THESIS

INVESTIGATING THE REGULATORS OF CYTOPLASMIC DYNEIN

Submitted by

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ABSTRACT

INVESTIGATING THE REGULATORS OF CYTOPLASMIC DYNEIN

Organization of the cell is a dynamic and complex process that is often underappreciated. To accomplish this, cells use motor proteins to move different cargo to their destination. Cytoplasmic dynein is one such motor protein that uses filaments called microtubules as tracks. However, there is only one cytoplasmic dynein to accomplish over forty tasks. To achieve this, the cell uses a complex array of cofactors and regulators to specifically control dynein. But the role of each of these cofactors and regulators in poorly understood. To better understand how dynein is regulated we turn to budding yeast that provides a simplified system where dynein only has one known function, this is to position the spindle in the division plane between two dividing cells. Localizing dynein is extremely important. One regulator of dynein is Pac1 which was recently found to also activate dynein motility *in vitro*. Pac1 works to localize dynein to microtubule plus ends where it can interact with dynactin and Num1. Ndl1 is known to interact with Pac1, knockouts of Ndl1 led to a mild phenotype mimicking a dynein knockout. But how Ndl1 functions is poorly understood. Dynactin is an essential regulator of almost all dynein's tasks in humans and dynein's only role in yeast. Without dynactin, dynein cannot reach Num1 patches at the cell periphery and pull the spindle. In this study we sought to better understand dynactin and Ndl1's role in dynein regulation using *in vitro* single molecule assays where the activity of dynein can be recorded. Initial attempts to purify dynactin for these assays failed. We then developed a cell lysis assay to study dynactin and other proteins role in dynein regulation. We found in preliminary results that dynactin increased dynein activity. We also attempted to use a protein known as Num1, that is essential to dynein localization and interacts with dynactin, to purify the dynein-dynactin complex. Preliminary results showed that this complex

was motile, indicating an intact complex. We also found that Ndl1 can bind motile dynein and increase run length using *in vitro* assays. We also were able to determine that Pac1 cannot bind dynein and Ndl1 at the same time indicating that there is a release mechanism for Pac1 from Ndl1 to bind dynein. We were able to map Ndl1's binding site to the N terminus of the dynein accessory chain Pac11. Then we tested to see if Ndl1 influence on Pac1-dynein interaction and found that Ndl1 was able to increase Pac1 comigrating with dynein in these assays. This work has opened new strategies for studying the regulators of dynein as well as better determined the interaction between Ndl1, dynein and Pac1. Further work will determine how each of these proteins affect dynein activity.

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Introduction and Background

Organization of the many different organelles, proteins and RNAs within a cell is a complex process. To accomplish this, cells employ motor proteins to traffic these cargos to their destination. There are three major types of motors: myosin, kinesin and cytoplasmic dynein, hereafter referred to as dynein. To complete their tasks, motors utilize either actin or microtubules (MT) as tracks to walk upon. Although kinesin and myosin have numerous members in their respective families to tackle their many tasks, dynein has only one[1–3]. Instead, to differentiate and accomplish its many tasks, many regulators are used to target and activate dynein.

Because of dynein's many tasks and due to its important regulatory mechanisms, there are many diseases associated with errors in dynein regulation. One of the sites were this occurs most commonly is in motor neurons, where cells stretch out long axons to commute. Once communication between cells is achieved transport from the axons to the cell body is essential. To achieve this long-range transport the cell uses the motors mentioned earlier. Specifically, for transport from the axon to the cell body dynein is a key factor[4]. Because of its critical role, errors in dynein itself can lead to many neuronal diseases[4,5]. Additionally, errors in regulatory protein interactions also cause disease, highlighting the importance of regulating dynein. One such protein is Lis1 whose name is derived from the disease Lissencephaly. This disorder results in a smooth brain phenotype and has been connected to dynein function[6].

Since most dynein transport leads to the nucleus many viruses have been shown to target dynein[7]. These viruses use dynein transport to reach the nucleus and either replicate their genome or integrate into the host genome[8–10]. Other viruses use dynein and its regulators in assembling or disassemble the virus capsids[11,12]. Dynein and its regulator proteins are clear targets for these different viruses, targeting dynein for transport to the nucleus as well as using

this motor for tasks involved in assembly or disassembly of their capsid. More work needs to be done to further understand dynein's many roles in virus targeting.

These diseases highlight the importance of dynein regulation using a mix of regulators and activators. As mentioned earlier regulation of dynein is key to function, however differentiating how dynein is regulated is difficult due to its various tasks in mammalian cells. To study how dynein is activated for these tasks, the Markus lab utilizes the budding yeast *Saccharomyces cerevisiae*, hereafter referred to as yeast. In yeast, dynein has only one known function in which it positions the spindle in the division plane of the dividing cell (Figure 1)[13]. To accomplish this, dynein is targeted to the cell cortex. Once at the cortex dynein will bind MTs and pull the spindle into the division plane between the mother and the daughter cell (Figure 1). Errors in the dynein pathway lead to an easily identified phenotype of binucleated mother cells[14].

But regulation of this process in yeast is only partially understood. Dynein is formed from a complex of four different proteins. Dyn1 is the major subunit of dynein that contains the motor, microtubule binding domain (MTBD), and tail domain where the accessory dynein chains bind[15]. In yeast these accessories include Dyn2, Dyn3, and Pac11. Pac11 is important for dynamization of two dynein legs and binding of many regulators of dynein. Dynein will then dimerize when the N terminal tails of two Dyn1 bind each other forming the dynein complex. Initially, dynein can adopt an auto-inhibited state by binding itself and forming a closed conformation, preventing its ability to walk on microtubules (Figure 2). Little is known of the function of this closed state but some studies in humans have found that loss of this inhibition leads to mitotic defects, indicating that overactive dynein can be harmful to cells (Figure 2)[16]. While in this closed state dynein can stochastically change to an open conformation. When this occurs, the interacting protein Pac1 can bind dynein; Pac1 binding seems to prevent dynein from re-entering the closed state due to a steric clashing between the adjacent dynein leg. This was shown by an increase in the length of dynein runs on MTs, a readout of dynein activity in single molecule assays, for wildtype

dynein in the presences of Pac1[5,17]. Mutants that cannot form the closed state showed an elevated run length that was not affected by Pac1 addition[17,18]. A known contributor to this mechanism is the protein Ndl1. It was found that in *ndl1*Δ cells there is an increase in binucleation of mother cells, this was rescued by overexpression of Pac1[14]. From these results it appears that Ndl1 somehow influences the recruitment of Pac1 to dynein. However, how Ndl1 accomplishes this is currently unknown. What is known is that Pac1 will target dynein to MT plus ends after binding. Once at the plus ends of MTs dynactin can then interact with dynein. Dynactin is key to dynein function, deletion of dynactin subunits results in a similar phenotype to dynein delete[19]. When a MT plus-end encounters a cortical patch of Num1, intact complexes of dynein-dynactin can bind Num1 and Pac1 is released (Figures 1 & 2)[20,21]. The purpose of this Pac1 release is not known. At the cortex dynein can then perform its function, to pull the spindle into the division plane of the dividing cell (Figure 1). How dynein is activated once it reaches the cortex and what part dynactin or Num1 could play in this is unknown.

Many questions remain regarding dynein regulation. Although it is known that dynein is active at the cortex it is not known how this activation occurs. One possibility is that binding to Num1 somehow activates dynein. An interesting finding revealed that a truncated version of Num1 was shown to activate dynein motility *in vivo* and components of dynactin were seen localized with this active dynein, indicating that Num1 interaction could activate dynein motility. They also found that deletion of dynactin components led to a decrease in this phenotype, indicating that dynactin is influencing this interaction as well[22]. To determine Num1 and dynactin's influence on dynein, we worked to generate a reconstituted *in vitro* motility assays to determine dynein's activity in the presence of these two proteins. In these single molecule assays, glass chambers are constructed and MTs are attached to the glass slide via antibodies. Using fluorescent microscopy and tagged dynein constructs, we can then determine motor activity based on run length and velocity of motors walking on the MTs. Interestingly, in these assays yeast dynein exhibits motility in the absence of cofactors such as dynactin and an

adaptor, yet human dynein requires both for processive movement[23,24]. Due to Num1's structure and its apparent influence on dynein activity, it is possible that Num1 could be acting as an adaptor. This would mean that dynactin and Num1 could be acting similarly to dynactin and adaptors in humans but more research needs to be done to address this. This study laid the ground work to better understand the influence dynactin and Num1 could play on dynein activity by generating different strategies to both purify dynactin and Num1 as well as developing an *in vitro* yeast lysate assay.

As mentioned earlier Ndl1 seems to work on the Pac1-dynein regulatory step by promoting the Pac1-dynein interaction. Whether this is really the case, and if so, how Ndl1 does this is unknown. Since Pac1 binding seems to prevent the closed state from occurring we can reason that Pac1 is regulating this state by preventing reentry. Because Ndl1 is involved in this pathway and has an impact on Pac1-dynein interaction it raises the question of what affect the inhibited state could play on Ndl1 interaction. It is interesting to speculate that there is a Ndl1-dynein interaction and that this interaction as well as the Ndl1-Pac1 interaction could be influenced by the inhibitory state of dynein. To determine how this interaction occurs we used a combination of competition, binding and single molecule assays. We found that Ndl1 and dynein share a similar binding site on Pac1 but not the same binding site. Because of this Pac1 cannot bind both Ndl1 and dynein at the same time. We also used single molecule assays to determine that the N terminus of Pac11 is important for Ndl1 binding. Finally, we determined that Ndl1 increases Pac1 comigrating with dynein using single molecules assays.

This research has begun to address how different aspects of dynein regulation occurs, what order it occurs in, and how this interaction influences dynein activity.

