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# Regulation of the Cytoplasmic Dynein Motor

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# Regulation of the Cytoplasmic Dynein Motor

Julia R. Kardon

## ABSTRACT

The large size and complex organization of eukaryotic cells necessitates active, directional transport for efficient relocalization of cellular components and fidelity in their placement. This transport is carried out primarily by motor proteins that move along cytoskeletal filaments. In the past few decades, biochemical and biophysical studies have added greatly to our understanding of the mechansisms of motility and force generation of cytoskeletal motors. Much less is known about how these motors are coupled to their cellular cargos, and how their activity might be regulated appropriately for each function. This is a particularly intriguing question for the microtubule minus-end directed motor cytoplasmic dynein, and is the focus of this dissertation. Only a single cytoplasmic form of dynein has been identified in any organism, and yet this single type of motor performs all cytoplasmic microtubule minus end-directed transport. In vivo, dynein forms a large complex with several other proteins and protein complexes that are required for dynein-mediated transport; these factors may help to adapt and regulate dynein for its cellular functions.

Chapter 1 provides a review of the current literature regarding the four major dynein adaptors, dynactin, Lis1-NudE/EL, Bicaudal-D, and RZZ-Spindly, and a discussion of how they might work together to facilitate dynein function in the cell. In the study presented in Chapter 2, we focused on the activity of dynactin, a ubiquitously

required dynein adaptor. Previous indirect observations of dynactin had suggested that it could increase dynein processevity by tethering dynein to the microtubule through its own microtubule binding domains. Using recombinant dynactin and dynein from *S. cerevisiae* for direct observation in a single molecule assay, we found that dynactin increases the run length of single dynein motors, confirming that dynactin is a processivity factor for dynein. We find that this enhancement of dynein processivity does not require the microtubule binding domains of dynactin, but instead require a projecting coiled-coil stalk. The dynactin coiled-coil stalk, but not its microtubule binding domains, is also required for dynein function in living cells.

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# Chapter 1

Adaptors for cytoplasmic dynein function in the cell

**ABSTRACT:**

Eukaryotic cells transport an hugely varied set of intracellular cargos, using motor proteins that move along cytoskeletal polymers. While two of the three types of cytoskeletal motor proteins, myosins (actin motors) and kinesin (plus end-directed microtubule motors), have evolved into highly diverse classes to carry out many different types of transport, only a single type of dynein (a minus end-directed microtubule motor) exists. How is the single cytoplasmic dynein motor coupled to its wide array of cargos, and regulated with spatial and temporal specificity? The answer may lie in the multiple adaptor proteins (most notably dynactin, Lis1-NudEL, Bicaudal-D, and RZZ-Spindly) that function with cytoplasmic dynein. This review surveys this set of dynein adaptor proteins and discusses what roles they may play in cargo binding and motor regulation. The baroque machinery coupling the single cytoplasmic dynein to its functions also suggests an opposing question: what is the benefit of maintaining a single type of cytoplasmic dynein, or what is the cost of its diversification? The general importance of dynein's adaptors suggests that animal cells may organize microtubule-based transport around a core centripetally directed transport complex.

**INTRODUCTION:**

To perform most cytoplasmic transport, and to organize and remodel their cytoskeletal structures, eukaryotic cells depend on motor proteins that walk along cytoskeletal tracks. There are three classes of cytoskeletal motor proteins: myosins,

which move along actin filaments, and kinesins and dyneins, which move along microtubules. Most kinesins (except the nuclear-localized, mitotic class 14 kinesins) move toward the fast polymerizing microtubule plus end, which, in most cells, generally extend toward the cell periphery. In contrast, all known dyneins move towards the microtubule minus ends, which are usually collected into a microtubule organizing center (MTOC) near the cell nucleus.

Multiple species of myosins and kinesins exist to help diversify them for their different functions; the highly conserved myosin and kinesin ATPase cores are attached to a wide variety of "tail" domains, which mediate cargo interactions both directly and by recruiting specific light chains. As many as 19 dynein species have been identified in some organisms, but the vast majority of them are immobilized within the axonemal structures of cilia and flagella, where they help to organize the structure of bundled microtubules, and to drive their beating by coordinated cycles of microtubule binding. Another species of dynein, called IFT dynein, dynein 1B or dynein2, functions exclusively in intraflagellar transport (IFT), moving cargos along the axoneme in cilia and flagella. All minus-end-directed transport within the cytoplasm, therefore, is carried out by a single cytoplasmic dynein (with the exception of higher plants, which have dispensed with dynein altogether (Vale, 2003)). Given the relative ease of gene duplication, this is somewhat surprising and suggests an evolutionary advantage in having a single motor for minus-end-directed transport, for reasons that are still unclear.

How is the single cytoplasmic dynein motor adapted to the wide array of microtubule minus end-direted transport required in eukaryotic cells? Although many aspects of this question remain unanswered, the work of the last few decades has yielded

a rough framework for this adaptation. The dynein motor itself seems to have intrinsically variable enzymatic function, that may be responsive to the demands of a particular transport process, and exists within a large assembly of smaller, noncatalytic subunits that provide specific points of attachment and regulation for some dynein cargos. Extrinsic to the dynein complex, dynein partners with several other proteins and complexes that are crucial to its function. Dynactin, Lis1-NudE/EL, Bicaudal-D, and the Rod-ZW10-Zwilch (RZZ) complex, the chief examples of these adaptors, were each initially discovered in association with one specific dynein function (vesicle transport for dynactin, nuclear migration for Lis1, mRNA transport for Bicaudal-D, and kinetochore function for the RZZ complex); however, further study has revealed them to be more broadly important for multiple dynein function, and in many cases their inhibition or depletion is phenotypically similar to a complete loss of dynein function. The requirement for multiple dynein adaptors, each crucial for multiple and overlapping processes, is intriguing, and the focus of this review will be on how these adaptors help to couple dynein to its cellular functions.

## THE CYTOPLASMIC DYNEIN COMPLEX

The cytoplasmic dynein heavy chain is a member of the large AAA+ family of enzymes (Neuwald et al., 1999), which mostly function as chaperones or protein unfoldases. Most AAA+ proteins self-assemble into and function as hexameric rings; dynein is somewhat unusual in that its six AAA modules are linked in a single polypeptide and are not identical. The first four AAA domains (AAA1-4) from the N-

terminus all are capable of binding ATP, while the C-terminal AAA5-6 are more divergent overall and have lost essential P-loop residues that are necessary for binding ATP (Neuwald et al., 1999). ATP hydrolysis in AAA1 and to a lesser degree AAA3 is crucial for motility, while AAA2 and AAA4 mutants exhibit more modest defects and may act to regulate the ATPase cycles of AAA1 and AAA3 and its transduction into movement along the microtubule (Cho et al., 2008; Kon et al., 2004; Reck-Peterson and Vale, 2004). The microtubule binding domain of dynein is a small, globular domain at the tip of an antiparallel coiled coil that emerges from the ring after AAA4 (Burgess et al., 2003; Gee et al., 1997). Recent structural and biochemical studies suggest that the ATPase state of the ring is communicated to the microtubule binding domain by a shift in the registry of the coiled coil, coupling ATPase cycling to rounds of microtubule binding and release (Carter et al., 2008; Kon et al., 2009). The mechanical element of dynein is believed to be the domain that links the C-terminal AAA 'head' domain of dynein with the N-terminal 'tail'; this linker sits on top of the AAA ring, and shifts its position during the ATPase cycle (Roberts et al., 2009).

The tail of the cytoplasmic dynein heavy chain is involved in its homodimerization and in the recruitment of tightly associated light chains. The cytoplasmic dynein holoenzyme contains up to five different light chains (possibly fewer in simple eukaryotes), which assemble as dimers on the tail of the dynein heavy chain dimer, and contribute more than half the mass of the 1.2 megadalton dynein complex. The dynein intermediate chain and light intermediate chain, the largest of the noncatalytic subunits, bind directly to the dynein heavy chain, while the smaller light chains, light chain 8 (LC), light chain 7/roadblock(LC7), and Tctex-1, assemble on the

intermediate chain. As discussed below, the associated chains are not necessary for cytoplasmic dynein motility in vitro, although they could possibly regulate dynein motility in the cell. Most current evidence suggests that the associated chains function as hubs for protein-protein interactions. Some specific links between dynein light chains and cargo proteins have been identified (Farkasovsky and Kuntzel, 2001; Purohit et al., 1999; Tai et al., 1999). In addition, many light chains are encoded by multiple genes. Different assemblies of light chain isoforms upon the dynein heavy chain may create functionally distinct dynein complexes, although this has not yet been demonstrated experimentally. As will be discussed in more depth later, the light chains also link dynein with the adaptor proteins, which are main focus of this review. For example, the intermediate chain binds the p150 subunit of dynactin, LC8 binds the Bicaudal-D partner Egalitarian, and both the intermediate chain and LC8 interact with NudE.

Dynein is a processive motor, capable of micron-scale movements along the microtubule without dissociation (Mallik et al., 2004; Wang et al., 1995). Dimerization of the two dynein motor domains is required for processive motion, although the light chains and the native dimerization domain are not required (Reck-Peterson et al., 2006) High spatial precision tracking studies of single dynein motors suggests that dynein moves by alternating steps of the dynein heads past each other, conceptually similar to the hand-over-hand model for processive kinesin motility (Reck-Peterson et al., 2006). This model implies coordination between the two heads, such that one remains bound while the other moves forward; optical trap studies of dynein force production suggest that this coordination is carried out by strain transmitted through the linkage between the two heads (reviewed in(Gennerich and Vale, 2009)).

The stepping behavior of dynein along the microtubule is more variable than that of kinesin. Dynein predominantly takes 8 nm-long steps, but occasionally takes steps of up to 32 nm, and backwards steps towards the plus end of the microtubule, while kinesin steps are invariably 8 nm long and plus end-directed (Gennerich et al., 2007; Mallik et al., 2004; Reck-Peterson et al., 2006). Dynein also often takes steps sideways to an adjacent protofilament (Reck-Peterson et al., 2006), which rarely occurs for kinesin. Although it is difficult to observe dynein motility in vivo directly, indirect observations have also indicated variability in stepping behaviour during physiological dynein transport (Nan et al., 2008), but whether this variability is exploited or regulated in vivo is unknown.

Optical trap experiments have indicated that the stepping behavior of dynein is responsive to load. Increasing the load experienced by dynein increases the proportion of smaller steps and backwards steps (Gennerich et al., 2007; Mallik et al., 2004). Higher loads also induce periods of oscillatory forward-backward stepping with no net movement, a phenomenon that is so far unique in observations of cytoskeletal motors (Gennerich et al., 2007). As dynein can respond in vitro to different loads by altering its stepping behaviour, it seems possible that it could respond autonomously or under the control of a regulatory factor to the demands of a particular in vivo transport function. It is also important to note that while observations of single dynein motors will continue to be essential for understanding the mechanism of dynein motility, most dynein cargos in cells probably are powered by a few dynein motors at a time. Theoretical and in vitro studies of the behavior of multiple dyneins working on a single cargo have suggested

several possible models for their cooperation and the properties of multi-motor motility (Ross et al., 2008).

