Mud binds the kinesin-14 Ncd in *Drosophila*

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**A B S T R A C T**

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^CTm) 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 mitotic/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 cross-linking 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

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molecular models for these functions remain incomplete [11,12]. We delimit this interaction to a short Mud coiled-coil domain (MudCC) that directly binds to the NcdnMBD domain, notably with a higher affinity than to the full-length Ncd protein (NcdCTm). We also find that the NcdnMBD directly interacts with the NcdCTm 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 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.

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 MudCC 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

![Diagram](image-url)
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 [16,17], 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 NcdMBD being most susceptible likely due to its lack of significant globular structure [17]. These MBP fusion baits were then immobilized on solid amylase resin and used 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). In contrast, a high affinity, nanomolar interaction was measured with the isolated MBP:NcdMBD domain (Fig. 1C). No binding was detected to MBP:NcdCC at any Mud concentration tested (Fig. 1D). Binding to MBP:NcdCTm 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 NcdMBD, and (3) Mud binding to the NcdCTm domain appears to be attenuated within the context of the NcdFL protein.

The NcdCTm 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) NcdMBD mutants and found that neither single mutant nor a double mutant significantly affected affinity for MudCC 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 MudCC directly binds the NcdMBD domain in a manner that is likely independent of its phosphorylation status.

### 2.2. NcdMBD and NcdCTm self-associate in vitro

The ability of MudCC 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 NcdMBD (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 NcdMBD and NcdCTm domains could directly interact in trans as isolated recombinant proteins. Indeed, soluble NcdMBD bound to MBP:NcdCTm 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 NcdCTm binding to MBP:NcdMBD (Fig. 2D), further validating this 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 with binding to their respective counterpart domain as an isolated fragment (Fig. S3). These results are consistent with interactions between NcdMBD and NcdCTm domains within full-length proteins bound to the resin competing against binding to isolated domains in solution.

To map a putative interaction site 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 [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 NcdCTm3A 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 NcdCTm3A to bind to NcdMBD 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 NcdMBD 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 MudCC binding (Fig. S4), suggesting the low-affinity binding to NcdCTm occurs at a site that overlaps with its MT binding as well as with NcdMBD. However, our results cannot rule out an alternative possibility that the 3A mutation allosterically induces a conformational change at an alternative site on NcdCTm, distinct from the MT-binding site, that binds NcdMBD and MudCC. Nevertheless, these results suggest that the site of MudCC binding and NcdMBD self-association could potentially overlap with the MT binding surface of the NcdCTm 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 NcdMBD

Having established that NcdMBD directly binds both MudCC and NcdCTm, and that Mud and NcdMBD each had reduced binding affinity to the NcdCTm3A mutant (Figs. 1C and 2, and S4), we next examined whether these interactions are mutually exclusive. To do this, we immobilized MBP:NcdCTm on amylase resin and examined how its interaction with a single concentration of NcdMBD (at the ~Kd of 2 μM) is affected by addition of increasing concentrations of MudCC. As shown in Fig. 3, binding of MudCC resulted in a concentration-dependent reduction in the interaction between NcdCTm and NcdMBD, demonstrating that MudCC directly competes against NcdMBD/NcdCTm 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 MudCC binding influences the interaction between NcdMBD and taxol-stabilized MTs. Surprisingly, association with MudCC increased MT binding to MBP:NcdMBD (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 NcdMBD 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 NcdMBD 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 MudCC/NcdMBD/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.

### 3. Discussion

Maintenance of proper spindle structure throughout mitosis is essential for correct and efficient segregation of replicated chromosomes.
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 amyllose 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 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 amyllose 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 NcdCTm (red bar) was immobilized on amyllose 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.)
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. 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.)
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\textsuperscript{nMBD}/Ncd\textsuperscript{CTm} and Mud\textsuperscript{CC}/Ncd\textsuperscript{MBD} 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′-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 1–204; 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′-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.

### 4.2. Protein purification

All proteins were expressed in BL21(DE3) E. coli under induction of isopropyl-β-D-thiogalactoside (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 pH8, 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 NcdC™-containing proteins (ATPase motor domain), final storage buffers included 2 mM MgCl2 and 100 μM ATP.

### 4.3. Pulldown 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 MgCl2 and 100 μM ATP for reactions involving the NcdC™ 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 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 MudC™ 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.

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### Appendix A. Supplementary data

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

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