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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 direct Ncd binding partner. We demonstrate this interaction involves a short coiled-coil domain within Mud (Mud^{CC}) binding the N-terminal, non-motor microtubule-binding domain of Ncd (Ncd^{nMBD}). We further show that the C-terminal ATPase motor domain of Ncd (Ncd^{CTm}) directly interacts with Ncd^{nMBD} as well. Mud binding competes against this self-association and also increases Ncd^{nMBD} microtubule binding *in vitro*. Our results describe an interaction between two spindle-associated proteins and suggest a potentially new mode of minus-end motor protein regulation at mitotic spindle poles.

1. 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 [1]. Motor activity must be tightly controlled to ensure proper execution of specific tasks, and kinesins have evolved several mechanisms to achieve this goal [2]. Among them, auto-inhibition 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 [2]. 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 (Ncd^{CTm}). These motors also display 'reversed' directional movement along MTs toward the minus-ends rather than plus-ends seen with most other kinesins [3]. The N-terminal 'tail' domain of several kinesin-14s acts as an additional, non-motor MT-binding domain (Ncd^{nMBD}), thus allowing MT crosslinking and sliding functions essential for spindle assembly [4–7]. To prevent these activities on cytoplasmic interphase MTs, an N-terminal nuclear localization sequence (NLS) signals for importin-mediated nuclear sequestration [3]. Upon mitotic entry and nuclear envelope breakdown (NEBD), Ran-dependent disruption of importin binding, which occludes MT binding to the nMBD [8], is thought to be an important step in kinesin-14 activation [9]. 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 [10]. 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 [11,12]. We delimit this interaction to a short Mud coiled-coil domain (Mud^{CC}) that directly binds to the Ncd^{nMBD} domain, notably with a higher affinity than to the full-length Ncd protein (Ncd^{FL}). We also find that the Ncd^{nMBD} directly interacts with the Ncd^{CTm} *in trans*. Mutation of putative 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 Ncd^{CTm} with Ncd^{nMBD} and also increases MT association to the isolated Ncd^{nMBD}. 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.

2. Results

2.1. 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 [13]. As coiled-coil domains are well-characterized protein interaction platforms [14], we performed mass spectrometry on samples isolated from an unbiased GST pulldown of Mud^{CC} bait with *Drosophila* S2 whole-cell lysate prey to identify previously unknown 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). Fig. S1 illustrates the five unique peptides
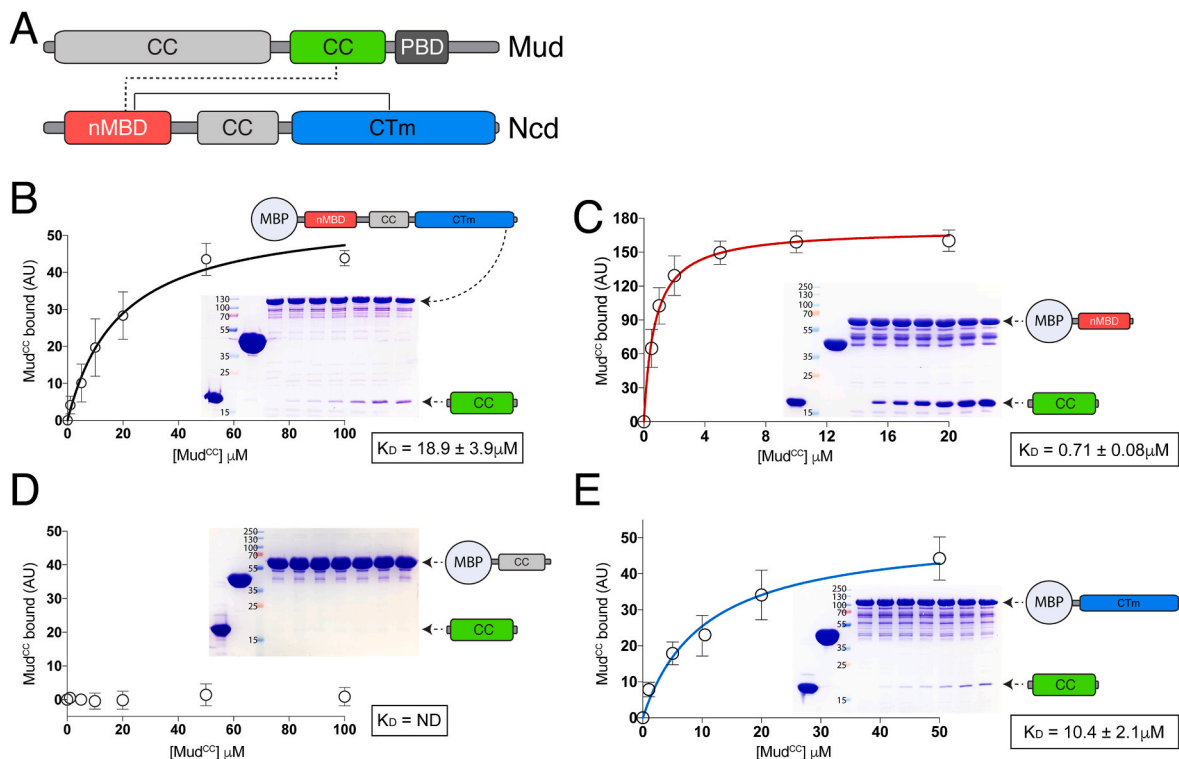