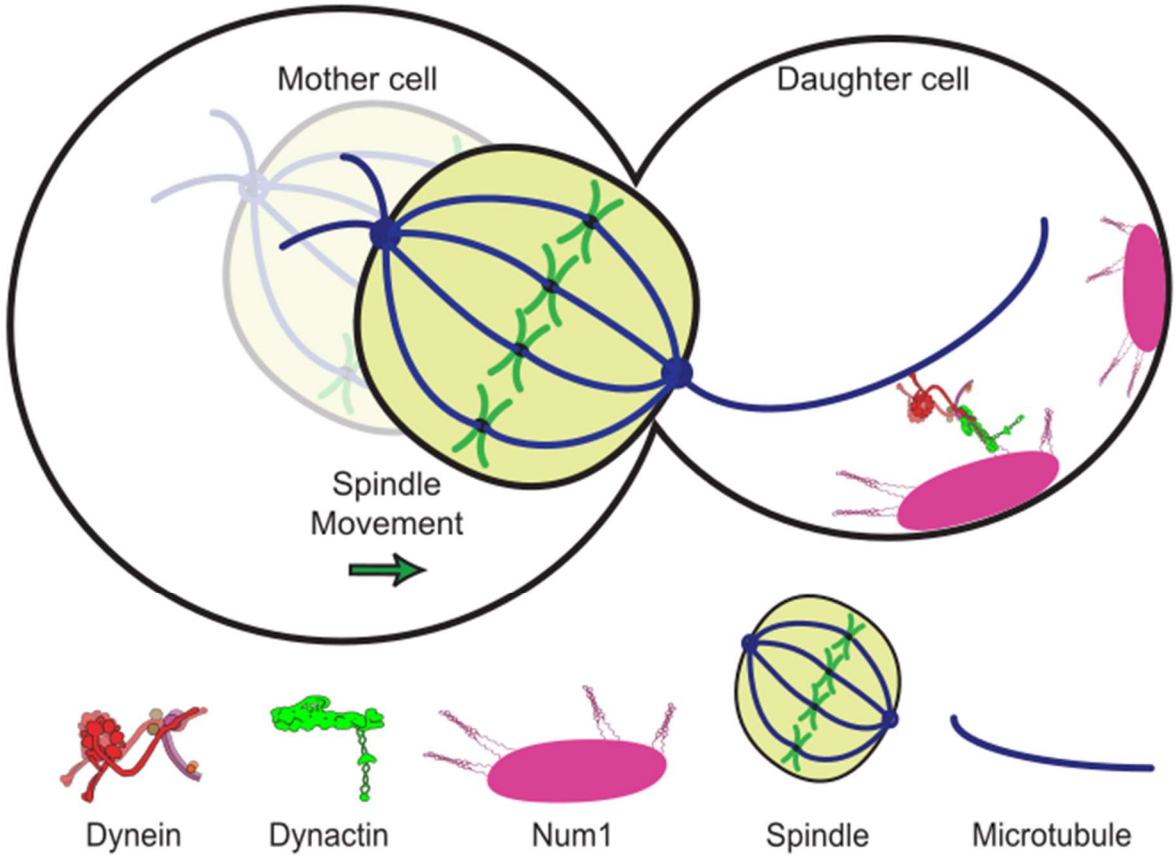


Figure 1. Dynein positioning the spindle in budding yeast

Dynein is localized to microtubule plus ends where dynein-dynactin complexes can then bind Num1 patches at the cell cortex and pull the spindle into the division plane between two dividing cells.

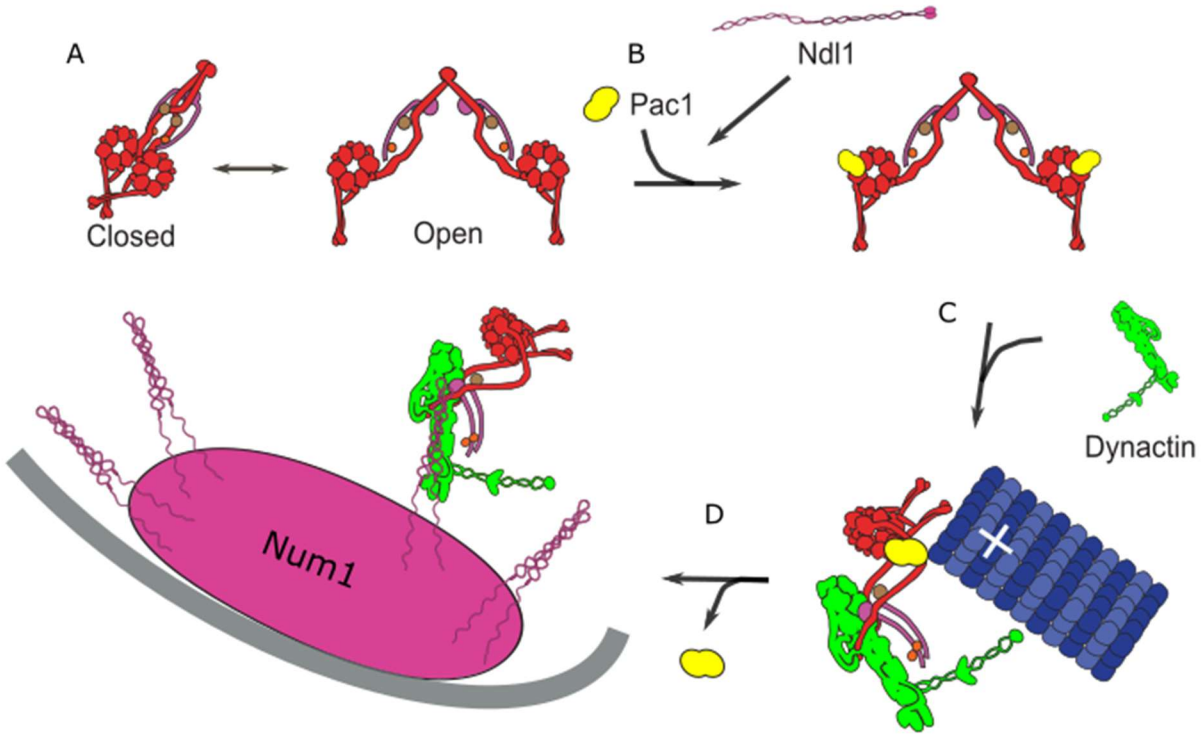


Figure 2. Regulation of dynein in budding yeast

- **(A)** Dynein can form the autoinhibited state on the left where the dynein tails and heads are stacked on top of each other. Through an unknown mechanism this inhibition can be released and dynein can adopt a more open conformation.
- **(B)** While in the open conformation Pac1 can then bind dynein and prevent reentry into the inhibited state. Ndl1 is known to contribute to this part of the pathway but the mechanism is unknown.
- **(C)** While in this open state Pac1 can relocate dynein to MT plus ends. While at the plus end dynactin can bind.
- (D) When the plus end encounters a Num1 patch at the cell cortex Pac1 is released and dynein-dynactin binds Num1. At his point dynein is now active and able to complete its task outlined in Figure 1.

Chapter 1: Developing methods to study dynactin‘s influence on dynein activity

Background

As mentioned earlier, dynactin is essential for nearly all of dynein’s functions in mammalian cells and essential for dynein function in yeast. While earlier studies worked to determine dynein’s many functions, recent studies have focused on understanding the regulators of dynein. An important development of these studies was to determine the structure of dynactin and the function of its many subunits. In a remarkable series of studies the Carter lab have been able to provide a structure for dynactin alone as well as the structures of dynein and dynactin in complex. The focus of these studies provided a structure for most of the dynactin complex and how it interacts and influences dynein. These studies were able to determine that dynactin orients the dynein motor heads through binding sites in the tail domain of dynein and is thought to produce a more processive motor. They were also able to determine how dynactin can bind two dynein dimers and how this increases activity of the complex. Their work also was able to explain the role of adaptors, which are essential for proper dynein-dynactin interaction in mammalian cells. Adaptors run along the binding surface of the dynein-dynactin interface, making contacts with both proteins in several places, presumably stabilizing the interaction[17,25,26].

Dynactin structure

Dynactin is a large 1 MDa protein complex comprised of many different components. From Cryo-EM mentioned earlier, the Carter lab was able to determine the exact structure of dynactin in mammals. In humans, dynactin has 23 subunits comprised of 11 different proteins. From Cryo-EM data, the Carter lab found that the core of dynactin is made up of a short actin filament made of the protein Arp1. It was also a shock to find a single β-actin nestled near the

pointed end of the Arp1 filament. This filament is then capped at the barbed end by the capping proteins CapZαβ(Cap1/2 in yeast). Another capping complex exists at the pointed end made up of four proteins. This complex comprises Arp11(Arp10 in yeast), p62,p25, and p27. A shoulder is formed near the barbed end made up of many different proteins. Dynamitin(Jnm1 in yeast) is one of these proteins and is present in four copies in each dynactin complex. It is thought, based on the structure, that the arms of Dynamitin reach down from the shoulder of dynactin and stabilize the Arp1 filament by direct interaction. Dynamitin in the shoulder is thought to interact with an extended arm made up of a dimer of p150 (Nip100 in yeast) and stabilized by the interaction of p24(Ldb18 in yeast)[26]. The p150 subunit has also been shown to interact with dynein at a separate binding site located at its first coiled-coil which interacts with the accessory chain DIC2 (Pac11 in yeast)[27]. This could indicate that subunits of dynactin can interact with dynein independent of the entire dynactin complex. An important aspect of p150 is its CAP-Gly domain that allows it to bind microtubules and is important for different dynein functions in humans as well as yeast[28,29].

In yeast, less is known of dynactin's structure and composition. For instance, homologs of p62,p25 and p27 have not been identified in yeast. It is also unknown if any β-actin is part of this complex. Additionally, the Cap1,2 proteins are dispensable as they have not been seen to localize to the plus end of microtubules or the cortex, two locations dynactin and dynein are known to be[28]. Similarly, deletion of these do not lead to a dynein null phenotype that is seen in deletion of all other dynactin components except Arp10. Although Arp10 has been shown to be localized correctly in cell, its deletion also does not lead to a dynein null phenotype[28].

Num1's structure

As mentioned earlier Num1 is an essential component for the dynein pathway in yeast. Num1 is a large protein that contains close to 3000 amino acids. At the N terminus it contains two coiled-coil domains, one of which was found to be essential binding to dynein-dynactin.

Adjacent to these is an EF domain followed by a large tandem repeat (TR) domain. At the C terminus there is a PH domain involved in lipid binding and essential for localization[30,31]. Many studies have sought to understand how Num1 functions in the dynein pathway. In a series of studies the Lee lab looked at different truncations of Num1 and how they affected function. They found that the central TR domain as well as the EF domain are dispensable for Num1 localization to the cell cortex. Truncations were also made to determine that the first coiled-coil of Num1 is important in dynein pathway function. An important construct that was made contained only the N terminal coiled-coil domains and the C terminal PH domain and produced viable cells with a functioning dynein pathway[30,32]. A previous paper from our lab utilized this information and made a similar construct of Num1 containing this coiled-coil domain but lacking the C terminal PH domain, called Num1CC hereafter, and expressed it *in vivo*. From this they saw a large shift of dynein localized at the plus ends redistributed to the minus end located at the spindle pole body (SPB) and along the microtubules lattice. They were also able to capture movies visualizing dynein walking towards the SPB. Additionally, they saw this same localization pattern for Jnm1, an important subunit of dynactin. In cells lacking important dynactin components they noticed that this activity and localization for dynein was diminished. This indicates that Num1CC was able to activate dynein activity and allow dynein, and dynactin, to walk to the minus end of microtubules[33].