## DYNACTIN

Dynactin was identified as an activator of dynein-mediated, minus end-directed vesicle transport (Gill et al., 1991; Schroer and Sheetz, 1991), and was named accordingly (*dyn*ein *act*ivator). The entire complex is nearly as large as the cytoplasmic dynein complex itself, with a molecular mass of over 1 MD. Dynactin has been found to be essential for nearly every cellular function of cytoplasmic dynein (Karki and Holzbaur, 1999; Schroer, 2004), such that overexpression of the p50 subunit of dynactin is often used as a tool to disrupt dynein function (Burkhardt et al., 1997; Echeverri et al., 1996). Dynactin seems to be important both for providing and regulating attachment of dynein to its cargos, as well as perhaps for regulation of dynein motor function itself. As might be expected for such a large and baroquely structured complex, dynactin carries out these activites through a wide array of interactions and several mechanisms, many of which remain little understood.

### Molecular structure and interactions

The complex is organized around a short (~40 nm) filament of the nonconventional actin Arp1, with subcomplexes at either end. The subcomplex at the barbed end of the Arp1 filament is composed of a dimer of the largest dynactin subunit,

p150 (also p150$^{Glued}$, from the previously isolated *Drosophila* allele), a tetramer of p50 (also termed 'dynamitin,' as its overexpression dissociates the shoulder from the rest of the dynactin complex), the smallest dynactin subunit, p24, and a conventional actin capping protein heterodimer, although capping protein may not incorporate into the dynactin complex in all organisms (Eckley et al., 1999; Gill et al., 1991; Moore et al., 2008; Schafer et al., 1994). The most N-terminal coiled coil (CC1) of p150 most likely forms the 24 nm projection from the Arp1 filament that is visible by EM; this projection is called the sidearm, and correspondingly the barbed end subcomplex the shoulder, of dynactin. p150 contains N-terminal MT binding domains that probably form the globular domain observed at the distal end of the sidearm (Quintyne et al., 1999; Schafer et al., 1994). The only known contacts with dynein (to the dynein intermediate chain) are also contained within the p150 subunit (Deacon et al., 2003; King et al., 2003; Vaughan and Vallee, 1995). The pointed end subcomplex contains another actin related protein, Arp11; p62, which may mediate dynactin interaction with cortical actin structures; and two more subunits, p25 and p27(Eckley et al., 1999; Garces et al., 1999; Lee et al., 2001; Schafer et al., 1994).

**Dynein-dynactin cellular function**

Dynactin was originally isolated based on its ability to activate dynein-mediated vesicle transport in vivo, and early studies of its subcellular function identified many organelles whose transport is disrupted upon p50 overexpression. In order for dynein to carry out productive tranport, dynein must be targeted to the appropriate site, bound to its

cargo, and its activity regulated as appropriate for that cargo. Dynactin contributes at each of the steps (although perhaps not all steps for all cargos).

The first step in organelle transport, targeting, has been best studied in fungi, in the context of nuclear migration. In *Saccharomyces cerevisiae*, dynein bound to the bud cortex translocates astral microtubules along the cortex, thereby pulling the nucleus into the bud neck and ensuring that after cell division, both the mother and daughter cell receive a nucleus(Adames and Cooper, 2000; Lee et al., 2005). In this process, the cell cortex is conceptually similar as a dynein cargo to a membrane-bound organelle. Dynein appears first at the plus ends of astral microtubules, and then in faint punctae at the bud cortex, suggesting that dynein is transported to the cortex on the microtubule plus end and then transferred to its binding partner at the cortex, Num1(Farkasovsky and Kuntzel, 2001; Lee et al., 2005). Dynactin seems to be important for this transfer process; deletion or mutation of many dynactin subunits causes dynein to accumulate at the plus end of microtubules and abolishes cortical punctae of dynein, prevent dynein-mediated nuclear movements (Kardon et al., 2009; Lee et al., 2003; Sheeman et al., 2003).

In *A. nidulans*, nuclear migration occurs after germination, when the spore must distribute its multiple nuclei along the hyphae. Screens for factors required for this process identified the *nud* genes, many of which encoded dynein and dynactin subunits (Xiang et al., 1994; Xiang et al., 1999). Nuclear migration in filamentous fungi may proceed similarly as in *S. cerevisiae*. GFP-tagged NUDA (dynein heavy chain) and NUDM (dynactin p150) localize to the plus ends of microtubules in growing hyphae, and a homologue of Num1, ApsA, is required for proper nuclear positioning, but dynein and dynactin have not been observed at the cell cortex. Dynein and dynactin are mutually

required for plus end localization (Fischer and Timberlake, 1995; Xiang et al., 2000; Zhang et al., 2003); whether dynactin also functions with dynein after plus end localization is unknown.

Dynactin also mediates plus end targeting of dynein in metazoan cells. p150 can bind via its CAP-Gly domain to a C-terminal acidic motif found in α-tubulin, EB1, and CLIP-170, and this binding is strengthened by a secondary CAP-Gly contact with EB1 and CLIP-170 (Hayashi et al., 2007; Hayashi et al., 2005; Honnappa et al., 2006; Lansbergen et al., 2004; Valetti et al., 1999; Weisbrich et al., 2007). The complex network of interactions between these proteins has made it difficult to determine their exact hierarchy and order in plus end binding, and it is likely that these interactions are dynamically remodeled in response to the polymerization state of the plus end and cargo binding by dynein-dynactin. Dynein is difficult to observe at the plus ends of microtubules in metazoan cells, but at reduced temperature colocalizes there with p150 (Vaughan et al., 1999). Although this has not been directly tested, this localization is most likely mediated by dynactin. This plus end localization seems to help dynein initiate cargo transport; observations of Golgi vesicles in live COS-7 cells revealed an interaction with a GFP-p150-labeled microtubule plus end immediately prior to the initiation of minus end directed movement (Vaughan et al., 2002). A mutation in the p150 CAP-Gly domain (G59S) that disrupts microtubule binding is linked to human motor neuron disease, and disrupts retrograde transport in mouse neurons (Lai et al., 2007; Puls et al., 2003). This disruption could be due to loss of dynein plus end targeting, although the p150 CAP-Gly domain has also been suggested to regulate dynein motility (discussed below). Phosphorylation of the p150 CAP-Gly domain negatively regulates

p150 plus end binding, which could be a means of downregulating dynein plus end targeting, or of releasing dynein-dynactin from the plus end after cargo loading. Although these data fit an appealing model for how dynein is targeted to its cargos, the functional relevance of plus end targeting by dynactin has not been confirmed, and is contradicted by some studies. p150 constructs lacking CAP-Gly domains, one of which occurs as a natural isoform in neurons, were capable of supporting normal Golgi, lysosome, and peroxisome transport in HeLa and *Drosophila* S2 cells, suggesting that plus end loading of dynein may not be required for cargo capture, or that alternate mechanisms of plus end interaction, perhaps mediated by LIS1 (discussed below) may be sufficient (Dixit et al., 2008; Kim et al., 2007; Tokito et al., 1996).

Once at the plus end (or when encountering a cargo membrane from the cytosol), dynactin also helps dynein interact with the surface of the membrane to form a productive and specific link to the cargo. One dynactin-membrane link is between the spectrin network that coats the cytosolic surface of many cellular membranes, and the Arp1 filament. Overexpressed Arp1 and the βIII isoform of spectrin colocalize at Golgi membranes, and Arp1 binds βIII spectrin at both the conserved actin-binding motif, as well as the region containing the phospholipid-binding PH domain (Holleran et al., 2001). Squid axoplasm can support the minus end directed motility of synthetic liposomes containing a mixture of acidic phospholipids. This motility could be disrupted with high concentrations of the Arp1-binding domain of spectrin, and could be reconstituted, albeit less robustly, using partially purified dynein-dynactin and βIII spectrin (Muresan et al., 2001). This indicates that the Arp1 filament-spectrin interaction forms a core link in vesicle transport that could be generalized. The spectrin family is

large, and different isoforms are associated with various membranes in the cell, and even the βIII isoform occurs on several membrane compartments other than the Golgi (De Matteis and Morrow, 2000). Through this diversity, the Arp1-spectrin link theoretically could be modified to facilitate dynein transport of many cargos, although whether this is the case has yet to be demonstrated. The dynactin subunits that cap the Arp1 filament may help to regulate its interactions with membranes. In Neurospora, mutants in all components of the pointed end complex, as well as truncation of the C-terminus of p150, were shown to enhance dynein-dynactin binding to isolated membranes. Addition of the C-terminal domain of p150 in trans could also release dynactin from membranes, suggesting that this domain might mask Arp1 membrane binding sites unless this autoinihibition was relieved (Kumar et al., 2001; Lee et al., 2001).

The C-terminal domain of p150 itself has been shown to mediate specific interactions with some cargos. One such interaction helps to facilitate ER-to-Golgi transport. Specialized exit sites from the ER bud off vesicles, coated with CopII, that are subsequently transported toward the Golgi by dynein. The trajectories of p150, carried at the tips of growing microtubules, often tracks through these ER exit sites. The p150 C-terminus binds specifically to the CopII subunit Sec23, and overexpression of the C-terminal domain alone strongly inhibits secretory protein delivery to the Golgi. Interestingly, the p150-C-Sec23 interaction is quite weak compared to the strong effect caused by its disruption, and the CopII coat is known to rapidly dissasemble from vesicles after they bud; this suggests that the p150-C-Sec23 interaction functions to activate dynein-cargo binding, which is then maintained by some more stable link (Watson et al., 2005). A similar, transient set of interactions was found to govern dynein

transport of late endosomes. The Rab7 effector RILP recruits p150 to late endosomes and a Rab7 complex via the p150 C-terminus. RILP also enhances Rab7 interaction with another Rab7 effector, ORP1L. This set of interactions is crucial to dynein-driven endosome transport, but not sufficient; βIII spectrin RNAi caused the dispersal of RILP-p150-labeled late endosomes, indicating disruption of dynein-mediated transport. The authors hypothesized that the RILP-p150 interaction could recruit dynein-dynactin to late endosomes, and that subsequent interaction with spectrin is required for transport, although a direct link between these two dynein cargo adaptors and their temporal relationship was not established (Johansson et al., 2007). Combined with previous work that established the Arp1-spectrin link and its inhibition by the p150 C-terminal domain, these studies provide evidence for an elegant model for initiation of dynein-driven vesicle transport: specific interaction of a cargo protein with the p150 C-terminus could relieve its inhibition of Arp1 binding to spectrin, allowing a tight link to form between dynein-dynactin and the membrane only at specified cargos.