Fig. 1. **Mud directly binds Ncd** *in vitro*.
(A) Domain architectures of Mud (*top*) and Ncd (*bottom*). Mud is a large coiled-coil (CC) domain protein, with the C-terminal of these CC domains (*green*) representing the focus of this study. This Mud^{CC} domain precedes the Pins-binding domain (PBD). 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 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 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.)

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 several of its essential functions [3,11], suggesting the 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 [15], 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 [10,16], 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 Ncd^{nMBD} being most susceptible likely due to its lack of significant globular structure [17]. These MBP fusion baits were then immobilized on solid amylose resin and used in *in vitro* pulldown experiments with purified Mud^{CC} as soluble prey. Binding was quantified across a range of Mud^{CC} concentrations and equilibrium dissociation binding constants (K_D) were calculated for each MBP:Ncd protein tested. Mud^{CC} binding to $MBP:Ncd^{FL}$ was modest and had a calculated affinity in the micromolar range (Fig. 1B). In contrast, a high affinity, nanomolar interaction was measured with the isolated $MBP:Ncd^{nMBD}$ domain (Fig. 1C). No binding was detected to $MBP:Ncd^{CC}$ at any Mud concentration tested (Fig. 1D). Binding to $MBP:Ncd^{CTm}$ was similar to full-length, also showing a relatively weak dissociation constant (Fig. 1E). 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 Ncd^{nMBD}, and (3) Mud binding the Ncd^{nMBD} appears to be attenuated within the context of the Ncd^{FL} protein.

The Ncd^{nMBD} 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 [10]. To determine if such modifications affect Mud binding, we tested phosphomimetic (serine-to-aspartate) Ncd^{nMBD} mutants and found that neither single mutant nor a double mutant significantly affected affinity for Mud^{CC} binding (Fig. S2), suggesting that Mud binding is either insensitive to phosphorylation or occurs at a site distinct from these modifications. Although it remains possible that natively phosphorylated Ncd in cells may display altered binding, we conclude that Mud^{CC} directly binds the Ncd^{nMBD} domain in a manner that is likely independent of its phosphorylation status.

2.2. Ncd^{nMBD} and Ncd^{CTm} self-associate in vitro

The ability of Mud^{CC} to directly bind 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 Ncd^{nMBD} (Fig. 1B and C). Such self-associations between distinct kinesin domains have been described for several other subfamilies and often substantiate mechanisms of regulation [2]. To test this hypothesis, we first examined whether the Ncd^{nMBD} and Ncd^{CTm} could directly interact *in trans* as isolated recombinant proteins. Indeed, soluble Ncd^{nMBD} bound to MBP: Ncd^{CTm} in a dose-dependent manner with a low-micromolar affinity (Fig. 2C). Similar results were obtained when inverting the interaction order and instead examining soluble Ncd^{CTm} binding to $MBP:Ncd^{nMBD}$ (Fig. 2D), further validating this interaction. We then examined the

interaction of these isolated domains with immobilized $MBP:Ncd^{FL}$ and found that each had significantly reduced binding affinity when compared with binding to their respective counterpart domain as an isolated fragment (Fig. S3). These results are consistent with interactions between Ncd^{nMBD} and Ncd^{CTm} domains within full-length proteins bound to the resin competing against binding to isolated domains in solution.