To better understand the influence dynactin and Num1 could have on dynein activity, we set out to generate an *in vitro* single molecule assay from purified components. In the process we laid the groundwork for a yeast lysate system that can be used to quickly study the effect on dynein activity by different proteins, including dynactin. This approach coupled with the power of yeast genetics will also allow us to quickly determine what proteins are important in dynein activation and activity in an easily manipulated *in vitro* setting. We also established a possible pathway for purifying dynactin using a truncated version of Num1.

Chapter 1 Results

Attempts to purify yeast dynactin

While much of the work on yeast dynactin had been done *in vivo*, limited work has been done *in vitro.* One study has claimed to have purified dynactin using a tag located on the N-terminus of Arp10[34]. Because of this, a previous graduate student in our lab attempted to replicate their results by purifying dynactin using the same tag on Arp10 and conducting single molecule assays with dynein. After many experiments the student found that the comigration of dynactin with dynein seen in the aforementioned paper was due to contaminating dye from the dynactin prep labeling dynein, giving the appearance that dynactin was comigrating with dynein. When she swapped the fluorescent tags to one that was different than what dynein was labeled with or to a fluorescent protein, such as GFP, to eliminate dye contamination no comigration was seen. Therefore, we sought to purify yeast dynactin for the first time.

To do this we first added an HZZ-Halo tag at the N-terminus of Arp10. We also hoped to increase the amount of dynactin in the cell by overexpressing each subunit using a galactose inducible promoter. Using an earlier study that explored the importance of different dynactin components we also added Nip100, Jnm1, Arp1, and Ldb18 to the plasmid containing Arp10. Initial purifications did not show any dynactin components beyond the tagged Arp10 subunit by protein gel and no comigration with dynein in single molecules assays (data not shown). In these initial studies we had grown the yeast for 24 hours to a dense culture in media inducing expression of the dynactin components to allow for the maximal amount of dynactin to be produced. We then hypothesized that purification of dynactin may not be occurring due to the complex not assembling properly. Another graduate student in the lab also had issues with overexpressing dynein for extended periods of time leading to changes in dynein activity. We therefore reasoned that dynactin could be experiencing the same issues as dynein upon extended overexpression. Additionally, several papers have pointed to the components of the

dynactin complex appearing in different areas of the cell throughout the cell cycle, indicating that the components aren't assembled immediately after translation[28,35]. Because of this we decided to reduce the time of expression of dynactin to only two hours, similar to our dynein overexpression, and harvest cells while they were still dividing. Thus, we attempted to purify dynactin using these new parameters. Unfortunately, these purifications still proved to be unsuccessful. How the dynactin complex is formed is still unknown and could be hindering our ability to purify this complex.

Using Num1CC to isolate dynein-dynactin complexes

Due to the initial issues in purifying dynactin we decided to turn to the literature to find a better approach to isolate dynactin. In a paper from the Lee lab they were able to purify dynein heavy chain, Dyn1, using a truncated version of Num1 that included the coiled-coil domain shown to be important for dynein-dynactin localization and dynein function. In this paper they also noticed that deletion of Nip100, a component of the dynactin complex, abolished their ability to purify dynein[30,32]. This indicated that dynein's interaction with Num1CC is dependent on Nip100, suggesting that this could be a possible mechanism of purification for dynactin as well. Additionally, our lab published a paper looking at this truncated Num1's effect in cells. To do this they placed a galactose inducible promoter upstream of the Num1 locus and truncated the C terminus of Num1 to produce the aforementioned Num1CC. This construct contained the coiled-coil for dynein-dynactin interaction but lacked the PH domain that is important in Num1 localization to the cell cortex thus it was presumably diffusive in the cytoplasm. Upon induction they saw a change in dynein and dynactin localization from the plus end of microtubules to the SPB. They concluded that this was because Num1CC could be acting as an adaptor, similar to the adaptors in humans, and led to dynein walking along the microtubules to the minus end[33]. Because of these two lines of evidence we reasoned that Num1CC could be acting as an adaptor, due to the presence of a coiled-coil which all known dynein adaptors contain, in yeast

and could be necessary for proper dynein-dynactin interaction. To do this we generated a yeast strain containing a Gal inducible Num1CC with an N terminal HZZ tag for purification. This strain also contains a fluorescently labeled Jnm1 to visualize dynactin migration in single molecule assays. We grew up one liter of cells and induced expression for two hours then harvested the cells and purified similarly to overexpressed dynein. Although the prep wasn't clean enough to determine the components by protein gel, we were able to visualize motile Jnm1 by single molecule assay (Figure 3a,b). Indicating that dynein could be present and transporting Jnm1 and possibly the entire dynactin complex. Analysis of the speed and run length of the motile Jnm1 showed it to be in the range of dynein (Figure 3c,d).

Developing an *in vitro* yeast lysate system to study dynein regulation

Due to the purification of dynactin being problematic we began to develop an *in vitro* assay to study dynactin's influence on dynein activity. This was a technique that Dr. Markus began to develop during his postdoc and involved flowing clarified lysate into imaging chambers. Initially, we used a strain containing Jnm1-3mCherry, to label dynactin, and Dyn1-3GFP, to label dynein, for visualizing comigrating complexes. Importantly, this strain was also she1Δ. The reasoning behind this was due to an earlier paper showing that She1 could be a regulator of dynein-dynactin interaction. The Barnes lab showed that in she1Δ cells more dynactin localized to the plus end of microtubules where it interacts with dynein[35]. Using this yeast line we arrested cells in mitosis using hydroxyurea, a commonly used inhibitor of DNA replication, and were able to take clarified lysates from yeast extracts and flow them into a single molecule chamber. Upon quantification of this we found few but still some dynactin moving with dynein (Figure 4a,b). From this we observed 6% comigration of dynactin and dynein together. Although the data set was limited for moving dynactin, we did find a difference in the activity of dynein comigrating with dynactin determined by the run length of dynein but not the velocity of dynein (Figure 4c,d).

Chapter 1 Discussion and Future Directions

In the pursuit of purifying dynactin we followed three separate routes. Initially, we tried to purify dynactin directly by using an affinity tag on the Arp10 subunit. Unfortunately, altering growth and expression conditions did not result in purification of the dynactin complex. This could indicate that Arp10 is not a good target for dynactin purification. Finding a suitable target for dynactin purification is difficult due to the structure of dynactin and the multiple protein interactions. In most places the tag could interfere with protein interactions within the complex, thus preventing complex formation. There is also the possibility that dynactin formation is actively regulated and thus purifying the intact complex is difficult without meeting the required criteria, through addition or subtraction of regulatory elements, before purification. One paper has proposed that posttranslational modifications (PTMs) occur on Ldb18. In their paper Amaro et al. saw that only a larger shifted Ldb18 band co-migrated with dynactin[36]. They also found that Ldb18 was important in proper dynactin formation since it was able to pull out all other components of the dynactin complex[36]. This could indicate that PTMs play an essential role in dynactin complex formation through Ldb18. This would point to Ldb18 being a potential target for future studies of purifying dynactin directly and a possible regulator of dynactin complex formation through its PTMs. But more needs to be known of the dynactin complex before we can begin to purify it for use in *in vitro* assays.

A promising new direction for this project is the co-purification assays using Num1CC. Although this assay was only run once it shows the feasibility of purifying an intact dynein-dynactin complex in which dynein is active. A tandem purification step could lead to a cleaner protein prep, thus allowing us to send samples for mass spectrometry analysis. This would benefit us in two key ways. First, we would be able to determine the exact components of the

dynactin complex. This would allow us to identify if there are homologs of p62, p25, and p27. This would also indicate if Ap10 and the Cap proteins are essential for dynactin formation. Secondly, it would allow us to understand if any of these components, such as Ldb18, are being regulated by PTMs. To accomplish this we have been exploring the use of magnetic beads, rather than Sepharose beads, for purification. In an unrelated study, researchers were able to use magnetic beads to not only improve the amount of the protein complex purified but also eliminate nonspecific binding, allowing for a much cleaner protein preparation as seen by protein gel[37]. This would allow us to determine if there are any other proteins involved in proper dynactin formation. Potentially, this could also shed light onto the interesting finding regarding Ldb18 mentioned earlier and help us to determine if PTMs play a role in dynactin formation and function.

After isolation of dynactin was proving to be difficult, we turned to generating a yeast lysate system to quickly study the affect dynactin could have on dynein activity. Initial experiments proved the viability of this approach but improvements in frequency of dynein-dynactin comigration need to be made to determine dynactin's influences on dynein. For instance, we may see more dynein-dynactin comigrating if we included the truncated Num1CC mentioned above. If this is working similarly to humans and Num1 is acting as an adaptor, as shown by evidence provided earlier, then dynein-dynactin interaction would need Num1 to stabilize the interaction and lead to more instances of comigration. We would also like to determine if deletion of She1 is necessary for dynein-dynactin interaction in these assays or if its absence just increases the occurrence of dynein-dynactin interaction. Finally, we would like to better understand how different components of dynactin influence dynein activity and its ability to comigrate with dynein. It would be interesting to see if Nip100 is able to bind dynein, similar to p150 binding in humans, and if this can comigrate with dynein and what effect this has on dynein activity in the absence of other dynactin components[27]. Additionally, this would allow

us to understand if there are subcomplexes of dynactin being formed and if they comigrate with dynein as well.

From work in this study we have outlined many different paths for studying yeast dynactin. This project is poised, with many different tools, to study the effect dynactin and Num1 could have on dynein activity to further our understanding of how this interaction occurs, the importance of these two components to dynein activation and activity, and the potential identification of the first dynein adaptor in yeast.