The p50 subunit has also been demonstrated to assist in binding to several dynein cargos. Both p50 and Golgi-associated Rab6 bind another dynein adaptor, Bicaudal-D, by the Bicaudal-D C-terminus, suggesting possible models for dynein recruitment to cargo (discussed further in Bicaudal-D section). The p50 N-terminus is not important for dynactin assembly, but it is highly conserved, and its overexpression disrupts secretory organelle distribution, suggesting that it may mediate several cargo interactions (Valetti et al., 1999). One important interaction of the p50 N-terminus is required not for dynein organelle transport, but for recruitment of dynein to the kinetochore (discussed further in RZZ section).

Finally, there is some evidence that the p62 subunit of dynactin could link dynein to actin-rich structures. p62 exogenously expressed in COS-7 cells is recruited to focal adhesions, dependent on its N-terminal LIM/RING domain (Garces et al., 1999). *Neurospora crassa* cells mutant for a homologue of p62, *ro-2*, exhibit a marked increase in cortical actin patches(Vierula and Mais, 1997). However, the functional significance of these phenotypes to dynein function remains unclear.

**Dynactin modulation of dynein motility**

The initial discovery of the dynactin complex as an activator of dynein transport of vesicles suggested that dynactin might serve to activate dynein motility itself. Although the importance of dynactin for proper dynein-cargo interaction has subsequently been established, and most likely accounts for some of the effects observed in these initial experiments, there is substantial evidence that dynactin may also serve to modulate dynein motor activity as well. The discovery of a CAP-Gly microtubule binding domain at the N-terminus of the p150 subunit let to the hypothesis that dynactin could act as a tether for dynein to the microtubule; if dynein itself unbound from the microtubule during transport, dynactin could keep dynein associated with the microtubule so that it could rebind, thereby increasing the processivity of dynein movement in the cell (Waterman-Storer et al., 1995).

Evidence for such an activity came first from bead motility assays, but subsequent experiments have demonstrated that while dynactin can act as a processivity factor, it acts through some mechanism other than tethering. Dynein-adsorbed polystyrene beads

(estimated to contain no more than one dynein motor each) moved further along a microtubule before dissociating when incubated with dynactin than when assayed alone. This increase in run length was abrogated by incubation with an antibody to the N-terminus of p150, suggesting that it was mediated by microtubule binding by p150 (King and Schroer, 2000). A later study identified a second microtubule binding domain in the N-terminal globular region of p150, termed the 'basic domain,' as it is defined by an enrichment of basic residues that likely interact with the strongly acidic surface of the microtubule. Protein constructs containing this domain alone supported 1-D diffusion of beads along the microtubule, in contrast to static binding induced by isolated p150 CAP-Gly domain. The basic domain, when adsorbed to dynein beads, could also increase the run length of these beads, lending further support to the model that dynactin acts as a processivity factor by tethering dynein to the microtubule, in this case by a diffusive interaction (Culver-Hanlon et al., 2006). However, an in vivo study of dynein motility using high precision tracking of dynein cargos found that microtubule binding domain-truncated p150 could support normal dynein cargo movement in *Drosophila* S2 cells, with similar frequency, velocity and run lengths as in wildtype cells (Kim et al., 2007). This finding suggested that either the N-terminal microtubule binding domains of p150 are not required for dynactin to enhance dynein processivity, or that the in vitro activity of dynactin as a processivity factor is not relevant for physiological transport of dynein cargo. A subsequent study of dynein and dynactin complexes isolated from budding yeast demonstrated by direct observation of single, fluorescently labeled motor complexes that dynactin can act as a dynein processivity factor. The authors used homologous recombination to generate dynactin complexes containing N-terminally

truncated p150, and found that dynactin lacking p150's microtubule binding domains retained wildtype activity to increase dynein run length (Kardon et al., 2009). p150 CC1 truncation caused a strong decrease in dynein-dynactin processivity, and truncation of this domain blocked dynactin activity in living yeast, as overexpression of dynactin CC1 in metazoan cells has been demonstrated to do (Kardon et al., 2009), reviewed in(Schroer, 2004). This study indicated that dynactin does not require a microtubule tether to act as a processivity factor, and indicated that some aspect of this regulation of motor function could be relevent for in vivo function, as the domains required for processivity enhancement were also required in vivo. Another group, looking in detail at the exaggerated dynein-driven oscillation of the yeast nucleus that occur in cells lacking a component important for pre-anaphase nuclear positioning, found that truncation or mutation of the p150 CAP-Gly domain caused a decrease in the persistence of thse oscillations (Moore et al., 2009). The authors hypothesized that the CAP-Gly domain, while dispensible for some modes of dynein motility, was important to maintain its association with the microtubule under high load, such as when pulling the yeast nucleus through the constricted bud neck, although they could not determine in this complex, in vivo system whether the reduced oscillations they observed resulted from a true defect in dynein motility. Further detailed mechanistic studies will be important to determine exactly how dynactin contributes to dynein motility and force generation.

In a study of the single molecule motility of dynein-dynactin complexes containing GFP-p50, purified from transgenic mouse brain, Ross et al. observed bidirectional motility of dynein-dynactin, with runs sometimes exceeding 1 μm toward the plus end of the microtubule. This was very surprising, as dynein had previously been

observed to move only toward the minus end (except for occasional nm-scale steps toward the plus end), and Ross et al. hypothesized that this backwards movement was due to the activity of dynactin. As the fluorescent label was located on the dynactin complex, however, in this system it was not possible to observe dynein movement on its own. In addition, dynein movement has a significant diffusive component, and it was not possible to exclude the possibility that the plus end-directed movements were purely diffusive. In addition, a subsequent study (discussed above) of yeast dyenin-dynactin reported only minus end-directed movements above the nm scale(Kardon et al., 2009). Further work is required to determine the source of this bidirectional movement.

A role for dynactin in regulating bidirectional transport has been proposed, by which dynactin contacts with kinesin allow it to coordinately regulate which motor is active. A few physical interactions of dynactin with the kinesins Eg5(Blangy et al., 1997) and kinesin II (Deacon et al., 2003) have been demonstrated, and dynactin could also increase the processivity of kinesin II-adsorbed beads (Berezuk and Schroer, 2007). For many bidirectional cargos, interference with either the plus- or the minus-end directed motor, including by dynactin disruption, completely disrupts motility, strongly suggesting a tight, interdependent link between dynein and kinesins, which dynactin could conceivably provide. Aside from these observations, however, there is little evidence for how dynactin might help to form and regulate a bidirectional transport complex.

**LIS1-NUDE/EL**

Lis1 was first identified as a gene tightly linked to the human neurodevelopmental disease lissencephaly(Dobyns et al., 1993). Sporadic mutations of Lis1 cause severe defects in the structure of the brain cortex, a result of disrupted neuronal mitoses and migration. Studies of nuclear migration in fungi identified fungal homologues of Lis1, and indicated its molecular role as a dynein cofactor (Geiser et al., 1997; Xiang et al., 1994; Xiang et al., 1995). These studies also led to the identification of an important partner to Lis1, NudE, for which two closely related homologues, NudE and NudEL, were later identified in metazoan organisms (Efimov and Morris, 2000; Feng et al., 2000; Niethammer et al., 2000; Sasaki et al., 2000). Since the initial discovery of the link between dynein and Lis1 in nuclear movement, Lis1-NudE/EL has been demostrated to be essential to a wide array of dynein functions, suggesting that it may be as ubiquitous an adaptor for dynein as dynactin. Lis1-NudE/EL affects dynein localization to the microtubule plus end and to many sites of activity, but very few direct cargo interactions are known. In addition, there is strong evidence that Lis1-NudE/EL regulates dynein enzymatic activity.

**Molecular structure and interactions**

Lis1 is composed of an N-terminal coiled coil linked to seven WD-40 repeats at the C-terminus. The N-terminal coiled coil mediates dimerization of Lis1, while the WD40 domain binds directly to the AAA1 domain of dynein. Yeast two-hybrid data also suggests that the Lis1 WD-40 domain interacts with the dynein intermediate chain and the dynactin subunit p50 (Tai et al., 2002). Lis1 acts in a dynein-independent role as a

regulatory subunit for the platelet-activating factor acetylhydrolase (PAF-AH) (Hattori et al., 1994), and binds to PAF-AH through its C-terminus, overlapping the dynein binding site (Tarricone et al., 2004). Lis1 interaction with NudE/EL is mutually exclusive with PAF-AH binding, suggesting that NudE/EL binding may help direct Lis1 to its dynein-related functions (Tarricone et al., 2004). Lis1 binds the N-terminus of NudE/EL, and the NudE/EL C-terminus itself has been reported to bind dynein independently, both through the dynein AAA1 domain and through the light chain LC8 and the intermediate chain (Efimov and Morris, 2000; Sasaki et al., 2000; Stehman et al., 2007). Depletion of NudE/EL reduces Lis1-dynein interactions (Shu et al., 2004; Yan et al., 2003). NudE/EL phosphorylation in metazoans strengthens its interaction with Lis1, and may favor binding of the Lis1/Nde complex with dynein (Hebbar et al., 2008; Niethammer et al., 2000; Sasaki et al., 2000; Yan et al., 2003). It would be nearly impossible for the myriad interactions between Lis1, NudE/EL, and dynein to exist simultanously, so it is likely that many of them are regulated, or NudE/EL isoform specific.

**Lis1-NudE/EL cellular functions**

Studies in organisms across the phylogenetic tree have demonstrated that Lis1-NudE/EL regulation of the dynein motor is central to dynein-mediated positioning of the nucleus and centrosome. In higher eukaryotes, Lis1-NudE/EL is also important for dynein kinetochore function, and has a less-characterized but essential role in dynein-driven transport of organelles (Liang et al., 2004; Smith et al., 2000) and mRNA particles (Swan et al., 1999).

In the filamentous fungi *Aspergillus nidulans*, as well as the budding yeast *Saccharomyces cerevisiae*, Lis1 and NudE are required for dynein-mediated nuclear positioning, where they modulate the association of dynein with the plus end of microtubules and its subsequent nuclear translocation. Like dynein, NUDF and NUDE localize to the plus ends of microtubules in the hyphal tip (Han et al., 2001; Xiang et al., 2000). Loss of NUDE or NUDF function causes an increase in GFP-NUDA intensity at the plus end of microtubules (Efimov, 2003; Zhang et al., 2003); this may indicate that NUDE and NUDF are required to activate dynein to move toward the microtubule minus end. Overexpression of NUDF or the NUDF-interacting N-terminus of NUDE is sufficient to suppress *nudE* mutants, but only the C-terminus of NUDE can localize to microtubule tips on its own (Efimov, 2003). This suggests that NUDE can function as a transient activator of NUDF, which then could activate dynein.