To map a putative interaction sight within Ncd^{CTm}, we introduced a triplet of alanine mutations in the L12 loop of the Ncd motor domain (Ncd^{CTm3A}, H619A/R623A/H629A; Fig. 2A). These residues lie within the predicted MT-binding site [18–20], with related mutations in kinesin-1 having also been shown to reduce MT interaction [21]. Although not completely devoid of MT interaction, this Ncd^{CTm3A} mutant showed reduced binding to taxol-stabilized MTs *in vitro* (Fig. 2B), consistent with Ncd L12 loop involvement in MT binding [22]. We next tested the ability of Ncd^{CTm3A} to bind to Ncd^{nMBD} *in trans* to determine if the L12 loop is also important for the Ncd self-association. Indeed, the 3A mutant had significantly reduced binding affinity to Ncd^{nMBD} when tested as either the soluble or MBP-immobilized fraction (Fig. 2C and D). Interestingly, we found that the 3A mutant also had a significant impairment for Mud^{CC} binding (Fig. S4), suggesting the low-affinity binding to Ncd^{CTm} occurs at a site that overlaps with its MT binding as well as with Ncd^{nMBD}. However, our results cannot rule out an alternative possibility that the 3A mutation allosterically induces a conformational change at an alternative site on Ncd^{CTm}, distinct from the MT-binding site, that binds Ncd^{nMBD} and Mud^{CC}. Nevertheless, these results suggest that the site of both Mud^{CC} binding and Ncd^{nMBD} self-association could potentially overlap with the MT binding surface of the Ncd^{CTm} domain and imply that a network of interactions may play an important role in regulating Ncd function.

2.3. Mud competes against Ncd self-association and enhances MT interaction with Ncd^{nMBD}

Having established that Ncd^{nMBD} directly binds both Mud^{CC} and Ncd^{CTm}, and that Mud and Ncd^{nMBD} each had reduced binding affinity to the Ncd^{CTm3A} mutant (Figs. 1C and 2, and S4), we next examined whether these interactions are mutually exclusive. To do this, we immobilized $MBP:Ncd^{CTm}$ on amylose resin and examined how its interaction with a single concentration of Ncd^{nMBD} (at the ~K_D of 2 μM) is affected by addition of increasing concentrations of Mud^{CC}. As shown in Fig. 3, binding of Mud^{CC} resulted in a concentration-dependent reduction in the interaction between Ncd^{CTm} and Ncd^{nMBD}, demonstrating that Mud^{CC} directly competes against Ncd^{nMBD}/Ncd^{CTm} binding. This result suggests that Mud binding could act as a mechanism to disengage the Ncd self-association to regulate its function.

Finally, to explore the impact of Mud on Ncd function, we examined how Mud^{CC} binding influences the interaction between Ncd^{nMBD} and taxol-stabilized MTs. Surprisingly, association with Mud^{CC} increased MT binding to $MBP:Ncd^{nMBD}$ (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 Ncd^{nMBD} 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 Ncd^{nMBD} has been shown to contain two MT-contacting sites [16]; 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 Mud^{CC}/Ncd^{nMBD}/MT trimeric complex is not only possible but may exist as a high-affinity complex. We conclude that Mud^{CC} reduces self-association between Ncd domains while also enhancing its MT binding through direct interaction with the nMBD.