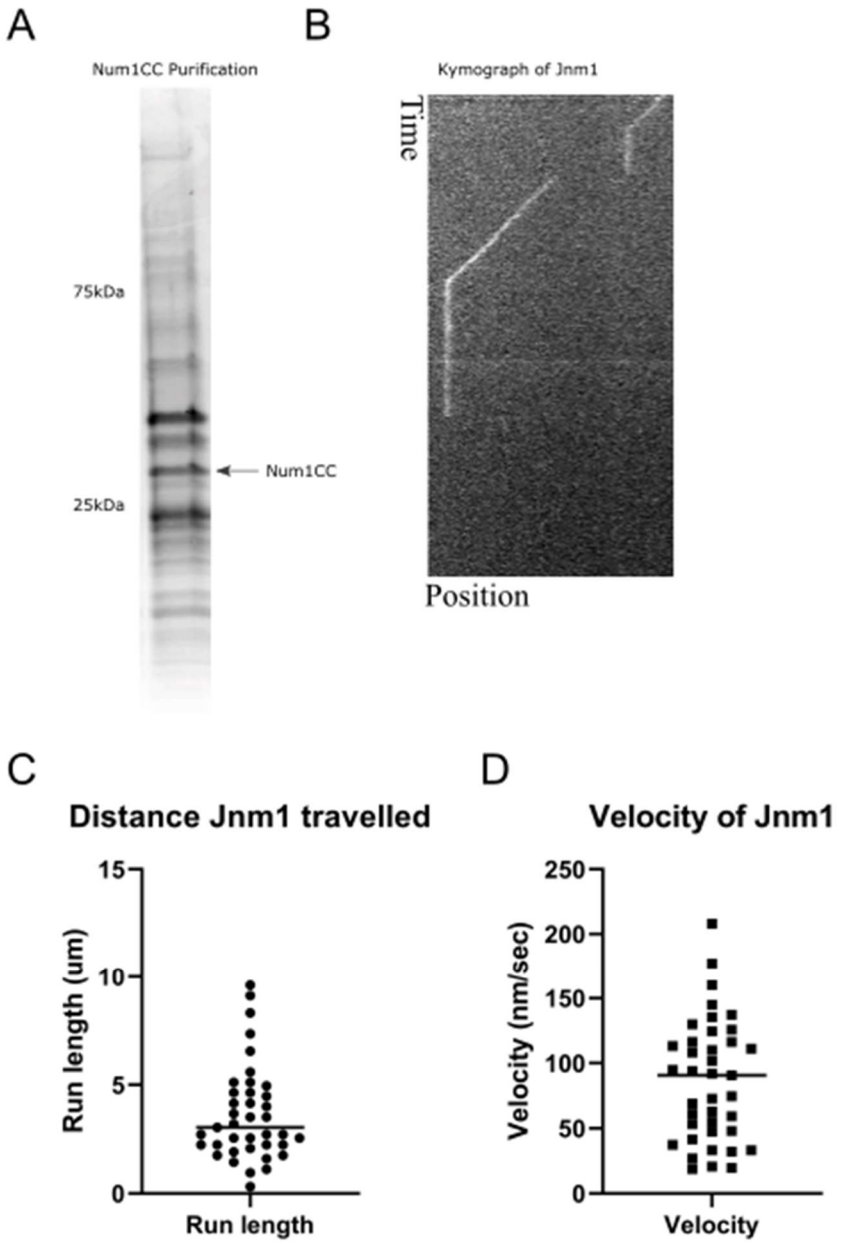


Figure 3. Using Num1CC to pulldown Jnm1

(A) Protein gel (10%) stained with SYPRO Ruby dye of cleaved supernatant from one liter of SMY2334
(B) Representative kymograph of single molecule assays imagining Jnm1-3YFP
(C) Distance Jnm1 travelled in single molecule experiments (n=1)
(D) Velocity of Jnm1 in single molecule experiments (n=1)

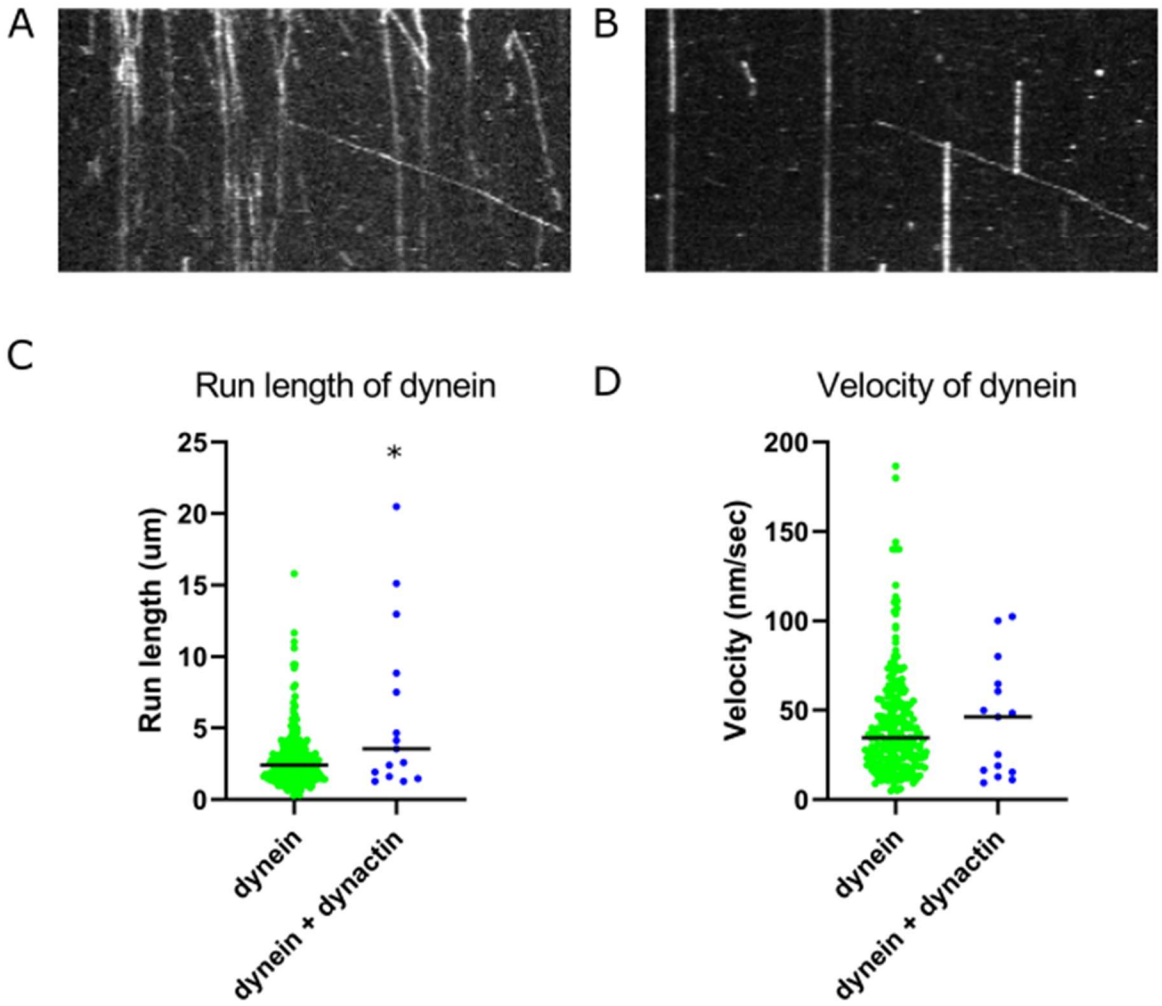


Figure 4. Yeast lysate system in single molecule assays to visualize dynein-dynactin interaction

(A) Representative kymograph of dynein (WT) in single molecule assay

(B) Representative kymograph of dynactin, Jnm1 contains label, in single molecule assay

(C) Analysis of run length of dynein (WT) and dynein-dynactin (WT+Dynactin) data represents an (n = 1)

(D) Analysis of velocity of dynein (WT) and dynein-dynactin (WT+Dynactin) (n = 1)

Chapter 2: Investigating Ndl1's role in dynein regulation

Background

As it was highlighted above, Pac1 regulation of dynein is key to the dynein pathway in yeast. Less is known about Ndl1 but many studies in mammalian cells focused on NudE and NudEL, mammalian homologs of Ndl1. In these studies they have shown the importance of this protein in dynein regulation. In mammalian cells NudE and NudEL, hereafter referred to as NudE/L, have been shown to be important in localization of dynein and Lis1 to sites such as the nuclear envelope, prior to breakdown of the envelope, and to the kinetochore[38]. It has also been shown to work alongside Lis1 and allows dynein to produce a prolonged force producing state, indicating that the Lis1-NudE/L interaction could have activities other than preventing the inhibited state of dynein[39]. Another important note is that in mice NudE mice showed lower brain mass while NudEL mice were not viable[40,41]. Thus, mutations in this protein can be detrimental to mammalian health as well.

As mentioned earlier, little is known of yeast Ndl1. In budding yeast one study found that strains lacking Ndl1 showed only a slight phenotype related to the dynein pathway being disrupted. When researchers over-expressed Pac1 in these strains they saw this phenotype rescued to wildtype levels[14]. This indicated that Ndl1's role in yeast is to increase the Pac1-dynein interaction. This is supported by Ndl1 not being present at the cell cortex and only occasionally at MT plus ends[14]. There is still much to learn about how Ndl1 interacts with dynein and Pac1 and how it could influence the dynein-Pac1 interaction. Additionally, yeast provide a unique system to study Ndl1. This is because NudE/L and dynactin compete for the same binding site on dynein[42]. Since human dynein requires dynactin for motility *in vitro*, the effect that NudE/L has on dynein activity cannot be studied[23,24]. Since yeast dynein is active in the absence of dynactin, we can thus investigate how Ndl1 could affect dynein activity *in vitro*.

Ndl1 structure and interaction with dynein and Pac1

The structure of Ndl1 and NudE/L are very similar. NudE/L proteins are long structures with two coiled-coil domains, Ndl1 is shorter and lacks the C terminal coiled-coil domain but retains the N terminal coiled-coil. This N terminal coiled-coil is thought to allow Ndl1 to dimerize, researchers showed this by differentially labeling Ndl1 and preforming pull-down assays[14]. More recent studies have focused on how NudE/L interacts with both Pac1/Lis1 and dynein. It was found that in the C terminal domain of NudE/L there is a binding site for dynein and in a recent study mapped this site to the first seventy amino acids of the intermediate chain of dynein (Pac11 in yeast)[41,42]. However, it has not been shown if Ndl1 can interact with dynein directly in yeast. Much more is known of the Pac1-Ndl1 interaction. Mammalians experiments have been conducted that looked to map the Lis1 binding site on NudE/L. They found that the amino acids 8-192 in NudEL were essential for Lis1 binding[43]. In yeast, the Cooper lab was also able to pullout Pac1 with Ndl1 and vice versa showing a clear interaction between Ndl1 and Pac1[14]. This is most likely a conserved site in the N terminal coiled-coil that is shared with mammalian NudE/L.