In *Saccharomyces cerevisiae*, Lis1 (named Pac1) also localizes to the plus end of cytoplasmic microtubules. Deletion of Pac1, however, instead of increasing the amount of dynein at the plus end of the microtubule, nearly abolishes localization of dynein to the plus end and subseqently to the cortex (Geiser et al., 1997; Lee et al., 2003). No innate microtubule binding of Lis1 to the microtubules has been detected, so it may facilitate dynein plus end loading through its interaction with CLIP-170, which also helps bring dynein to the plus end (Carvalho et al., 2004; Coquelle et al., 2002; Sheeman et al., 2003), or by a conformational effect on dynein. The yeast NUDE homologue, Ndl1, is required for dynein localization and function, although overexpression of Pac1 rescues the *ndl1Δ* phenotype. Ndl1 depends on Pac1 for localization to microtubule tips, but this localization is transient (Li et al., 2005). In both *S. cerevisiae* and *A. nidulans*, the

uncoupled function and localization of Ndl1/NUDE, and the replacement of its function by mass action with Lis1, suggest that Ndl1/NUDE may function catalytically, as an activator of Lis1. Ndl1/NUDE could perform this activation by promoting Lis1- dynein interaction, as metazoan NudEL was observed to do in the biochemical assays discussed previously. A recent study demonstrated that the head domain of yeast dynein required Lis1, but not Ndl1, to be recruited to the astral microtubule tips, suggesting that the dynein tail might be inhibitory to Lis1 binding or activity, and Ndl1 could relieve this inhibition(Markus et al., 2009). Whether Lis1-Ndl1/NUDE are involved only in the initiation of dynein motility, as an activator or recruitment factor, or also in dynein translocation along microtubules is unknown.

The role of metazoan Lis1, NudEL, and dynein in nuclear and MTOC positioning has been best studied in two types of migratory cells, cortical neurons and fibroblasts. NudE functions with Lis1 and dynein to drive nuclear oscillations and position the spindle properly in neural progenitor cells before their asymmetric division (Feng et al., 2000; Feng and Walsh, 2004; Shu et al., 2004; Siller and Doe, 2008). In the resulting newborn neurons, dynein and Lis1, at this stage working with NudEL, drive the migration-coupled movements of the centrosome and the nucleus (reviewed in (Vallee and Tsai, 2006); (Shu et al., 2004; Tanaka et al., 2004; Tsai et al., 2007; Tsai et al., 2005)).

In lissencephaly, the earlier mitotic defects probably cause the overall deficiency of cortical neurons, while abnormalities of their later migration likely contribute to the characteristic cortical malformation. In migrating fibroblasts, dynein and Lis1/NudEL are required to maintain the position of the MTOC during initial polarization, while the

nucleus is driven by actin retrograde flow toward the rear of the cell (Dujardin et al., 2003; Gomes et al., 2005; Shen et al., 2008). When these cells divide, the alignment of the mitotic spindle with the substratum is also controlled by dynein and Lis1 (Faulkner et al., 2000). Lis1-NudE/EL may help control the localization of dynein in metazoan cells as well; overexpression or depletion of Lis1 disrupts dynein localization to the cell cortex and the centrosome, locations from which it may anchor and organize interphase microtubule arrays and the spindle, and pull on the nucleus, respectively (Cockell et al., 2004; Faulkner et al., 2000; Smith et al., 2000).

Lis1 and NudE/EL are also essential for normal dynein function at the metazoan kinetochore; disruptions of these proteins lead to chromosome misalignment and incorrect attachment, and to spindle assembly checkpoint arrest, similar to that observed in dynein null cells. Lis1-NudE/EL help to recruit dynein to kinetochores, possibly through interaction with the RZZ complex (discussed below) but also through independent recruitment of NudE/EL to the outer kinetochore protein CENP-F (Liang et al., 2007; Soukoulis et al., 2005; Vergnolle and Taylor, 2007). NudEL and NudE have separable roles at the kinetochore; NudEL depletion disrupts chromosome alignment and kinetochore attachment, while NudE depletion displaces dynein from the kinetochore and causes prolonged activation of the spindle assembly checkpoint, probably resulting from lack of dynein transport of kinetochore proteins towards the spindle poles (Vergnolle and Taylor, 2007).

The studies discussed here have clearly delineated many dynein processes to which Lis1 and NudE/EL are essential; however, a molecular understanding of how Lis1 and NudE/EL contribute to dynein function in the cell has been elusive. Although Lis1

or NudE/EL disruptions sometimes affect the localization of dynein, no specific receptors for these proteins (with the notable exception of NudE/EL binding proteins at the kinetochore) are known. It seems likely that Lis1 and NudE/EL do not function purely to form a dynein-cargo link, and there is some evidence from cell biological and genetic studies that Lis1-NudE/EL regulate dynein motor activity. The dynein plus end accumulation observed in *A. nidulans* NUDE and NUDF mutants suggests that Lis1-NudE/El could activate dynein motility. Additionally, a suppressor mutation of *nudF* deletion was mapped to the AAA4 domain of dynein, which seems to act at a regulatory step in the dynein ATPase cycle (Zhuang et al., 2007). Lis1 overexpression in fibroblasts causes dynein, dynactin, and Golgi vesicles to redistribute to near MTOC, indicating possible hyperactivated dynein motility(Smith et al., 2000). This in vivo evidence for regulation of dynein motility has been recently supported by biochemical studies of dynein with Lis1 and NudEL.

**Lis1-NudE/EL regulation of dynein motor activity**

Recent in vitro studies have provided more direct evidence that Lis1 and NudEL affect dynein motor function, but the effects they observe are contradictory. One study of purified brain dynein and recombinant Lis1 demonstrated that Lis1 increases the microtubule-stimulated ATPase activity of dynein without affecting dynein binding to microtubules, suggesting that Lis1 directly activates the dynein ATPase. This increase required the N-terminal region of Lis1, which mediates its dimerization(Mesngon et al., 2006). Another study of recombinant Lis1 and NudEL with purified brain cytoplasmic

dynein found that incubating Lis1 or NudEL with dynein had various opposing effects on dynein ATPase activity, MT binding, and MT gliding, and that co-incubation of Lis1 and NudEL with dynein restored these activities to dynein-alone levels (Yamada et al., 2008). Lis1 and NudEL likely are involved in regulation of dynein motor function, but further work will be required to better elucidate this regulation and to connect it with the in vivo function of Lis1 and NudEL.

**BICAUDAL-D**

Bicaudal-D (BicD) is another multipurpose adaptor of dynein, but unlike dynactin and Lis1, seems to exist solely in metazoan organisms. BicD was identified through genetic screens in *Drosophila* for genes involved in embryogenesis and pattern formation(Mohler and Wieschaus, 1986; Steward and Nusslein-Volhard, 1986). BicD and its mammalian homologues, BICD1 and BICD2, have been best characterized in localization of RNA by dynein throughout *Drosophila* oogenesis and development, and in Golgi vesicle transport in mammalian cells, but they contribute to other dynein-mediated processes as well, including nuclear positioning and microtubule organization (Fumoto et al., 2006; Oh and Steward, 2001; Pare and Suter, 2000; Swan et al., 1999). BicD/BICD may facilitate dynein interactions with cargo as well as with dynactin, and may also regulate dynein activity, but its activity is less well characterized than either dynactin or Lis1. Whether it functions as ubiquitously with dynein as these adaptors do is unknown.

**Molecular structure and interactions**

The mammalian homologues of BicD, BICD1 and BICD2, are highly similar by sequence and function, and here will be referred to collectively as BICD. BicD/BICD is composed largely of three coiled coil domains that mediate binding to dynactin and the dynein motor complex, as well as dimerization and also possibly an inhibitory intramolecular interaction of BicD/BICD (Hoogenraad et al., 2001; Stuurman et al., 1999). In *Drosophila*, BicD forms a complex with another protein, Egalitarian (Egl), which binds to the dynein light chain, and is obligatory for BicD localization and function in dynein mediated-transport during oogenesis and development, but which is not found in vertebrates (Mach and Lehmann, 1997; Navarro et al., 2004). BICD itself binds the dynein-dynactin complex; the C-terminal coiled-coil (CC3) binds to the p50 subunit of dynactin, and the N-terminal coiled-coil (CC1) interacts with the dynein motor complex through an undetermined subunit (Hoogenraad et al., 2001).

**Bicaudal-D in mRNA transport**

In *Drosophila* oogenesis, BicD and Egl are required for the dynein-mediated transport of mRNA into the developing oocyte from the surrounding nurse cells, helping to cement its fate and promote its development as the oocyte. After fertilization, these proteins participate in a similar transport process to asymmetrically localize various RNAs, including the mRNA encoding BicD itself, throughout the development of the embryo (Bullock and Ish-Horowicz, 2001; Delanoue and Davis, 2005; Navarro et al.,

2004; Swan et al., 1999). In situ hybridization combinded with immunoelectron microscopy was used to observe the localization of a transported mRNA (*grk*), dynein, BicD, and Egl relative to microtubules in oocytes. These components localized to small, non-membrane bound 'transport particles' often containing several mRNA copies, which may be general transport complexes for mRNA in oocytes (Delanoue et al., 2007). It is not known whether the BicD-Egl complex recruits dynein to mRNA cargos or regulates motor activity; dynein localization and comigration with mRNA has not been examined in the absence of BicD-Egl function, and no interaction between either of these proteins and localized mRNAs has been demonstrated. If BicD functions to recruit dynein to cargo, as its mammalian homologues do in somatic cells (discussed below), it probably interacts with other proteins in the transport particles, rather than with specific mRNAs.

A study of the motility of mRNA particles in vivo provides some evidence for BicD-Egl regulation of dynein motility. Fluorescently labeled mRNAs were injected into *Drosophila* syncytial blastoderm embryos, and their rates of transport and frequency of transition between apical (minus end-directed) and basal (plus end-directed) transport were monitored. In embryos overexpressing BicD or Egl, transitions to apical transport were favored, suggesting that BicD-Egl could function to switch on dynein transport. Although a role for BicD-Egl in dynein-cargo attachment was not excluded, the lifetime of the transport complexes was much longer than their rate of transition, suggesting that changes in transport direction were induced by regulation of motor activity rather than recruitment to cargo (Bullock et al., 2006).