3. Discussion

Maintenance of proper spindle structure throughout mitosis is essential for correct and efficient segregation of replicated chromosomes
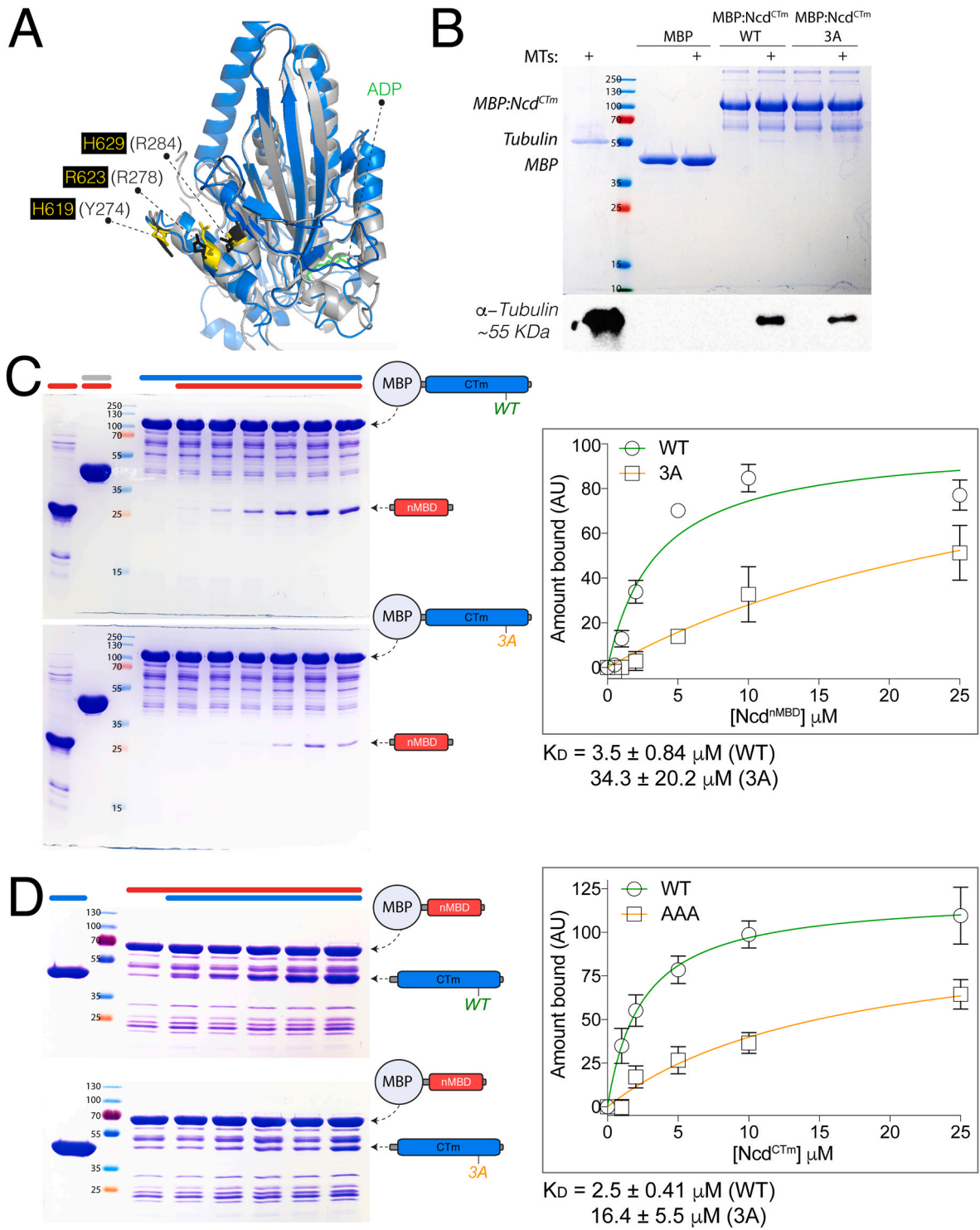
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Fig. 2. Ncd MT-binding domains self-associate *in vitro*.

(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 Ncd^{CTm} 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 [20–22]. Gel shown is representative of 3 independent experiments.

(C) MBP alone (*grey* bar, *second lane*) or as a fusion to Ncd^{CTm} (*blue* bar; WT, *top* or 3A, *bottom*) was immobilized on amylose resin and incubated in the absence or presence of increasing concentrations of Ncd^{nMBD} (*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 Ncd^{nMBD} 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 Ncd^{nMBD} (*red* bar) was immobilized on amylose resin and incubated in the absence or presence of increasing concentrations of Ncd^{CTm} (*blue* bar; 1–25 μM). *Left*: gels shown are representative of 4 independent experiments. *Right*: saturation binding curves show average ± standard deviation values for Ncd^{CTm} 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 Ncd^{CTm} binding to MBP:Ncd^{nMBD} (D), particular attention was carefully given to background subtraction of the obscuring band that runs at a similar molecular weight as Ncd^{CTm}.

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.)

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 [23]. Herein, we have described an interaction between two prominent spindle-associated proteins, the structural protein Mud and the kinesin-14 motor Ncd. Mud^{CC} directly binds the non-motor MT-binding domain at the Ncd N-terminus with a sub-micromolar affinity, which is ~25-fold greater compared to that measured with the full-length Ncd protein. Furthermore, we show a direct self-association between the Ncd^{nMBD} domain and the C-terminal ATPase motor domain and demonstrate that Mud binding competitively uncouples this Ncd self-association. Both Mud^{CC} and Ncd^{nMBD} binding to the Ncd^{CTm} are impaired by mutations to the putative MT-contacting L12 loop. 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 [24].