Pac1 structure and dynein interaction

Much more is known of the Pac1-dynein interaction in yeast. The structure for Pac1 includes a dimerization domain and pull-down assays were used to determine that dimerization truly occurs using two different tags, similar to how they proved Ndl1 dimerizes mentioned above[14]. The binding site for dynein on Pac1 has also been mapped and point mutants in Pac1 have been identified that interfere with dynein binding. The binding site for Pac1 on dynein has also been mapped to the AAA3 and AAA4 domains located within the motor[44]. This interaction can be disrupted by altering two amino acids within this region of Pac1[45]. Similarly, mutations in the AAA3/4 domain of dynein leads to a loss in Pac1 binding[44].

Although the binding of Pac1 to dynein is well understood the actual implications on dynein activity is controversial. Some studies have reported that Pac1 acts as an inhibitor or a clutch for dynein activity[39,44,46]. However, recent work from our lab and others has shown that Pac1 could be acting as an activator by preventing the auto inhibited state of dynein from forming [18,47–49].

While the Pac1-dynein interaction is well understood how Ndl1 influences this interaction and how Ndl1 could influence dynein activity isn't well studied. This work set out to begin to answer some of these questions by providing a better understanding how Ndl1 interacts with both dynein and Pac1.

Chapter 2 Results

The location of dynein binding on Pac1 overlaps with the Ndl1 binding site

To better understand how the Ndl1-Pac1-dynein interaction occurs we first wanted to ask if Pac1 could interact with Ndl1 and dynein at the same time. This would allow us to determine when Ndl1 is released from dynein and how Ndl1 releases Pac1. We know that Ndl1 is the first protein in this complex to be released since it has been shown that the Pac1-dynein interaction occurs more frequently at microtubule plus ends[14]. Thus we used a truncated version of dynein lacking the tail domain but including the motor domain and dimerized using a GST tag, hereafter referred to as GST-Motor. This construct of dynein is motile and includes the Pac1 binding site, allowing it to bind Pac1 in pulldown assays but lacks the presumed Ndl1 binding site (Figure 5a, 7a-c). To begin we first bound Flag-Pac1 to magnetic Flag beads. We then bound GST-Motor to these beads and applied either buffer or Ndl1 to these reactions. Surprisingly, Ndl1 was able to compete off GST-Motor from these beads (Figure 5a-b). We then wanted to know if Ndl1 shared the same binding site for dynein on Pac1. To do this we expressed and purified two Pac1 mutants altered at R378A and W419A from yeast. These

mutants could not bind GST-Motor but were able to bind Ndl1 (Figure 5c-f). Indicating that the binding sites for dynein and Ndl1 on Pac1 overlap but are not the same.

Ndl1 comigrates with dynein in single molecule assays

To better determine the binding site of Ndl1 on dynein we first asked if Ndl1 can comigrated with wildtype dynein. To do this we used a single molecule assay and visualized dynein and Ndl1 using two colored imagining. In a series of experiments we determined that Ndl1 can comigrate with dynein (Figure 6). To quantify this we first measured dynein in a single molecule chamber alone, this is labeled dynein – Ndl1 (Figure 6c,d). We then preincubated Ndl1 and dynein for 5-10 minutes on ice and added this to a single molecule chamber. Quantification was carried out by measuring the comigrating Ndl1-dynein, labeled comigrating, or what appeared to be dynein migrating alone in this chamber, labeled dynein + Ndl1 below (Figure 6c,d). In three experiments we found that Ndl1 was found to comigrate with dynein on average 21% of the time (data not shown). However, one experiment showed comigration at an extremely high rate (44%). While the other two experiments showed low levels (less than 10%) of comigration. It isn't clear why there was such a striking difference between the three experiments. We did notice a decrease in the velocity of dynein when Ndl1 was added (Figure 6c). There was also an increase in dynein run length when Ndl1 was added (Figure 6d). We then looked to see if Ndl1 could be causing dynein to aggregate. We measured the intensity values of dynein alone or dynein walking in complex with Ndl1 (Figure 6e-f). We found that there was not a statistical difference between these two populations indicating that Ndl1 was not causing dynein to aggregate.

Ndl1 binding site is located at the N terminus of dynein intermediate chain

We then asked if the Ndl1 binding site on dynein was conserved with mammalian NudE/L. To do this we used GST-Motor that lacks the Ndl1 binding site and looked to see if

Ndl1 comigrated with it. We saw zero instances of Ndl1 comigrating with GST-Motor (Figure 7a-c). Additionally, we saw no difference in either the run length or the velocity of GST-Motor when Ndl1 was added (Figure 7b-c). To further determine the Ndl1 binding site on dynein we used a full-length dynein that contained a Pac11 mutant lacking the first 43 amino acids, hereafter referred to as 1-43ΔIC. As mentioned earlier dynactin and NudE compete for binding to the same region on the IC/Pac11[42]. To determine if Ndl1 can comigrate with this mutant we ran single molecule assays similar to those described above using the 1-43ΔIC mutant. We again observed zero instances of Ndl1 comigrating with 1-43ΔIC (Figure 7d-f). Indicating that this short region contains the binding site for Ndl1 in yeast. Additionally, we also did not see a change in velocity or run length of the 1-43ΔIC mutant when Ndl1 was added. This was consistent with our findings using the GST-Motor construct. It is important to note that the percentage of these motors that were active was far lower than those compared to wildtype dynein (37% vs 72%). This could indicate that the N terminus of the IC is important to dynein function as well.

Ndl1 increases the frequency of Pac1-dynein comigration

We then wanted to ask if Ndl1 could be acting as a tether for Pac1 to bind dynein by looking at the instances of Pac1-dynein comigration in single molecules assays (Figure 8a). In the absence of Ndl1, Pac1 and dynein were found to comigrate about 30% of the time (Figure 8b). When 5nM Ndl1 was added we saw an increase of Pac1-dynein comigration to 46% (Figure 8b). We thought we could further increase this effect by addition of more Ndl1. But upon addition of 20nM Ndl1 we saw a decrease in the Pac1-dynein comigration similar to 0nM Ndl1 (Figure 8b). As a control we used the mutant dynein 1-43ΔIC and looked to see if there was still an increase in Pac1-dynein interaction in the presences of Ndl1. In this experiments Ndl1 did not seem to have any effect on Pac1-dynein comigration rates (Figure 8c).

Chapter 2 Discussion and Future Directions

To better understand Ndl1's role in yeast we first wanted to better understand how it interacted with Pac1 and dynein. We used competition assays with a dynein that lacked the mapped binding site for Ndl1 and found that Ndl1 can compete off this dynein from Pac1 coated beads. This indicated that Ndl1 and dynein share the same binding site on Pac1 and that Pac1 can only bind one of these proteins at a time. Since it is known that Pac1 binding to dynein is regulated by the inhibited state and also regulates this state by preventing reentry, and because Ndl1 works in this pathway, it is intriguing to consider that Ndl1 could also play a role in dynein's inhibited state. This could occur in many ways. The inhibited state of dynein could lead to different amounts of Ndl1 binding. From data provided in this study and previously, Ndl1's main role is to tether Pac1 to dynein. It is intriguing to speculate that Ndl1 could bind with higher affinity to the inhibited dynein, thus allowing Ndl1 to increase the concentration of Pac1 at inhibited dynein[44,50]. To study this, simple pull-down assays can be performed with a constitutively open dynein, studied before in our lab, or a crosslinked constitutively closed version of dynein could be engineered and used[5,18]. This would allow us to understand if Ndl1 prefers a certain state of dynein. We predict that it would prefer dynein in the inhibited state. It would then stand to reason that the stochastic opening of the inhibited state could trigger release of Pac1 from Ndl1 to bind open dynein.

Additionally, how Ndl1 is released from dynein is not understood either. From a study conducted in the Cooper lab they showed that Ndl1 was found less often at MT plus ends when compared to dynein and Pac1[14]. This indicates that Ndl1 release occurs either prior to or upon localization to the plus ends of MTs. It is possible that another dynein binding partner found at MT plus ends could cause this release of Ndl1. The obvious possibility is that Nip100 binding to dynein displaces Ndl1. In a study in 2012 the Vallee lab showed that a coiled-coil domain from p150(Nip100 in yeast) was able to compete off dynein from NudE coated beads. Another

interesting finding from this paper was also that NudE could outcompete the full dynactin complex for dynein binding[42]. It would be interesting to replicate these experiments with the yeast components. The implications of p150, but not the full dynactin complex, ability to outcompete NudE for dynein binding is not understood. In the future it would be interesting to see the difference in Ndl1 and Nip100 comigration rates with dynein in a competitive single molecule assay. It would also be interesting to see how the dynein inhibited state could affect this competitive binding interaction. But as it stands it appears that Nip100 or dynactin binding could be the mechanism for release of Ndl1 from dynein.

We also saw that Ndl1 did increase the instances of comigration between Pac1 and dynein (Figure 8). We then hypothesized that high levels of Ndl1 would lead to more comigration of Pac1 with dynein. However, high levels seemed to decrease Pac1-dynein comigration to levels seen in the absence of Ndl1 (Figure 8). This was an anomaly we did not expect. However, this could be simply explained by how the experiment was carried out. To reach the higher levels of Ndl1 in these experiments we had to replace most of the buffer we were diluting the protein into during the incubation phase before addition to the chambers. The buffer that Ndl1 was purified in has a different concentration of salts and detergents than the buffer we used to dilute the other experiments. We also knew that detergents are very important in Ndl1 purification as excluding it does not allow us to purify this protein. Therefore, The change in buffer components could be leading to the Ndl1 to aggregate and thus unable to bind dynein or Pac1. Purifying more concentrated Ndl1 would allow us to assay if this is true. But it would be interesting to see if higher amounts of Ndl1 do lead to more Pac1-dynein interactions. To determine if the increase in Pac1-dynein interaction was due to Ndl1 binding to dynein we used the mutated dynein that does not contain the Ndl1 binding site. This mutant does not comigrate with Ndl1 in single molecule assays (Figure 7d-f). We predicted that this dynein mutant would show the same rates of Pac1-dynein comigration in the presence or absence of

Ndl1. This held true as no statistical difference was found between these two conditions (Figure 8c). To further test this we can employ a mutant Ndl1, mutated in the predicted Ndl1-Pac1 binding motif, and see if we still see an increase in Pac1-dynein interaction. This would indicate if the comigration increase if because a second binding site is simply being added for Pac1 by addition of Ndl1 or if Ndl1 does lead to an increase in Pac1-dynein interaction. This agrees with findings *in vivo* that Ndl1 contributes to the Pac1-dynein interaction but is not necessary for the interaction to occur[14].