**BICD in organelle transport**

Studies of the mammalian homologues of Bicaudal-D in tissue culture cells have indicated that BICD may function as a general regulator of the dynein motor. BICD is important at least for dynein function in Golgi-ER transport and at the centrosome (Fumoto et al., 2006; Hoogenraad et al., 2001). Overexpression of the putative dynein-interacting domain of BICD, CC1, disperses the Golgi from its perinuclear position, indicating a disruption of dynein-mediated transport (Hoogenraad et al., 2001). BICD localizes strongly to the Golgi, and CC3 is necessary and sufficient for this localization (Hoogenraad et al., 2001) through its interaction with membrane-localized Rab6. GTP-bound Rab6 binds specifically to BICD CC3 in vitro and recruits BICD to the Gogli in cells, while expression of GDP-locked Rab6 or Rab6 RNAi disperses BICD. Overexpression of BICD increased recruitment of dynein and dynactin to Rab6-positive vesicles and induced the tubulation of these vesicles toward the Golgi, suggesting hyperactivation of dynein(Matanis et al., 2002).

BICD CC1 can also serve as an artificial cargo adaptor for dynein. Fusion of CC1 with mitochondrial or peroxisomal targeting sequences induces the relocalization of the corresponding organelles to the MTOC. Interestingly, expression of these fusion proteins increased localization of dynein and dynactin to the MTOC as well, suggesting that in addition to providing a link to cargo, BICD can somehow activate dynein motility (Hoogenraad et al., 2003). These experiments demonstrate that BICD could serve as a general dynein adaptor for multiple types of cargo by interaction with different cargo receptors, but natural BICD receptors other than Rab6 have not been identified.

BICD regulation of dynein function at the centrosome has not been as extensively described, but seems to be important for dynein transport of the microtubule-anchoring protein ninein to the centrosome. GSK-3β-dependent phosphorylation of BICD is important for BICD-dynein interaction, as well as for recruitment of BICD and ninein to the centrosome (Fumoto et al., 2006).

These studies, in *Drosophila* oocytes and in somatic cells, map out a complex set of interactions between BICD, dynein, dynactin, and cargo, but how these interactions are ordered during the process of dynein recruitment to cargo and activation, is unclear. BICD dimers conceivably could bind to cargo, dynein, and dynactin simultaneously. The observation of hyperactivated dynein transport induced by BicD/BICD overexpression and BICD N-terminal fusions suggests, however, that the full length molecule may help to coordinate a more regulated series of interactions between motor and cargo, as well as to switch on dynein motility.

## ROD-ZW10-ZWILCH (RZZ) AND SPINDLY

Three proteins conserved in metazoans, Rough deal (Rod), Zeste-white 10 (ZW10), and Zwilch form a complex (called the RZZ complex) that localizes to the kinetochore throughout mitosis, and is essential to the kinetochore localization of dynein as well as the spindle assembly checkpoint proteins Mad1 and Mad2 (reviewed in (Karess, 2005)). Together with a recently discovered protein, Spindly (Griffis et al., 2007), RZZ functions as a central mediator of dynein function at the kinetochore, linking dynein and other dynein adaptors to the kinetochore and to the checkpoint proteins, both

of which become dynein cargos. ZW10 may also participate in interphase dynein transport independently from the RZZ complex, and there is some indication that Spindly has interphase function as well.

**Molecular structure and interactions**

The RZZ complex is located in the outermost part of the kinetochore, the fibrous corona, likely attached there by ZW10 interaction with Zwint-1 in vertebrates, although no homolog for this protein has been identified in lower eukaryotes (Famulski and Chan, 2007; Kops et al., 2005). All three components are mutually dependent for localization to the kinetochore (Williams and Goldberg, 1994; Williams et al., 2003). Based on the apparent mass of the RZZ complex, it contains two copies of each component, but nothing is known of the structure or organization of the complex. RZZ functions with Spindly to recruit dynein to the kinetochore; Spindly depends on RZZ for kinetochore localization, but their precise molecular link is unknown.

RZZ-Spindly is required to recruit dynein to the kinetochore, but does so only indirectly, through a network of interactions for which the timing and hierarchy is still unclear. ZW10 interacts with the dynactin p50 subunit and NudE (Inoue et al., 2008; Starr et al., 1998) (Stehman et al., 2007). NudE/EL localization to the kinetochore seems to precede RZZ-Spindly arrival, so they may help to co-recruit RZZ-Spindly and Lis1 and by extension dynactin and dynein (Gassmann et al., 2008; Stehman et al., 2007; Vergnolle and Taylor, 2007). RZZ-Spindly is also required for the checkpoint proteins Mad1 and Mad2 to localize to the kinetochore, but the only direct interaction known

(between Mad1 and Spindly) is present in *C. elegans* embryos, but not *Drosophila* S2 cells (Griffis et al., 2007; Yamamoto et al., 2008). Although these interactions have been documented clearly, their importance and hierarchy of recruitment to the kinetochore has varied between systems. RZZ and Spindly are essential for dynein kinetochore recruitment in all systems where they have been examined, but Lis1 is only sometimes required for recruitment (Coquelle et al., 2002; Dzhindzhev et al., 2005; Griffis et al., 2007; Siller et al., 2005), and dynactin-indepndent kinetochore dynein recruitment has also been observed (Whyte et al., 2008). In addition, in the few studies of Spindly to date, its place in the hierarchy of recruitment to the kinetochore has differed; in *D. melanogaster* S2 cells, Spindly recruitment required dynactin for recruitment, while in *C. elegans* embryos, Spindly itself was required for dynactin as well as Mad1 (via a direct interaction) and Mad2 localization to the kinetochore(Gassmann et al., 2008; Griffis et al., 2007; Yamamoto et al., 2008).

**Dynein function with RZZ and Spindly**

In prometaphase, chromosomes display rapid (nearly 1 μm/s) movements along astral microtubules toward the spindle poles before they finally align at the metaphase plate. In the absence of dynein function, this poleward movment is not observed, astral microtubule capture by kinetochores is less frequent, and some chromosomes fail to align and form improper attachments before anaphase onset (Li et al., 2007; Yang et al., 2007). These phenotypes support a model in which dynein captures astral microtubules as they approach the kinetochore, and then transports the attached chromosome poleward; this process is thought to be important for the conversion of the 'side-on' dynein-microtubule

attachment into a stronger 'end-on' microtubule attachment by the KMN complex, or for KMN capture of other astral microtubules. The RZZ complex is required for this movement, and chromosome alignment delay and misalignment at metaphase were also observed in Spindly-deficient cells (Gassmann et al., 2008; Griffis et al., 2007).

RZZ and Spindly contribute to dynein-mediated chromosome movements during alignment at least as a physical link between dynein and its chromosome cargo, and perhaps have a regulatory function in this process as well. Although no significant defect in load-bearing, end-on microtubule-kinetochore attachment (as monitored by intracentromere tension) was observed in Spindly-depleted S2 cells(Griffis et al., 2007), formation of these attachments was severely delayed in Spindy-null *C. elegans* embryos, and the overall chromosome alignment and segregation defect in these cells was much more severe than in RZZ mutants(Gassmann et al., 2008; Yamamoto et al., 2008). Double mutants of RZZ and Spindly, however, had a milder defect, similar to that caused by RZZ mutations alone(Gassmann et al., 2008). Gassman et al. hypothesized that the RZZ complex inhibits KMN microtubule binding, and that Spindly relieves this autoinhibition, perhaps in response to the increased load dynein would experience once a microtubule met with KMN end-on, thereby promoting the addition of microtubules to end-on attachments, and disfavoring the weaker attachment that KMN side-on binding would produce. Although this model explains the stronger Spindly phenotypes and their suppression by RZZ mutation, a dynein load-responsive element in Spindly, and the mechanisms of RZZ-KMN repression and relief by Spindly, have not been described. As Spindly is a recently discovered regulator of kinetochore dynein, further experiments

will likely continue to elaborate how it acts with RZZ to mediate kinetochore dynein function.

Once a chromatid pair becomes bioriented, dynein removes Mad1, Mad2, and BubR1 from its kinetochores by transporting it toward the spindle poles, thereby silencing the spindle assembly checkpoint and permitting anaphase onset once all chromosomes are bioriented. RZZ facilitates both checkpoint activity and checkpoint silencing; it is required for Mad1 and Mad2 kinetochore localization and thus activity, but also recruits dynein to the kinetochore. Before biorientation or in the absence of functional dynein, RZZ, Mad1, Mad2 and BubR1 levels are high at the kinetochore; after biorientation, these proteins stream in dynein transport particles toward the spindle poles. RZZ, therefore, both recruits dynein to the kinetochore and couples dynein to its checkpoint protein cargo during transport. Spindly, like RZZ, accumulates at kinetochores and then streams poleward with dynein, but only in *C. elegans* has been observed to recruit Mad1 and Mad2, as discussed above(Gassmann et al., 2008). RZZ and Spindly are also good candidates to help transmit the yet-unknown signal for dynein to begin transport of checkpoint proteins.

A candidate for this signal is a mitotic phosphorylation of the dynein intermediate chain. Whyte et al. generated an antibody specific to this phosphorylation, at a highly conserved threonine, and observed that this phospho-species of dynein exists primarily at unattached kinetochores, and disappears after attachment(Whyte et al., 2008). Phosphorylation reduces intermediate chain binding to the p150 subunit of dynactin in vitro, but strongly enhances a direct interaction between ZW10 and phosphorylated intermediate chain; recruitment of phospho-dynein (but not total dynein) to kinetochores

was also independent of dynactin. PP1γ phophatase inhibition blocked the loss of phospho-dynein and the transport of checkpoint proteins. These results suggested an intriguing model, in which PP1γ switches dynein from prometaphase transport of unaligned chromosomes to metaphase transport of checkpoint proteins by inducing a switch from direct ZW10 binding to binding dynactin.

Dynein also may help to power the poleward movement of the chromosomes themselves in early anaphase, although this activity is controversial, and in many systems, dynein inhibition does not disrupt chromosome separation(Savoian et al., 2000; Sharp et al., 2000). Although RZZ levels at the kinetochore fall rapidly after metaphase due to dynein transport, a more modest pool of RZZ has been observed to persist at the kinetochore through anaphase. RNAi of ZW10, like depletion of dynein, reduces the velocity of chromosome poleward transport in early anaphase(Li et al., 2007; Yang et al., 2007). This suggest that kinetochore dynein directly powers chromosome separation, and that RZZ links dynein to its cargos throughout mitosis.

**A possible interphase role for ZW10 and Spindly**

While RZZ complex function is restricted to mitosis, ZW10 seems to have an additional role in the secretory pathway during interphase. ZW10 levels are constant throughout the cell cycle, unusual for a checkpoint protein, and half of the cellular pool is insoluble until treatment with nonionic detergent, suggestive of strong membrane association (Starr et al., 1998; Varma et al., 2006). A more specific membrane link has been documented by Hirose et al., who demonstrated that ZW10 is localized to the

endoplasmic reticulum, as part of an complex with the t-SNARE syntaxin 18, which is involved in ER-Golgi tranfficking. ZW10 and its partners in this complex were stripped from syntaxin 18 by NSF and α-SNAP, as expected for functional SNARE complex members, and disruption of cellular ZW10 caused the dispersal of ER, Golgi, endosomes and lysosomes (Hirose et al., 2004; Varma et al., 2006). ZW10 RNAi also reduced the amount of dynein associated with the Golgi, and specifically reduced the minus end-directed motility of Golgi, endosomes, and lysosomes (Varma et al., 2006).These dynein-like phenotypes, combined with the known physical interaction between ZW10 and dynactin, suggested that ZW10 is involved in linking dynein-dynactin to secretory organelle transport.