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 [25]. Alternatively, low-affinity Mud binding to Ncd^{CTm} 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 Ncd^{nMBD}. The existence of an apparent Mud^{CC}/Ncd^{nMBD}/MT complex yielding increased MT binding (Fig. 4) is consistent with this model, and is similar to regulation of the human kinesin-14, HSET (see below [26]). 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 [23,27]. Kinesin-14s such as Ncd are no exception to this rule, with excessive activity leading to altered spindle geometry and chromosome dynamics [9]. In contrast, reduced kinesin-14 function leaves the opposite force generating kinesin-5 unopposed, leading to outward forces that unfocus spindle poles [28,29]. These findings 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 [8,30,31]. 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 Mud^{CC} domain examined in our study [32,33]. As the active Ran gradient dissipates toward spindle poles [34,35], 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 Ncd^{nMBD} 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 [10]. 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 [27,36]. The interaction between the Ncd^{nMBD} and Ncd^{CTm} 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 [37]. 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 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) [26]. CEP215, also known as CDK5RAP2, is the human ortholog of *Drosophila* centrosomin (Cnn), a large coiled-coil protein critical for centrosome assembly and maintenance [38]. 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 [26], 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 [26]. 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 Ncd^{nMBD} simultaneously, or if their interactions are mutually exclusive, could offer additional insight into the specific role for each in Ncd function. It is also possible that Mud is a functional ortholog of CEP215 here, rather than Cnn. Whether the MT-binding domains of HSET self-associate and if CEP215 could impact such interaction will also require future studies. 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
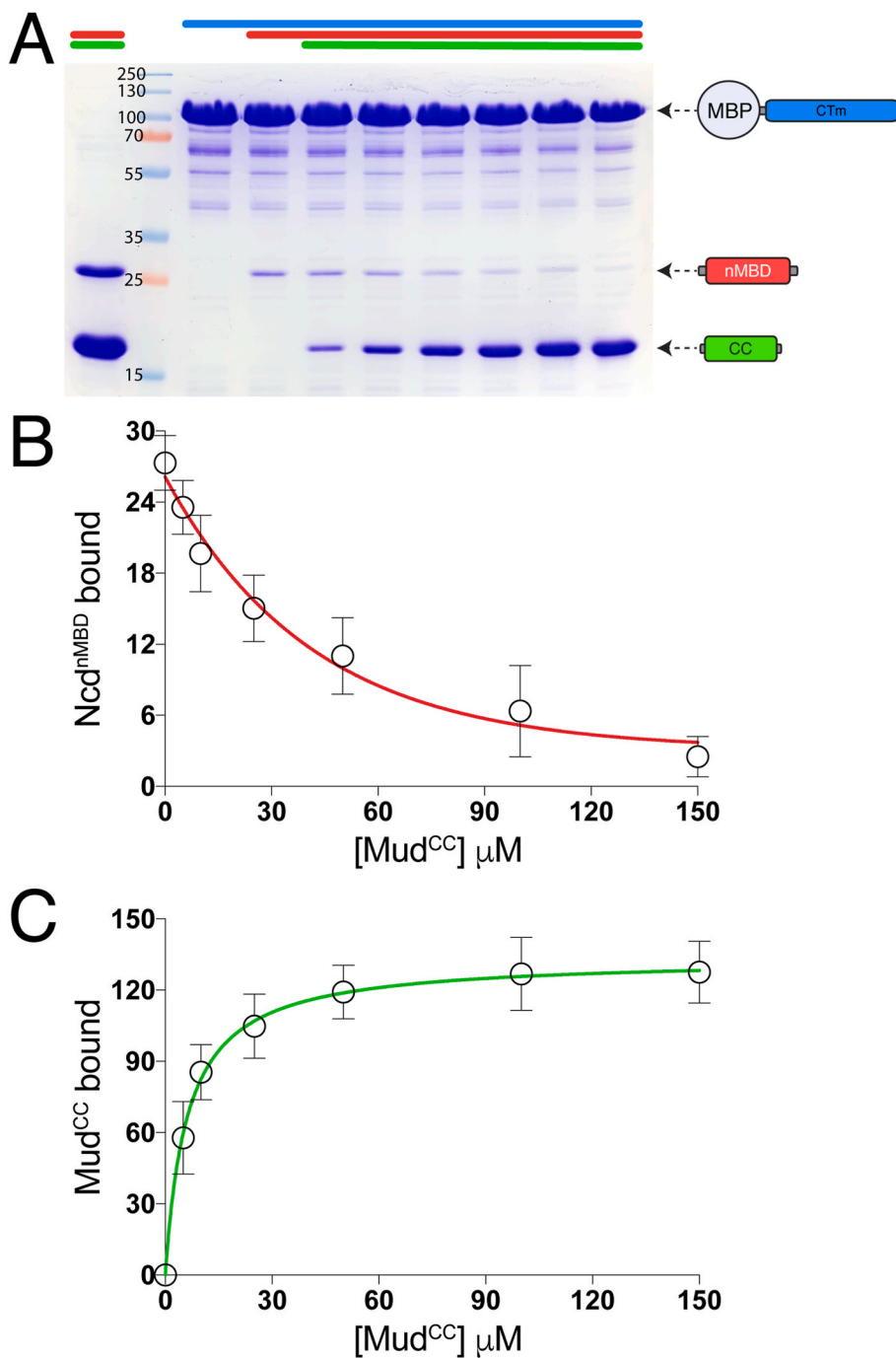
Fig. 3. Mud directly competes against Ncd self-association.
(A) MBP-fused Ncd^{CTm} (*blue*) was immobilized on amylose resin and subsequently incubated without or with 2 μM NcdnMBD (*red*) and increasing Mud^{CC} (5 μM–150 μM; *green*). Gel shown is representative of 5 independent experiments. Molecular weight standards in each gel are labeled in kilodaltons (kD).
(B) Effects of Mud^{CC} on the interaction between MBP:Ncd^{CTm} and Ncd^{nMBD}. Curve plots the average ± standard deviations for the amount of Ncd^{nMBD} bound to MBP:Ncd^{CTm} (as a function of Mud^{CC} concentration for 5 independent experiments.
(C) Curve plots the average ± standard deviations for the amount of Mud^{CC} bound to MBP:Ncd^{CTm} as a function of Mud^{CC} concentration in the presence of 2 μM Ncd^{nMBD} 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.)