In this work we have begun to better understand how the Ndl1-Pac1-dynein interaction occurs. We have also investigated the effect that Ndl1 binding could have on dynein activity and found that Ndl1 does increase the instances of comigration between dynein and Pac1.

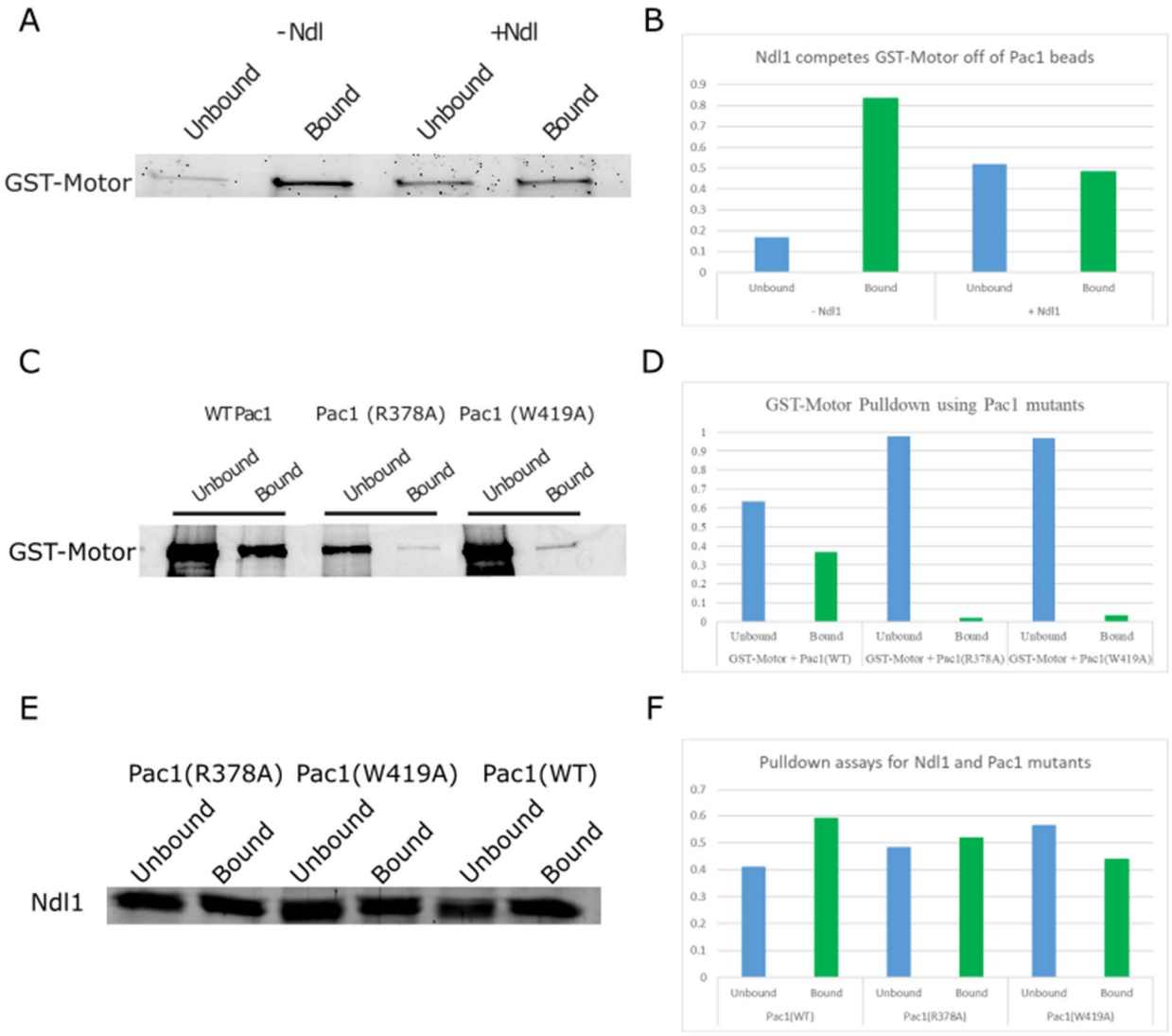


Figure 5. Ndl1 and dynein binding sites on Pac1 overlap

(A-B) Representative image and quantification of competition assay of Ndl1 eluting GST-Motor from Pac1 beads

(C-D) Representative image and quantification of pulldown assays of GST-Motor using Pac1 wildtype and mutant coated beads

(E-F) Representative image and quantification of pulldown assays of Ndl1 using Pac1 wildtype and mutant coated beads

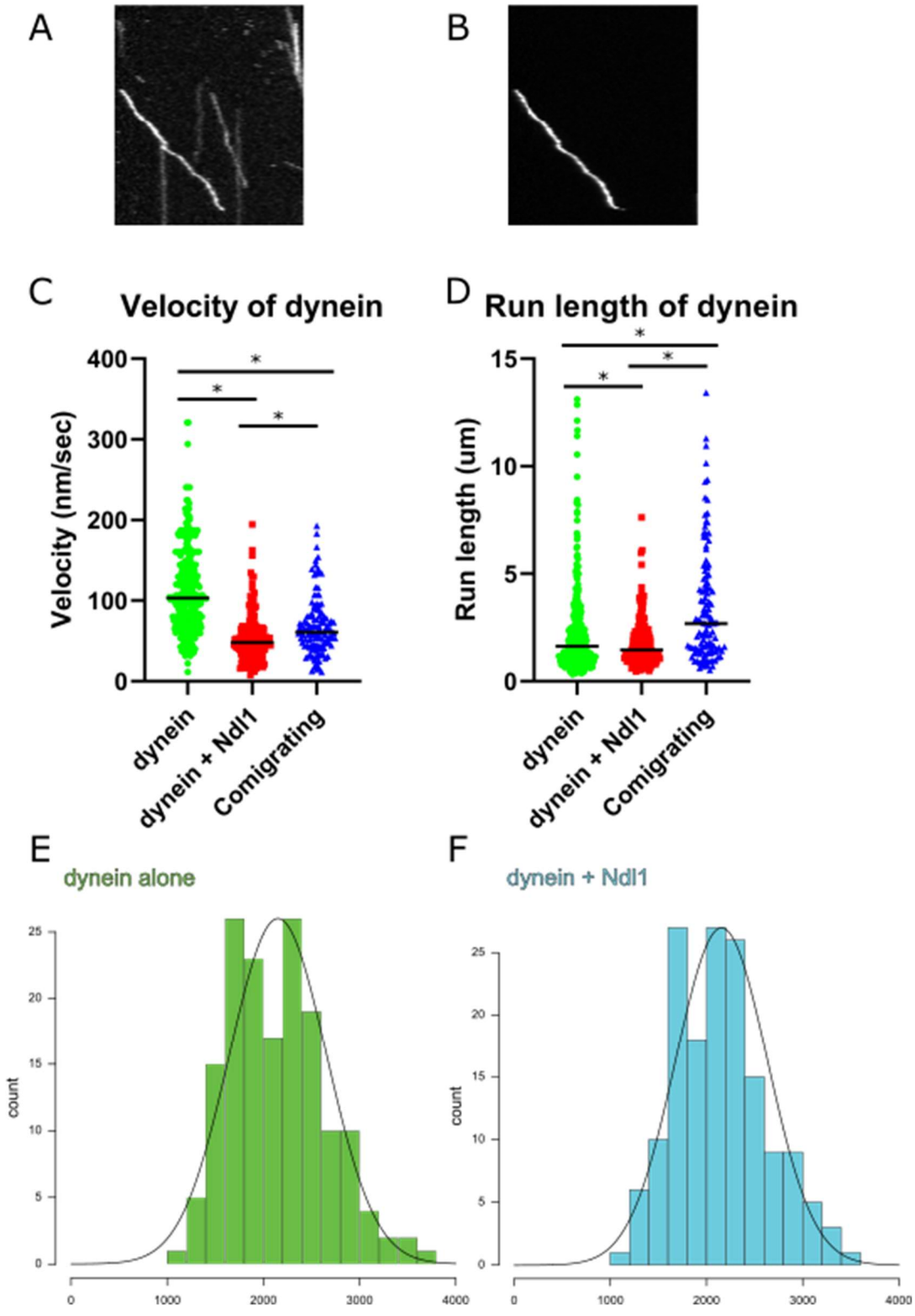
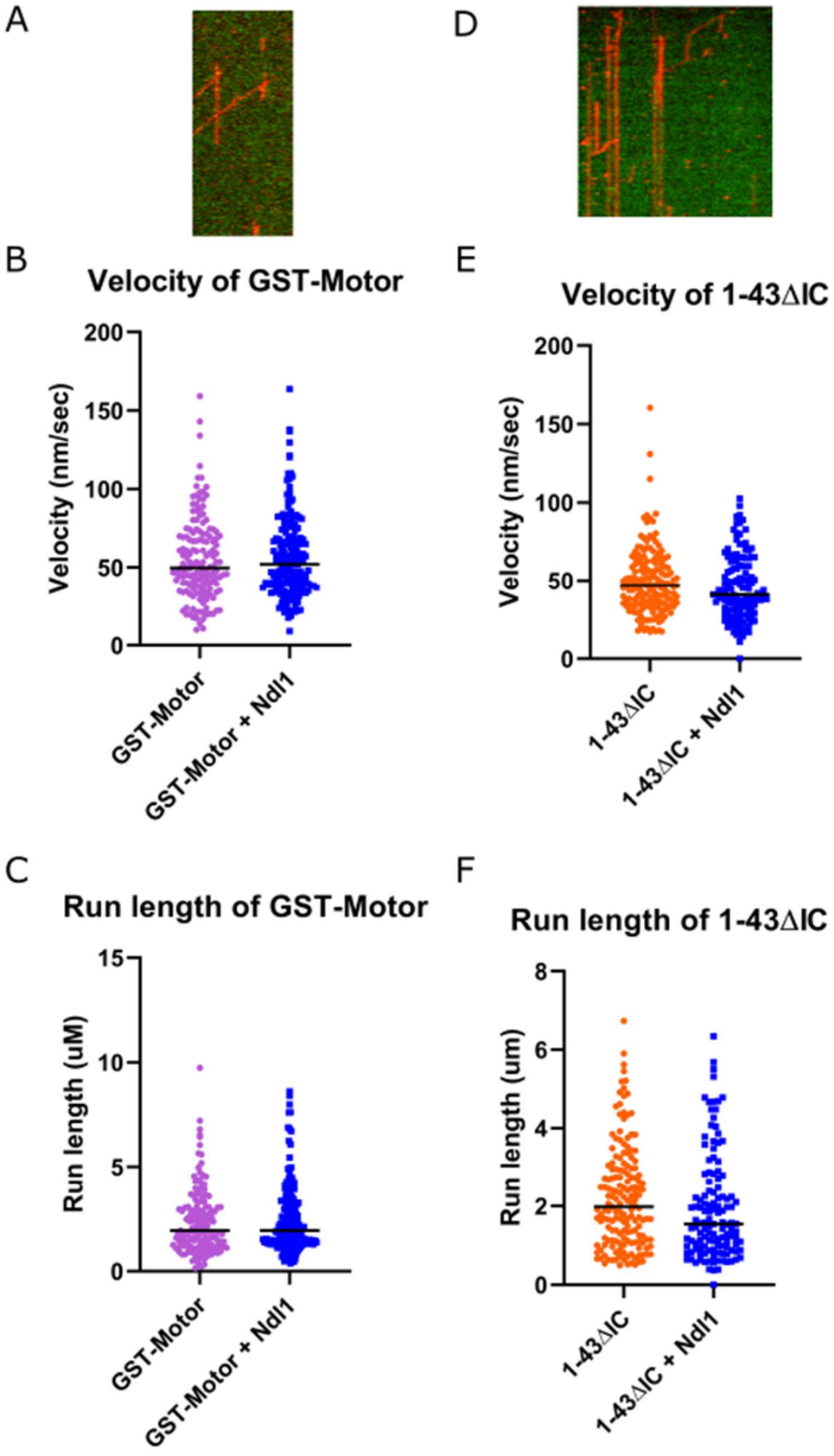