Although no concrete link between Spindly and dynein interphase function has been demonstrated, Spindly was identified in a screen for genes controlling interphase morphology as well as a screen for mitotic factors(Griffis et al., 2007). Spindly RNAi S2 cells exhibited long, microtubule-rich projections from the cell body, strikingly different from the characteristic round, flattened morphology observed in wildtype cells. Spindly was also observed at microtuble plus ends in interphase cells. Exactly what interphase function Spindly performs, however, and whether this function might also be coupled to dynein, is unknown.

## TOWARDS AN INTEGRATED MODEL FOR DYNEIN ADAPTATION

A remarkable characteristic of the dynein adaptors discussed here is their ubiquity and overlapping roles in dynein function. In particular, dynactin and Lis1-NudE/EL

seem crucial to every dynein-mediated process in which they have been tested in all eukaryotes, while Bicaudal-D is only somewhat restricted, to multicellular eukaryotes and perhaps only to interphase dynein function. The RZZ complex, while primarily important for dynein transport at the metazoan kinetochore, acts in part as a scaffold for other dynein adaptors. Since disruption of any one of the adaptors often causes a complete abrogation of dynein function and localization, it has been difficult to distinguish their individual contributions. What are the specific mechanisms by which these adaptors act? In addition to the large, possibly complete overlap of dynein functions in which these factors participate, they physically interact with each other, either directly or through contact with the dynein complex. Do the dynein adaptors act sequentially, or do they all function simultaneously with dynein as a meta-complex? How are their individual activities integrated to produce robust and finely regulated dynein transport? Although we presently have only a partial view of what each of these adaptors does, since their actions are likely coordinated, considering how they might act together could provide insight into their function and ideas for future investigation.

For any cellular function of dynein, its adaptation could be described in three stages: targeting or recruitment to cargo, formation of a transport complex, and transport. How do each of the dynein adaptors contribute to these stages?

**Dynein at the plus end**

A complex network of interactions between dynein, its adaptors, and other microtubule binding proteins lead to dynein recruitment to the plus end of microtubules.

Although direct proof of this model is still lacking, it is likely that dynein is delivered by growing plus ends to some sites of activity, such as the cell cortex and organelles. Both dynactin and Lis1 mediate dynein association with the plus end; dynactin p150 can associate directly with the tubulin and with the plus end binding protein EB1, but both dynactin and Lis1 probably associate with the plus end primarily through interactions with the C-terminal zinc knuckle domain of CLIP-170. NudE/EL is also important for dynein plus end localization, perhaps to relieve dynein tail inhibition of Lis1-dynein interaction. In vivo localization of these components in the background of different mutations and depletions has indicated a rough epistasis map of recruitment, but has not indicated what the dynamics of this recruitment might be. What sequence or rearrangement of these interactions leads to dynein association with the plus end, and to its eventual release for minus end-directed motility?

**Dynein-cargo interactions**

Whether delivered from the ends of growing microtubules or recruited from the cytoplasm, dynein must subsequently form a strong and regulated link with its cargo. While some direct interactions of dynein subunits with cargo proteins are known, this interactions are not sufficient for normal dynein transport. Do the dynein adaptors form a generalized platform for dynein interaction with cargo proteins? The dynactin complex has several possibly general interactions with cargo; the C-terminus of p150 and Arp1 bind to specific cargo receptors and spectrin, respectively, while the N-terminus of p50 binds to other adaptors (ZW10 and Bicaudal-D) which themselves interact with the

cargo. Lis1-NudE/EL localize to sites of dynein activity independently of dynein, and their depletion and overexpression strongly affect dynein localization, but no binding partners for Lis1-NudE/EL are known at these sites (except, as noted before, at the kinetochore). Lis1 seems unlikely to form a stable link for cargo transport, as Lis1 binds the dyenin motor domain and if directly linked to cargo during transport, would tether the mechanical element of dynein to the cargo.

For Lis1 in particular, but for the other dynein adaptors as well, some interactions with cargo may not constitute a structural element to maintain dynein attachment to cargo, but may form sequentially to regulate the initiation (or termination) of dynein transport. There is some evidence that the C-terminus of p150 may interact transiently with cargo, to prime a more stable Arp1-spectin interaction, as discussed previously. Bicaudal-D, through its interactions with dynein, dynactin and cargo, may also help initiate dynein-dynactin interaction with each other and with cargo.

**Regulation of dynein motility**

The activity of dynein within the cell could be regulated merely at the level of attachment to cargo; however, there is some evidence that the activity of the motor itself is regulated as well. Dynein is somehow held, inactive, at the plus ends of microtubules, and a significant fraction of dynein remains cytosolic, neither localized to cargo nor bound to microtubules. In addition, dynein cargos exhibit frequent pauses and movement in reverse, suggesting dynein inhibition and kinesin activation. Dynein does not exhibit obvious autoinhibition, as kinesin and myosin do; while the tail domains of kinesin and

myosin have an inhibitory interaction with the head domains that is relieved upon binding to cargo, and by truncation of the tail, full length dynein is fully active for motility on its own in vitro. The general dynein adaptors discussed here, either alone or in collaboration, could transduce binding of a variety of cargo proteins to a common inhibitory or activating signal to dynein.

There is direct evidence that both dynactin and Lis1-NudE/EL can affect dynein motility from in vitro motility studies, and in vivo studies suggested that N-terminal fusions of BICD and overexpression of Lis1 could hyperactivate dynein. Dynein activition or inhibition could be achieved by control of its connection with the microtubule, or by direct engagement with the dynein ATPase. Static binding by the CAP-Gly domain of p150 could hold dynein immobile on the microtubule, and some studies of Lis1 have suggested that it inhibits dynein motility in the absence of NudE/EL binding.

While it is nearly certain that dynein activity within the cell is regulated, it is not clear whether regulation is only at the level of activation/inhibition, or whether the motile parameters of dynein might be tuned. As discussed previously, the in vitro motility of dynein is unusually variable: its step size is variable, it takes occasional backwards and sideways steps, and its stepping behavior is responsive to load. Is this variability exploited and regulated for in vivo transport? What modulation of these parameters might be useful for more robust or finely regulated transport? Dynactin causes an increase in dynein processivity in vitro; This could reflect an enhanced affinity for the microtubule, which could be important for long range transport, or transport under high load; alternatively, this processivity increase could be a side effect of an unknown

regulatory function of dynactin. Lis1-NudE/EL, which seem to regulate the dynein ATPase, could modulate its level of activity or the coupling of ATP hydrolysis to motor stepping.

**Future directions**

The work discussed above has outlined the requirement (in several cases ubiquitous) for the dynein adaptors in the many cellular functions dynein carries out, and has indicated particular steps (such as cargo recruitment or motility) to which these adaptors contribute. However, we do not yet have a detailed model for the mechanisms by which the dynein adaptors coordinate and regulate the function of dynein in the cell. Several lines of experimentation seem especially likely to yield the information required to assemble this kind of model.

One promising methodological direction for studies of the combined action of dynein adaptors is the reconstitution of dynein microtubule plus end tracking and organelle transport. Recently, systems for *in vitro* reconstitution of microtubule plus-end tracking of EB1 and CLIP-170 have been developed; extension of these highly manipulable and reductive systems to include dynein and dynactin could provide a much more detailed view of the interaction of dynein and its adaptors with the plus end. Although attempts to develop a fully reconstituted system for organelle transport have been met with only limited success, partially reconstituted systems (i.e. with complex, undefined cargo but recombinant dynein and adaptors) could be useful.

In these partially or completely reconstituted systems, dynein and its adaptors could be mutated and their levels manipulated. Both at the plus end of microtubules and on dynein cargos, the ensemble and single molecule behavior of subcomplexes of dynein and its adaptors could be observed, which would provide insight into the dynamic assembly of these complexes and the specific mechanistic contribution of each factor. In addition, the molecular consequences of potential regulatory signals, such as NudE/EL phosphorylation, could be determined.

Recently developed techniques for the observation of fluorescent labels in vivo with high spatial precision (such as STORM (Bates et al., 2007; Huang et al., 2008) and PALM (Betzig et al., 2006; Shtengel et al., 2009)) could also be used to pick apart how the assembly and remodeling of supercomplexes of dynein and its adaptors coordinate dynein function in the cell. These techniques could potentially define the composition and architecture of dynein motor supercomplexes during transport, and detect remodeling of this architecture during different stages of transport.

Our understanding of the mechanism of dynein motility on its own is still incomplete, but great progress has been made in the past few years through the development of several recombinant systems by which dynein could be labeled and mutated for biochemical and single molecule studies in vitro. In a few cases, these systems have also been adapted to include dynein adaptors. Further study of the effect of the adaptors on dynein enzymatic activity and motility in vitro will be essential to understanding how they might regulate dynein activity in the cell.

## CONCLUSIONS

Dynactin, Lis1-NudE/EL, BicD/BICD, and RZZ-Spindly are required to couple the single cytoplasmic dynein motor to its hugely diverse cellular functions. These adaptors are not fully ubiquitous to dynein function, some some differential regulation could be achieved by different combinations of adaptors. However, perhaps a majority of dynein cargos seem to require the same supercomplex of dynein and its adaptors, leaving the specification of dynein for an individual cargo mostly to the cargo proteins.

A different way to view dynein function in the cell, rather than as a problem of adapting a single motor to so many functions, is that maintaining only one cytoplasmic dynein species may confer some advantage for the organization of cellular traffic. While the axonemal dyneins, kinesins, and myosins multiplied throughout the evolution of eukaryotes, and vary widely in number between organisms, the number of cytoplasmic dyneins remained at one (Vale, 2003), suggesting a particular selective advantage in maintaining a single cytoplasmic dynein. Perhaps a single cytoplasmic dynein supercomplex is important for coordinating bidirectional transport, allowing a cell to use a common core machinery for microtubule-based transport.

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# CHAPTER 2

## Regulation of the Processivity and Intracellular Localization of *S. cerevisiae* Dynein by Dynactin

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 I designed and performed the experimental work, with intellectual input from Dr. Reck-Peterson and Dr. Vale.