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 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 [3,6,23]. Loss of these functions can lead to multipolar divisions, chromosome segregation errors, and aneuploidy that affect cell viability [39]. In fact, inhibiting clustering in cancer cells with centrosome amplification has been suggested as a potential therapeutic avenue [39,40]. RNAi-based screening studies in both human and fly cells have identified numerous other genes necessary for this process, notably Ncd/HSET [41,42], 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 [43]. 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 [44]. A role for Dynein itself in centrosome clustering *per se* has also been subject of conflicting evidence, however [39,45–47]. 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 [43]. Thus, Mud (or Dynein) loss can be a contributor to the development of supernumerary centrosomes as well. Finally, multiple models have been proposed to
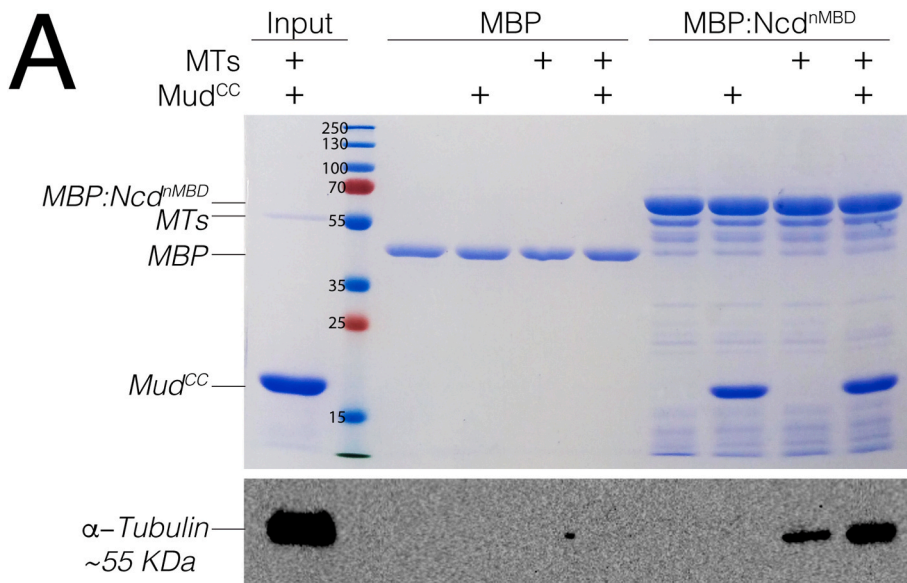
Fig. 4. Mud enhances MT binding to Ncd^nMBD *in vitro*.
(A) MBP alone or as a fusion to Ncd^{nMBD} was immobilized on amylose resin. Reactions were then incubated in the absence or presence of taxol-stabilized MTs, Mud^{CC}, or both as indicated. Coomassie stained gel (*top*) shows near equal levels of bait proteins as well as the Mud^{CC} interaction with MBP:Ncd^{nMBD}. Anti-αTubulin western blot (*bottom*) depicts an increased amount of MTs bound in the presence of Mud^{CC}. Molecular weight standards in the gel are labeled in kilodaltons (kD).
(B) Curves representing the effect of Mud^{CC} (10 μM) on concentration-dependent MT binding to MBP:Ncd^{nMBD}. 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.
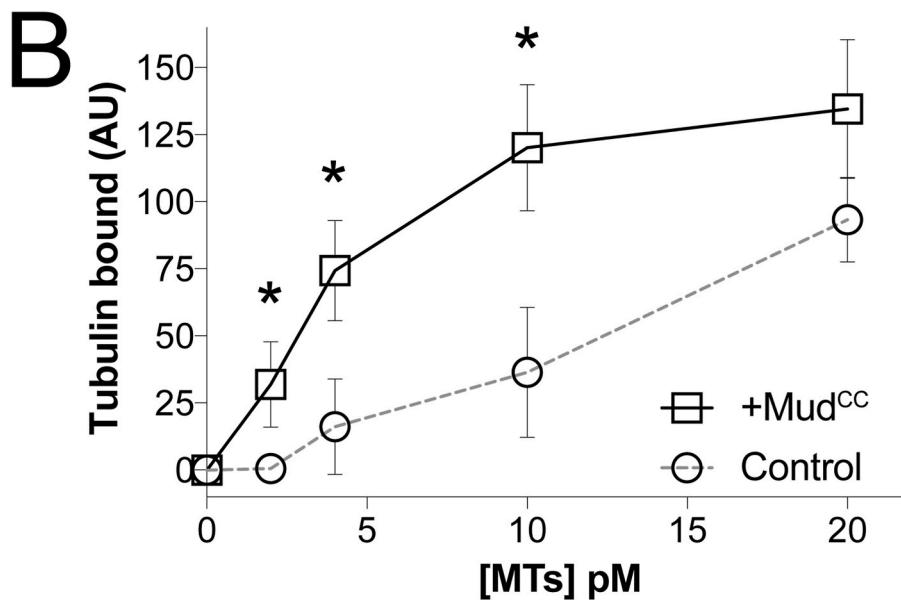