Figure 6. Ndl1 comigrates with dynein and affects its activity

(A) Representative kymograph of dynein motility
(B) Representative Kymograph of Ndl1 motility

(C) Quantitation of Velocity of dynein (WT) in the absence of Ndl1, in the presence of Ndl1 and comigrating with Ndl1 (n=3)
(D) Quantitation of Run length of dynein (WT) in the absence of Ndl1, in the presence of Ndl1 and comigrating with Ndl1 (n=3)
(E) Histogram of intensity of dynein motors (n=1)
(F) Histogram of intensity of dynein motors comigrating with Ndl1 (n=1)


A
D
B
Velocity of GST-Motor
Velocity (nm/sec)
200
150
100
50
0
GST-Motor
GST-Motor + Ndl1
E
Velocity of 1-43ΔIC
Velocity (nm/sec)
200
150
100
50
0
1-43ΔIC
1-43ΔIC + Ndl1
C
Run length of GST-Motor
Run length (uM)
15
10
5
0
GST-Motor
GST-Motor + Ndl1
F
Run length of 1-43ΔIC
Run length (um)
8
6
4
2
0
1-43ΔIC
1-43ΔIC + Ndl1

(continued from above)

Figure 7. Ndl1's binds to the N terminal of the dynein intermediate chain

(A) Representative kymograph of GST-Motor (red) and Ndl1 (green)

(B) Velocity of GST-Motor in the presence or absence of Ndl1 (n=2)

(C) Run length of GST-Motor in the presence or absence of Ndl1 (n=2)

(D) Representative kymograph of 1-43ΔIC (red) and Ndl1 (green)

(E) Velocity of 1-43ΔIC in the presence or absence of Ndl1 (n=2)

(F) Run length of 1-43ΔIC in the presence or absence of Ndl1 (n=2)

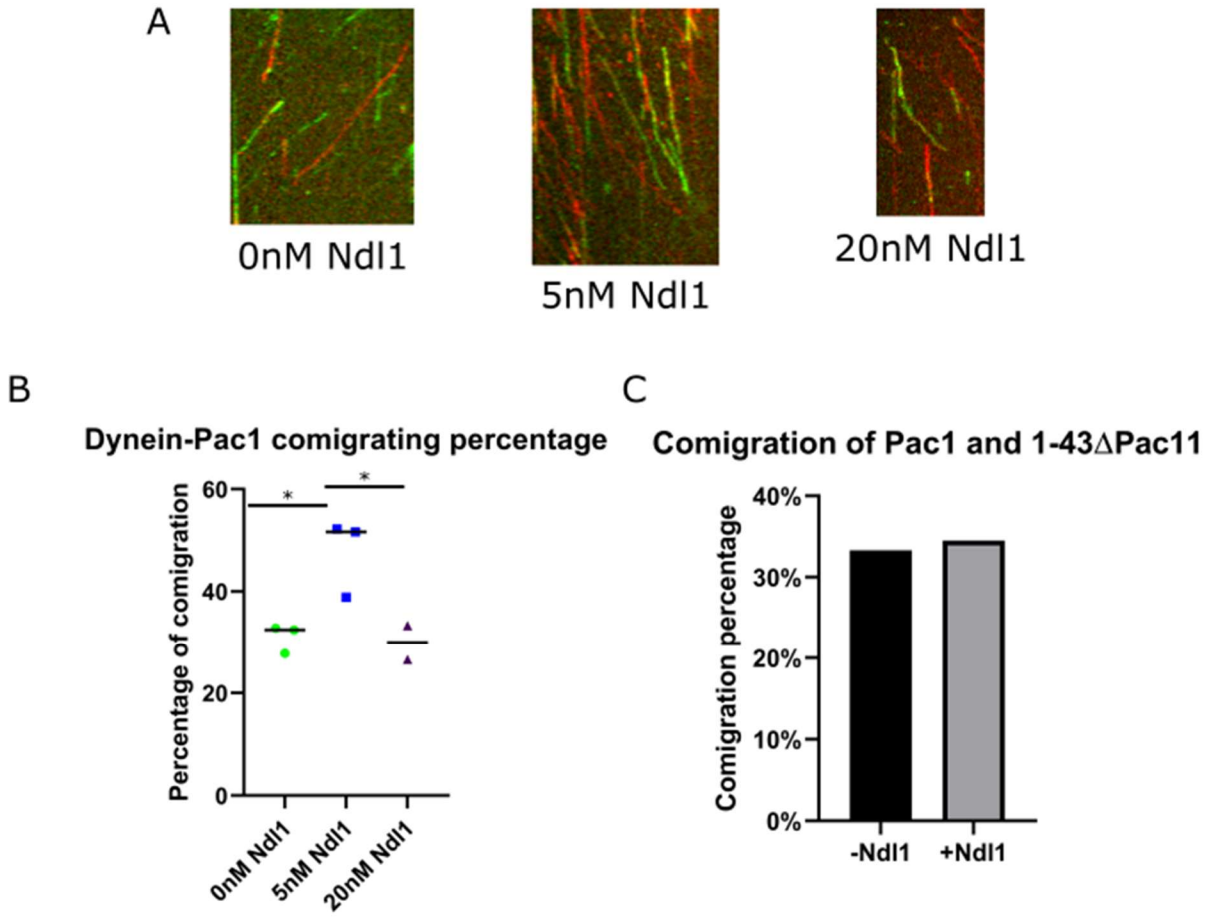


Figure 8. Low levels of Ndl1 increases the amount of Pac1-dynein comigration

(A) Representative kymographs of Pac1 (green) dynein (red) comigration
(B) Quantitation of comigration percentage for 0, 5, and 20nM Ndl1 (n=3)
(C) Quantitation of comigration for Pac1 and 1-43ΔIC in the absence or presence of 5nM Ndl1 (n=1)

Materials & Methods

Strain construction

Strain construction was carried out as previously described[18]. In brief, yeast strains are derived from either W303 or YEF473A[51]. Yeast strains were transformed using the lithium acetate method[52]. Mutant strains were constructed by PCR product-mediated transformation or by mating followed by tetrad dissection[53]. Proper tagging and mutagenesis was confirmed by PCR, and in most cases sequencing. Strains overexpressing the yeast dynein and dynactin complexes or Ndl1 were generated by transforming p8His-ZZ-SNAPf-Dynein, p8His-ZZ-HALO-Dynein, p8His-ZZ-2XTEV-StrepII-HALO-Dynactin, or pNdl1-FLAG-SNAPf-TEV-ZZ-8His (wild-type or mutants) linearized by digestion with ApaI (cuts within the URA3 gene, see figure 1A[18]). The mutant dynein strain (GAL1p-Dyn2:GAL1p-Dyn3:GAL1p-IC[Δ1-43]:GAL1p-8xHis-ZZ-2XTEV-HALO-Dyn1) was a kind gift of Andrew Carter. The Pac1 strain (8xHis-ZZ-TEV-Pac1-g-1xFLAG-gaSNAP) was a kind gift of Samara Reck-Peterson.

Plasmid engineering

A similar approach was used for Ndl1 (GAL1p:Ndl1-FLAG-SNAPf-TEV-ZZ-8His) strain construction as previously described for dynein plasmids[18]. To begin, a PCR product containing GAL1p:Ndl1-FLAG-SNAPf-TEV-ZZ-8His was recombined with a plasmid containing a URA cassette. This was integrated was achieved as described above.

Pac1 mutant plasmids were generated by site specific mutagenesis using PCR. Products were then assembled into plasmids as mentioned above and mutation was confirmed by sequencing.