**ABSTRACT**

Dynactin, a large multi-subunit complex, is required for intracellular transport by dynein, but its cellular functions and mechanism of action are not clear. Prior studies suggested that dynactin increases dynein's processivity by tethering the motor to the microtubule (MT) through its own MT binding domains, but this hypothesis could not be tested without a recombinant source of dynactin. Here, we have produced recombinant dynactin and dynein in *S. cerevisiae* and examined the effect of dynactin on dynein in single molecule motility assays. We show that dynactin increases the run length of single dynein motors, but does not alter the directionality of dynein movement. Dynactin enhancement of dynein processivity does not require the MT binding domains of Nip100. Dynactin lacking these MT binding domains also supports the proper localization and function of dynein during nuclear segregation *in vivo*. Instead, a segment of Nip100's coiled-coil is required for these activities. Our results directly demonstrate that dynactin increases the processivity of dynein through a mechanism independent of microtubule tethering.

**INTRODUCTION**

Dynactin, a large (~1.2 MD) complex, was first identified as an activator of dynein-mediated, minus-end-directed vesicle transport (Gill et al., 1991; Schroer and Sheetz, 1991) and has subsequently been shown to be essential for nearly every cellular function of cytoplasmic dynein (Karki and Holzbaur, 1999; Schroer, 2004). Several

dynactin alleles have been linked to human neurological disease, which most likely results from a defect in intracellular trafficking (Munch et al., 2004; Puls et al., 2003). Dynactin is composed of a filament of the actin-related protein Arp1, capped at each end by additional subunits. The barbed end subcomplex contains a dimer of the largest subunit, $p150^{Glued}$ (Nip100 in yeast), which binds to dynein directly (Schroer, 2004). The N-terminus of $p150^{Glued}$ contains a CAP-Gly domain and a basic region, both of which have been shown to bind microtubules (MTs) (Culver-Hanlon et al., 2006; Kobayashi et al., 2006; Waterman-Storer et al., 1995), followed by a coiled-coil that projects as a 24 nm stalk from the Arp1 filament (Eckley et al., 1999; Schafer et al., 1994).

Two general mechanisms have been proposed through which dynactin could aid dynein's cellular function. First, many studies have provided evidence that dynactin is important for localizing cytoplasmic dynein to its proper intracellular cargo (Schroer, 2004). Dynactin also might modulate dynein motor activity, an idea that has been explored through several *in vitro* motility assays. Dynactin has been proposed to increase dynein's processivity, based upon findings that dynactin increases the run length of dynein-coated beads in vitro (King and Schroer, 2000). Increased processivity might be important in the cell for uninterrupted transport over long distance or for transport under high load. However, since dynactin and dynein were nonspecifically adsorbed onto beads in this study, it could not be determined whether dynein-dynactin complexes were observed, or if dynactin bound separately from dynein on the bead surface and exerted its effects by interacting independently with MTs.

The MT binding domains of the dynactin subunit $p150^{Glued}$ have been proposed to play important roles in both dynactin functions. The MT-binding CAP-Gly domain of

p150$^{Glued}$ is required for dynactin to localize to MT plus ends in some cell types, and has been implicated in dynein recruitment and MT anchoring at the centrosome (Quintyne et al., 1999; Quintyne and Schroer, 2002; Vaughan et al., 2002). The initial discovery of the CAP-Gly MT binding domain at the N-terminus of p150$^{Glued}$ also led to the hypothesis that dynactin might increase dynein's processivity by tethering dynein to the MT, thus suppressing dynein release and the termination of a run (Waterman-Storer et al., 1995). Consistent with this idea, antibodies against the CAP-Gly domain were shown to abrogate the dynactin-induced increase in the run length of dynein-coated beads (King and Schroer, 2000), and short polypeptides containing the MT binding basic region of p150$^{Glued}$ increased bead run length when co-adsorbed with dynein onto beads (Culver-Hanlon et al., 2006). However, it was not possible to directly test the MT tethering hypothesis by assaying dynactin lacking its MT binding domains, as these studies were performed using native bovine dynactin, rather than dynactin from a recombinant source.

A recent study suggested that dynactin also might modulate the directionality of dynein. Dynein-dynactin complexes containing a GFP-tagged dynactin subunit were purified from transgenic mice, and their movements along MTs were observed by single molecule fluorescence microscopy (Ross et al., 2006). In striking contrast to previous observations, these dynactin-dynein complexes exhibited frequent plus-end-directed excursions (often greater than 1 μm), in addition to the minus-end-directed movement previously observed for dynein. The authors raised the possibility that dynactin might facilitate these reversals in the direction of dynein's movement, although the motility of the dynein alone was not observed in this study.

In this study, we have developed a new system for examining the effects of dynactin on single molecule dynein motility using purified, recombinant dynein and dynactin complexes from *S. cerevisiae*. We show that the dynein-dynactin complex is more processive than dynein alone, but does not exhibit more plus-end-directed motion. Dynactin does not require the MT binding domains of Nip100 (the yeast p150$^{Glued}$ subunit) to increase dynein processivity or for dynein localization and function in nuclear segregation. Instead, our data indicate that a coiled-coil region adjacent to the MT binding regions in Nip100 is critical for these activities. Therefore, dynactin is unlikely to act as a simple MT tether.

## RESULTS

### Yeast dynein-dynactin is a unidirectional motor complex with enhanced processivity

We have used the budding yeast *Saccharomyces cerevisiae* as a source of recombinant dynactin for our experiments, using a strategy similar to one previously used to purify dynein (Fig. 1A; purification shown in Fig. 2A) (Reck-Peterson et al., 2006). An N-terminal affinity tag was introduced at the genomic locus of *ARP10* (which encodes a protein that binds to the pointed end of the Arp1 filament, Fig. 1A). We also introduced a C-terminal HaloTag (Promega) at the genomic locus of *NIP100* (the yeast p150$^{Glued}$ subunit), which allowed us to site-specifically label the dynactin complex with a fluorescent dye (tetramethyl rhodamine (TMR)). Since the purification tag and fluorescent tag were placed on different subunits at opposite ends of the complex, only intact complexes were purified and labeled with TMR.

Using purified dynactin, we sought to determine the effect of dynactin on the motility of single dynein complexes, using total internal reflection fluorescence microscopy (TIRF). We found that TMR-labeled dynactin molecules displayed no detectable interaction with the MT, even when imaged at a high frame rate (30/s, data not shown). The lack of observable binding could reflect a very weak MT binding affinity of yeast dynactin, or that purified dynactin in the absence of dynein is in an inhibited state. However, when co-incubated with unlabeled recombinant yeast dynein, TMR-labeled dynactin now bound to MTs and moved processively toward the minus-end of axonemal MTs (Fig. 1B). Since TMR-dynactin alone did not move, and dynein was unlabeled in this assay, these moving particles must be dynein-dynactin complexes.

The *S. cerevisiae* dynein-dynactin-TMR complexes moved at a similar velocity to dynein-TMR alone (77 ± 37 nm/s, mean ± SD), compared to 87 ± 36 nm/s for dynein)(Fig. 3A). Notably, the dynein-dynactin complexes had a significantly longer run length (2.54 ± 0.17 μm, mean ± standard error) than dynein alone (1.14 ± 0.04 μm)(Fig. 3B; Fig. 6).

One possible explanation for this longer run length is that dynactin could link two or more dynein complexes together; one dynein motor in a multimeric complex might remain bound while another dissociated, leading to an apparent increase in run length. To determine the number of dynein motors within a moving dynein-dynactin complex, we analyzed photobleaching events of GFP-labeled dynein, with and without dynactin. When dynactin was present, we confirmed that GFP-dynein spots were bound to TMR-labeled dynactin by two-color observation. In both cases, we observed only one- and two-step photobleaching of dynein, indicating that the dynein-dynactin complex contains

a single dynein dimer (Fig. 3C, D). To determine the number of dynactin complexes bound to each dynein, we observed photobleaching of moving TMR-labeled dynactin spots. Again, we observed only one- and two-step photobleaching events, indicating that each dynein binds one dimer of Nip100, and thus a single dynactin complex (Fig. 3C, D). Therefore, a single dynactin complex can enhance the processivity of a single dynein motor.

A previous study of dynein-dynactin motility reported plus-end-directed runs exceeding one μm (Ross et al., 2006). From our kymograph analysis, we did not observe any plus-end-directed runs greater than 0.3 μm, the diffraction limit. To characterize the directionality of dynein-dynactin movement with higher spatial precision, we tracked dynein-dynactin complexes using Gaussian fitting, with a mean RMSD of 4.9 nm (Fig. 4) (Yildiz et al., 2003). Because this precision is lower than in previous studies of Qdot-labeled dynein, assigning individual steps was difficult, but within some traces, we observed stepwise movement of dynein-dynactin complexes that was grossly similar to the stepping behavior that has been observed for dynein alone (Reck-Peterson et al., 2006). To quantify plus- and minus-end-directed components of dynein-dynactin movement, we measured the length of each unidirectional segment of each run. Movement toward the plus-end persisted over much shorter distances than movement toward the minus-end; only 2% of plus-end-directed segments were greater than 24 nm long, compared to 36% of minus-end-directed segments (Fig. 4). Previously, dynein was shown to exhibit short plus-end-directed movement, taking only a few plus end-directed steps in a row (Reck-Peterson et al., 2006). We conclude from this that dynactin does not induce bidirectional movement in the yeast dynein motor.

**The CC1 domain, but not the MT binding domains, of dynactin is required for enhanced processivity**

Having shown that dynactin is a dynein processivity factor using a recombinant system, we next performed structure function studies to investigate the mechanism of this effect. The N-terminal CAP-Gly domain of $p150^{Glued}$ and an adjacent basic region (Culver-Hanlon et al., 2006; Kobayashi et al., 2006; Waterman-Storer et al., 1995) have been shown to interact with MTs, and it has been hypothesized that MT tethering by these domains might prevent dynein from dissociating from a MT and terminating a processive run. However, without a recombinant source of dynactin, it was not possible to test whether these domains are responsible for the effect of dynactin on dynein processivity. We truncated the CAP-Gly domain (ΔCAP-Gly) or the CAP-Gly domain and the adjacent basic region (Δbasic) from the single genomic copy of NIP100 and purified dynactin complexes containing these truncated Nip100 subunits (Fig. 5A). These truncated dynactins associated with dynein and moved along axonemes with similar velocities (Fig. 5B, C) to wildtype dynein-dynactin complexes. Notably, the run lengths were not significantly reduced by these truncations and remained two-fold longer than that of dynein alone (Fig. 5C). Therefore, dynactin does not require its MT binding domains to increase dynein's processivity.