explain the cooperative functions of NuMA, HSET, and Dynein in spindle assembly and chromosome dynamics [48]; the interaction between Mud and Ncd (and potentially conserved in NuMA and HSET) described here posits an additional mode by which these essential regulators may be linked. Thus, Mud may serve 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 processive motility? What is the role of the Mud/Ncd interaction *in vivo* and is it ubiquitous or tissue specific? Are the Ncd^{nMBD}/Ncd^{CTm} and Mud^{CC}/Ncd^{nMBD} interactions evolutionarily conserved mechanisms for kinesin-14 regulation? Is the Mud/Ncd interaction regulated by other cellular components?

4. Materials and methods

4.1. 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′-*Bam*HI and 3′-*Sal*I restriction sites, generating a TEV cleavable 6 × His fusion. Full-length Ncd (amino acids 1–700) or individual domains (nMBD: amino acids 1–204; coiled-coil: amino acids 205–332; 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′-*Kpn*I and 3′-*Sal*I or 5′-*Nde*I and 3′-*Sal*I restriction site combinations. Site-directed mutagenesis was carried out with a standard PCR protocol using KOD-XL DNA polymerase.

4.2. 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 OD_{600} ~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 Ncd^{CTm}-containing proteins (ATPase motor domain), final storage buffers included 2 mM $MgCl_2$ and 100 μM ATP.

4.3. 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 4°C 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, 120 mM NaCl, 1 mM DTT, and 0.2% Triton-X100; supplemented with 5 mM $MgCl_2$ and 100 μM ATP for reactions involving the Ncd^{CTm} 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 α/β-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 Chemi-Doc 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 Mud^{CC} 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.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101016.

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