Protein expression and purification

Expression of truncated yeast dynein (ZZ-TEV-6His-GFP-3HA-GST-dynein$_{MOTOR}$-HALO) was carried out as previously described[23]. Expression and purification of Pac1 (His-ZZ-TEV-Pac1-g-

1xFLAG-gaSNAP) was carried out as previously described[44]. Expression and purification of dynein (WT or mutants) and dynactin complexes as well as Ndl1 (ZZ-TEV-HALO-(or SNAPf)-Dynein, GAL1p-Dyn2:GAL1p-Dyn3:GAL1p-IC[Δ1-43]:GAL1p-8xHis-ZZ-2XTEV-HALO-Dyn1 8xHis-ZZ-2xTEV-StrepII-HALO-Dynactin, Ndl1-FLAG-SNAPf-TEV-ZZ-8His) with all genes under control of the GAL1p promoter was performed as previously described[18]. In brief, yeast cultures were grown in 2% galactose (for the GAL1p-inducible strains), harvested, washed with cold water, and then resuspended in a small volume of water. The resuspended cell pellet was drop frozen into liquid nitrogen and then lysed in a coffee grinder (Hamilton Beach). For most purifications we used the following procedure: after lysis, 0.25 volume of 4X dynein lysis buffer (1X buffer: 30 mM HEPES, pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate, 0.2 mM EGTA) supplemented with 1 mM DTT, 0.1 mM Mg-ATP, 0.5 mM Pefabloc SC (concentrations for 1X buffer) was added, and the lysate was clarified at 22,000 × g for 20 min. For Ndl1 purification we omitted Mg-ATP and for both the dynactin and Ndl1 purification we added 0.005% and 0.1% Triton X-100, respectively, to the clarified lysate and lysis buffer. The supernatant was then bound to IgG sepharose 6 fast flow resin (GE) for 1-1.5 hours at 4°C, which was subsequently washed three times in 5 ml lysis buffer, and twice in TEV buffer (50 mM Tris, pH 8.0, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 0.005% Triton X-100, 10% glycerol, 1 mM DTT, 0.1 mM Mg-ATP, 0.5 mM Pefabloc SC). Again we omitted the Mg-ATP in the TEV buffer for Ndl1 preparation. To fluorescently label the motors for single molecule analyses, the bead-bound protein was incubated with either 6.7 μM HaloTag-TMR (Promega), HaloTag-488(Promega), or SNAP-Surface Alex Fluor 647 (NEB), as appropriate, for 1-hour rotation at 4°C. The resin was then washed six more times in TEV digest buffer, then incubated in TEV buffer supplemented with TEV protease for 1-1.5 hours at 16°C. For Ndl1 digestion was left overnight to increase efficiency. Following TEV digest, the beads were pelleted, and the resulting supernatant was aliquoted, flash frozen in liquid nitrogen, and stored at −80°C. Previous to storage Ndl1 was applied to a size exclusion resin (superose 6;

GE). The gel filtration resin was equilibrated in TEV buffer mentioned earlier supplemented with 1mM DTT and 0.005% Triton X-100 using an AKTA Pure. Peak fractions (determined by UV 260 nm absorbance and SDS-PAGE) were pooled, concentrated, aliquoted, flash frozen, then stored at −80°C.

Num1CC preparation was grown and purified similarly to WT dynein mentioned above. In brief Expression of the Num1CC construct was restricted to two hours and cells were harvested. One liter of yeast cells were used for this protein preparation.

Single molecule assays

Single molecules were carried out as previously described[18]. In brief, flow chambers constructed using slides and plasma cleaned and silanized coverslips attached with double-sided adhesive tape were coated with anti-tubulin antibody (8 µg/ml, YL1/2; Accurate Chemical & Scientific Corporation) then blocked with 1% Pluronic F-127 (Fisher Scientific). Taxol-stabilized microtubules assembled from unlabeled and fluorescently-labeled porcine tubulin (10:1 ratio; Cytoskeleton) were introduced into the chamber. Following a 5-10-minute incubation, the chamber was washed with dynein lysis buffer (see above) supplemented with 20 µM taxol. Subsequently, purified dynein motors diluted in motility buffer (30 mM HEPES pH 7.2, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT, 1 mM Mg-ATP, 0.05% Pluronic F-127, 0.1% Triton X-100, 20 µM taxol, and an oxygen-scavenging system consisting of 1.5% glucose, 1 U/ml glucose oxidase, 125 U/ml catalase) were introduced in the chamber, and imaged.

To image comigrating Pac1-dynein, 200 nM Pac1-SNAPTMR (monomer concentration) and ~50 nM HALO488-Dynein were preincubated on ice for 10-15 minutes prior to a 20-fold dilution into modified motility buffer (30 mM HEPES pH 7.2, 150 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT, 0.1% Triton X-100, 1 mM Mg-ATP) supplemented with 150mM potassium

acetate, 0.05% Pluronic F-127, 20 μM taxol, and an oxygen-scavenging system (as above). These were done in the presence or absence of a final concentration of Ndl1 at 5nM or 20nM as mentioned in figures. The higher yield overexpressed dynein complex was needed for these assays given the low landing rate of dynein in the higher ionic strength buffers. We ensured that comigrating Pac1-SNAP647 spots were not due to bleed-through from the HALOTMR-dynein channel by performing two-color imaging with HALOTMR-dynein alone (no spots were apparent in the far-red channel in these cases).

To image comigrating Ndl1-dynein we followed a similar strategy as above. However, no Pac1 was used and 50nM to 400nM of Ndl1SNAP647 were preincubated with ~50 nM of HALO488/561-Dynein, or GFP-GST-Motor, or 1-43ΔIC-Dyn1-HALO488 for 10-15 minutes then diluted 20-fold into motility buffer mentioned above. To determine if Ndl1 was comigrating with dynein we did two color imagining as mentioned above.

TRIF imaging

TIRFM images were collected using a 1.49 NA 100X TIRF objective on a Nikon Ti-E inverted microscope equipped with a Ti-S-E motorized stage, piezo Z-control (Physik Instrumente), and an iXon X3 DU897 cooled EM-CCD camera (Andor). 488 nm, 561 nm, and 640 nm lasers (Coherent) were used along with a multi-pass quad filter cube set (C-TIRF for 405/488/561/638 nm; Chroma) and emission filters mounted in a filter wheel (525/50 nm, 600/50 nm and 700/75 nm; Chroma). We acquired images at 1, 2, or 3 second intervals for 8-10 min. Velocity and run length values were determined from kymographs generated using the MultipleKymograph plugin for ImageJ (http://www.embl.de/eamnet/html/body_kymograph.html). Those motors that moved for ≥ 3 time points were measured.

Competition and binding assays

To carry out competition assays, Magnetic Flag-beads were equilibrated in TEV buffer mentioned above supplemented with 0.005% Triton X-100, 1 mM DTT, and 0.1 mM Mg-ATP. His-ZZ-TEV-Pac1-g-1xFLAG-gaSNAP was then bound to beads by addition and rotation for 1 hour at 4°C. Beads were then rinsed in 5mL of TEV buffer twice. GST-Motor was then bound to Pac1 coated beads for 1 hour at 4°C. Beads were then rinsed once with 5mL of TEV buffer and Ndl1 was added and incubated for 1 hour at 4°C. Supernatant was collected, boiled and ran on a protein gel as previously described[23]. Eluate were obtained by addition of a Flag peptide for 30 minutes at 4°C. Eluate was also boiled and applied to a protein gel as previously described[23].

For binding assays, beads were prepared and Pac1 was bound as previously mentioned. Then Ndl1 or GST-Motor was then added for 1 hour at 4°C. Supernatant was collected and saved and beads were eluted with Flag peptide as mentioned above. Eluate and supernatant were boiled and ran on a protein gel as mentioned above.

Yeast lysate system

Growth of yeast lysates was carried out as follows. A dense culture of yeast was grown in YPA + 2% raffinose and were pelleted and resuspended in YPA + 2% galactose. Hydroxyurea was then added to a final concentration of 200mM to these cells and grown for 2.5 hours. Cells were then collected and wash first in dH_2O then in dynein lysis buffer mentioned earlier. Cells were pelleted and drop frozen in liquid nitrogen and crushed using a mortar and pestle. Dynein lysis buffer was added and supplemented with 1mM DTT, 0.5 mM Pefabloc SC, and 0.005% Triton X-100 and the lysate was clarified at 22,000 × g for 20 min on a chilled tabletop centrifuge. Supernatant was taken and again clarified at 22,000 × g for 10 min on a chilled tabletop centrifuge. Supernatant was then taken and diluted in a motility buffer mentioned with the

exception of Mg-ATP which was added to a final concentration of 10mM. This was then added to single molecule chambers containing tubulin assembled as mentioned above and imaged.

Statistical analysis

Statistical analysis was carried out at previously described[18]. In brief, statistical tests for run length and velocity were carried out using an unpaired student t test. T-tests were performed using Graphpad Prism. Z scores were calculated using the following formula:

$$Z = \frac{(\hat{p}_1 - \hat{p}_2)}{\hat{p}(1-\hat{p})\left(\frac{1}{n_1} + \frac{1}{n_2}\right)} \quad \text{where:} \quad \hat{p} = \frac{y_1 + y_2}{n_1 + n_2}$$

Z scores were converted to two-tailed P values using an online calculator.

Table 1

Strain number	Genotype	Yeast background
SMY238	Mata Jnm1-3mCherry::HIS3 DYN1-3GFP::TRP1 she1Δ::URA3 ura3-52 lys2-801 leu2-Δ1 his3-Δ200 trp1-Δ63	YEF473
SMY287	MataGAL1p:8xHis-ZZ-TEV-Pac1-g-1XFLAG-gaSNAP::KANR dyn1Δ::cgLEU2 ndl1Δ::HPH prb1Δ his3-11,15 ura3-1 leu2-3,112 ade2-1 trp1-1 pep4Δ::HIS5	W303
SMY1008	Mata *GAL1p:ZZ-TEV-6HIS-GFP-3HA-GST-dyn1$_{331}$-HaloTag::KAN^R prb1Δ pep4Δ::HIS5 his3-11,15 ura3-52 leu2-3,112 ade2-1 trp-1*	W303
SMY2144	Mata num1Δ::HPH 8His-ZZ-2XTEV-StrepII-HALO-Arp10/Nip100/Ldb18/Arp1/Jnm1:URA3 his3-11,15 ura3-1 leu2-3,112 ade2-1 trp-1 pep4Δ::HIS5 prb1Δ	W303
SMY2334	Mata KAN::GAL1p:8xHIS-ZZ-2xTEV-Num1CC(1-325)::TRP Jnm1-3YFP::LEU2 his3-11,15 ura3-1 leu2-3,112 ade2-1 trp-1 pep4Δ::HIS5 prb1Δ	W303
SMY2344	Mata Δnip100::LEU2 ura3-1::GAL1p-Dyn2:GAL1p-Dyn3:GAL1p-Pac11:GAL1p-8xHis-ZZ-2XTEV-HALO-	W303

	Dyn1::URA3 his3-11,15 ura3-1 leu2-3,112 ade2-1 trp-1 pep4Δ::HIS5 prb1Δ	
SMY2472	Mata his3-11,15 pBJ090:[B-PmeI-C]:GAL1p-Dyn2:GAL1p-Dyn3:GAL1p-IC[Δ1-43]:GAL1p-8xHis-ZZ-2XTEV-HALO-Dyn1::URA3 ura3-1 leu2-3,112 ade2-1 trp-1 pep4Δ::HIS5 prb1Δ	W303
SMY2485	Mata his3-11,15 ura3-1 leu2-3,112 ade2-1 trp-1 pep4Δ::HIS5 prb1Δ pBJ090:GAL1p:Ndl1-FLAG-SNAPf-TEV-ZZ-8His::URA3	W303

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