is responsible for proper tissue structure maintenance and cell differentiation factors rely on it to correctly determine cell fate during development (Lechler and Mapelli, 2021). Although much is now known about the mechanism behind spindle capture, some of the specific details of how this complex is formed remains elusive. Here, we show the beginnings of a possible interaction between Pav and Mud, and additionally propose that phosphorylation of Pav at S745 increases the affinity of this interaction.

In order to better understand possible functional relationships between Mud and Pav, we must first explore the known functional roles of Mud and Pav, as well as the role of their orthologs in other organisms. To begin, the *C. elegans* ortholog of Pav, known as ZEN-4, had been identified in previous studies to be required for polarization of epithelial cells because of an inability of the cell to target polarity cues such as the Par3/aPKC anterior polarity complex to the apical membrane cortex (Portereiko et al., 2004). Additionally, it has been shown that Pav localizes to both the spindle poles and to the cell cortex prior to its localization at the contractile ring in anaphase through telophase (Sommi et al., 2010; Tao et al., 2016). Since Mud is known to localize at both of these sites (Bowman et al., 2006; Dewey et al., 2015), but has no known motility that allows it to do so, Pav may represent one of the best candidates to provide Mud's localization to the cell cortex. This would also be consistent with the results seen in

previous studies, where both knockdown of Pav and Mud result in the loss of cell polarity during mitosis (Portereiko et al., 2004; Bowman et al., 2006).

In humans, the ortholog of Pav known as CHO1, and its splicing variant, MKLP1, are already known to have an effect on the human ortholog of NuMA (the human ortholog of Mud), by removing NuMA's presence from the equatorial region of the cell cortex (Kotak et al., 2014). Although it is not yet known whether CHO1/MKLP1 has a direct or indirect attachment to NuMA in humans, it is substantial enough to know that these two mitotic proteins do seem to interact in human cells as well. In addition to these studies on CHO1/MKLP1 and NuMA, it has also been shown that CHO1/MKLP1 is indeed regulated by phosphorylation by Lats1/2 (Warts in *Drosophila*), at a site conserved between CHO1/MKLP1 and Pav (Okamoto et al., 2015). This phosphorylation site's equivalence in Pav is the amino acid S743 mentioned previously and seems to indicate that both CHO1/MKLP1's and Pav's cargo may be regulated by phosphorylation of their cargo domains. Together these indicate a possible mechanism in which Pav is phosphorylated to induce Mud attachment in order to localize Mud to the cell cortex to carry out the process of spindle capture.

In order to determine whether this is true, a couple future experiments would need to be performed. Firstly, *in vivo* experiments using S2 cells transfected with Mud^{CC}-GFP with or without Pav RNAi treatment would allow us to determine whether Mud does indeed localize due to Pav, or if another kinesin is responsible for its localization at the cortex. Additionally, a similar experiment where Mud is fluorescently tagged would then have to be performed to tell us whether Pav is required for the transport of the

entire Mud protein, or if the presence of the other Mud domains also allows for Mud trafficking through some unknown interaction. Finally, it would also possibly be worth investigating if it is Pav that regulates Mud trafficking, or if the binding of Mud to Pav acts as the activator of Pav, as it was shown to likely be the case between Mud and Ncd (Cutillas and Johnston, 2021).

MATERIALS AND METHODS

Cloning and plasmid construction

Cloning was performed using PCR amplified fragments obtained from an S2 cell cDNA library template. The Mud coiled-coil domain (amino acids 1760–1906) was cloned into the bacterial expression pBH plasmid using 5’-BamHI and 3’-SalI restriction sites, generating a TEV cleavable 6×His fusion. Each Pav domain (NT: amino acids 1-529; CC: amino acids 530-678; CT: amino acids 679-887) were cloned as GST fusions by cloning into pGEX plasmids, using 5’-BamHI and 3’-XhoI restriction sites. Site-directed mutagenesis was carried out with a standard PCR protocol using KOD-XL DNA polymerase in order to create the S743D and S745D single mutations, as well as the S743D/S745D double mutant.

Protein purification

All proteins were expressed in BL21(DE3) E. coli under induction of isopropyl β-D-1-thiogalactopyranoside (IPTG) and grown in standard Luria–Bertani broth supplemented with 100 μg/ml ampicillin. Transformed cells were grown at 37°C to an OD600 ∼0.6 and induced with 0.2 mM IPTG overnight at 18°C. Cells were harvested by centrifugation (5000 × g for 10 min), and bacterial pellets were resuspended in lysis buffer and flash-frozen in liquid nitrogen. Cells were lysed using a Branson digital sonifier and clarified by centrifugation (12,000 × g for 30 min).

Pulldowns assays and microtubule interaction studies

Equivalent amounts of GST-fused Pav bait constructs were absorbed to glutathione sepharose for 30 min at room temperature and washed three times to remove unbound protein. These bait proteins represent the constant component in the binding experiments, and were kept at low concentrations (200-500 nM) relative to the variable component and dissociation constant. Subsequently, soluble untagged prey proteins were added at varying concentrations for 2 h at 4°C with constant rocking in wash buffer (20 mM Tris, pH 8, 100 mM NaCl, 0.2% Triton-X100; supplemented with 5 mM MgCl2 and 100 μM ATP for reactions involving the Pav^{CT} domain). Incubation for different times (e.g. 1 or 3 h at 4°C, or 1 h at RT) produced similar results, indicating that this experimental framework had established equilibrium binding conditions. Reactions were then washed four times in wash buffer, and resolved samples were analyzed by

coomassie blue staining of SDS-PAGE gels. All gels shown in figures are representative of at least 4 independent experiments.

All interactions were quantified using ImageJ software. Briefly, gel images were converted to greyscale and individual band intensities were measured using the boxed 'Measure' analysis tool. The size of measurement box was kept the same across all concentrations and was initially determined by the size of the largest bound band, typically at the highest concentration tested. To ensure accurate measurements of bound proteins, the intensities of bands for bound prey were normalized to that of the corresponding band for bait protein under each respective condition. For example, when calculating the affinity of Mud for Ncd, the intensity of the bound MudCC band at a given concentration was normalized to the MBP:Ncd band in the same gel lane. Binding curves shown in figures plot these normalized intensities (expressed as arbitrary units, 'AU') as a function of prey protein concentration. Dissociation binding constants were calculated in GraphPad Prism using a one-site binding isotherm regression analysis. All plots and statistics were also performed in Prism.

ACKNOWLEDGEMENTS

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V.C. contributed to experimental design, performed experiments, analyzed data, and wrote the manuscript. C.A.J. designed the research project, performed experiments, analyzed data, and prepared figures.

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