To identify a domain that might be required for this activity, we performed further truncations of the NIP100 gene. C-terminal to the CAP-Gly domain and basic region is a coiled-coil region that has been reported to bind to the N-terminus of the dynein intermediate chain (King et al., 2003). The first half of this coiled-coil is thought to form

the 24 nm projecting stalk seen by EM (Eckley et al., 1999; Schafer et al., 1994), while the second half may fold back into the shoulder of dynactin. We prepared dynactin complexes containing truncations of the first half (ΔCC1A) or the entirety (ΔCC1) of this coiled-coil domain, and examined how these alterations affected dynein-dynactin motility (Fig. 5A). We observed robust movement of dynein-dynactin co-complexes with these truncations, and dynein coimmunoprecipitated weakly with both wt and ΔCC1A-dynactin, indicating that CC1 truncation does not grossly perturb the interaction of dynactin with dynein. Co-complex formation instead may depend upon an interaction between the central region of Nip100 and the dynein intermediate chain (Deacon et al., 2003). Dynein bound to ΔCC1A-dynactin exhibited a longer run length (1.88 ± 0.10 μm, Fig. 5C) than dynein alone, although this activation was reduced compared with wildtype dynein-dynactin. However, dynein in complex with ΔCC1-dynactin had a mean run length of 1.42 ± 0.06 μm (Fig. 5C), approaching the run length of dynein alone (1.15 um ± 0.04 μm) (see Supplementary Methods showing that shorter run length is not due to premature dynactin dissociation from dynein). Therefore, CC1 is crucial to the enhancement of dynein processivity by dynactin.

We next wished to determine whether the activity of dynactin as a dynein processivity factor is solely contained in the Nip100-containing "shoulder" subcomplex of dynactin, or whether the Arp1 filament contributes to this activity. Deletion of Arp1 or overexpression of p50 (Jnm1 in yeast) separates the "shoulder" subcomplex of dynactin, containing $p150^{Glued}$, p50, and p24 (Ldb18 in yeast), from the rest of the complex (Burkhardt et al., 1997; Moore et al., 2008; Valetti et al., 1999), and causes strong perturbations of cellular dynein functions. We purified dynactin lacking the Arp1

filament, using an affinity tag on the Nip100 subunit in a yeast strain harboring a deletion in the Arp1 gene. Although deletion of Arp1 destabilizes $p150^{Glued}$ in some cell types (Haghnia et al., 2007; Minke et al., 1999; Moore et al., 2008), we found that Nip100 was stable in an *arp1Δ* background, as was previously observed in yeast (Moore et al., 2008) (Fig. 2B). ΔArp1-dynactin moved along axonemes with dynein, but the run length of this co-complex (1.35 ± 0.08 μm; Fig. 5C) was similar to dynein alone, indicating that an intact dynactin complex is necessary for increasing dynein processivity.

**Nip100 CC1, but not Nip100 MT binding domains, is required for dynactin function *in vivo***

Having determined which parts of the dynactin complex contribute to its *in vitro* enhancement of dynein processivity, we wished to test whether these domains are required for dynactin function in live cells. Dynactin is essential for dynein's only function in yeast, pulling the nucleus into the bud neck in early anaphase (Adames and Cooper, 2000; Eshel et al., 1993; Li et al., 1993). Cells with defects in dynein or dynactin function frequently missegregate their nuclei, retaining both in the mother cell. We found that nuclear segregation occurred normally in yeast harboring truncations of the N-terminal MT binding sites (ΔCAP-Gly and Δbasic) or the first half of CC1 (ΔCC1a) of *NIP100*, but was strongly perturbed in yeast with a complete CC1 truncation (Fig. 7A). In this *ΔCC1-NIP100* strain, a similar percentage of binucleate mother cells were observed as in a *nip100Δ* strain. Immunoblots revealed that the cellular levels of ΔCC1a-Nip100 and ΔCC1-Nip100 are reduced relative to wildtype Nip100 (approximately four-fold, Fig. 2C). However, since the levels of both CC1-truncated

proteins are similar, but only ΔCC1-Nip100 causes a cellular phenotype, this phenotype most likely arises from a specific requirement for CC1.

To perform its cellular function, yeast dynein must localize to the bud cortex, where it walks along the astral MTs, thereby pulling the nucleus towards the bud. The dynactin components Nip100 and Arp1 are required for this localization, as dynein accumulates at the plus-ends of astral MTs in strains bearing deletions of these genes (Lee et al., 2003; Sheeman et al., 2003). From this evidence, it has been proposed that dynactin may assist in transferring dynein from the plus-end of MTs to the cortex before motility begins. To determine whether the N-terminal domains of Nip100 are required for this transfer step, we observed the localization of Dyn1-3xGFP in the background of *NIP100* truncations. Dyn1-3xGFP localized normally in *ΔCAP-Gly-NIP100*, *Δbasic-NIP100*, and *ΔCC1A-NIP100* cells, exhibiting cortical spots of dynein and wildtype levels of fluorescence at astral MT plus-ends (Fig. 7B,C). In contrast, *ΔCC1-NIP100* cells lacked cortical dynein, and exhibited a two-fold increase in dynein at astral MT plus-ends, similar to that observed in *nip100Δ* cells (Fig. 7B,C). Thus, the first Nip100 coiled-coil, but not the N-terminal MT binding domains, is needed for proper localization of dynein during cell division.

## DISCUSSION

Dynactin is essential to dynein-mediated intracellular transport, but its size and complexity have hindered the elucidation of its function. Using dynactin purified from a recombinant source in a direct, single molecule assay, we have shown that dynactin

increases the processivity of single dynein motors. Previous studies of mammalian dynactin and dynein (Culver-Hanlon et al., 2006; King and Schroer, 2000) first showed that dynactin could increase the processivity of dynein bound to beads. However, these early experiments were performed by nonspecific absorption of the complexes to beads, which complicated mechanistic interpretation of these results. For example, dynactin and dynein might have bound separately on the bead and independently interacted with the MT, rather than operating as a dynein-dynactin co-complex. Here, we show through direct observation that a single dynein with a single bound dynactin moves for longer distances along a MT than dynein alone.

Dynactin has been proposed to increase dynein processivity by acting as a tether to the MT surface (Culver-Hanlon et al., 2006; King and Schroer, 2000; Kobayashi et al., 2006). This hypothesis is supported by observations that beads coated with mammalian dynactin or N-terminal fragments of $150^{Glued}$ bound to MTs and diffused one-dimensionally (Culver-Hanlon et al., 2006; King and Schroer, 2000; Kobayashi et al., 2006). A monoclonal antibody to the N-terminal MT binding region of dynactin also was found to reduce the processivity of dynein and dynactin-adsorbed beads (King and Schroer, 2000). However, by truncation of the Nip100 subunit within the intact dynactin complex, we find that Nip100's N-terminal MT binding regions are not required for dynactin to increase the processivity of yeast dynein. In addition, we have not observed dynactin binding to or diffusing along MTs on its own; this may reflect an inhibited state of dynactin, as suggested by some studies in yeast and mammalian cells (Moore et al., 2008; Vaughan et al., 2002). The MT interactions observed for bead-adsorbed dynactin and $p150^{Glued}$ fragments and the often pleiotropic effects of antibody inhibition also may

not reflect the behavior of the intact complex. Nevertheless, our results indicate that dynactin increases dynein's processivity by a mechanism other than MT tethering. The mechanism of this effect remains unknown; however, a possible model is that dynactin alters the coordination of dynein's two motor domains in a manner that reduces its probability of dissociation during processive motion.

A recent study suggested that dynactin can induce large plus end-directed movements of dynein, which the authors proposed was facilitated by MT tethering (Ross et al., 2006). We did not observe similar micron-scale plus-end-directed movements in our preparation of dynein-dynactin by kymograph analysis, and high precision tracking of dynein-dynactin did not show longer plus end-directed movements than were previously observed for dynein alone (36 nm maximum, compared to 70 nm previously measured)(Reck-Peterson et al., 2006). Therefore, our results indicate that the dynein-dynactin complexe functions essentially as a unidirectional motor that takes an occasional one or few backwards steps, and no additional mechanism is required to ensure minus end-directed transport by dynein in the cell. The difference between our observations and the study performed by Ross et al. may reflect different behaviors of dynein-dynactin from yeast and mammals, and will require further investigation.

Our in vivo studies also reveal that the known MT binding regions of dynactin are not required for its functions in yeast. This is surprising, given the high conservation of the $p150^{Glued}$ CAP-Gly domain across species. However, dynactin lacking the MT binding domains of $p150^{Glued}$ (CAP-Gly and basic regions) has been shown to support normal dynein-driven organelle motility in *Drosophila* S2 cells and in HeLa cells, indicating that the primary role of dynactin in cargo transport may not be as a MT tether

(Kim et al., 2007) (Dixit et al., 2008). However, mitotic functions of dynactin in S2 cells do require these domains (Kim et al., 2007). A neuronal isoform of $p150^{Glued}$ that lacks these MT binding domains incorporates into dynein-dynactin co-complexes and participates in axonal transport, again implying that some functions of dynactin may not involve MT tethering (Tokito et al., 1996, Dillman et al., 1996). Thus, the MT binding domains of dynactin may be necessary only for a subset of the processes in which it participates.

We also show that CC1, but not the N-terminal MT binding regions of Nip100, is required for the dynactin-mediated transfer of dynein from the plus-ends of MTs to the cell cortex. This requirement may reflect an undescribed role for dynactin as a cargo adapter at the cell cortex. However, since truncation of Nip100 CC1 disrupts dynactin activity both in vitro and in vivo, perhaps dynactin regulation of dynein motor function is important for the transfer process from the MT plus-end to the cortex. Supporting this idea, a small deletion (eight amino acids) in dynein's catalytic core also causes dynein to accumulate at astral MT plus-ends (Sheeman et al., 2003). The specific requirement for the CC1 domain of Nip100 for in vitro processivity enhancement and nuclear segregation in vivo suggests that dynactin regulation of dynein motor activity is important for dynein function in living cells.

## MATERIALS AND METHODS

### Yeast strains

Strains used in this study are listed in Table 1. Deletion, tagging, and truncation of dynein and dynactin subunits were performed at chromosomal loci using standard homologous recombination techniques. The genomic copy of Dyn1 was tagged with three tandem repeats of GFP using a plasmid provided by John Cooper (Washington University School of Medicine, St. Louis, MO) (Lee et al., 2003).

### Purification and fluorescent labeling of yeast cytoplasmic dynein and dynactin

Yeast cultures were grown in YPD to an $OD_{600}$ between 0.8 and 1. Cells were harvested by centrifugation and washed once with water; the pellet then was resuspended in residual water and frozen by drops in liquid nitrogen. The frozen cell pellet was lysed by grinding in a liquid nitrogen-cooled coffee grinder, and the resulting powder was thawed with 0.3 volumes of 4X lysis buffer (1X lysis buffer: 30 mM HEPES (pH 7.2), 50 mM KAcetate, 2 mM MgAcetate, 1 mM EGTA, 10% glycerol, 1 mM DTT, 0.1 mM Mg ATP, 1 mM Pefabloc, 10 μg/ml leupeptin, 10 μg/ml pepstatin A and 0.2% Triton X-100). The crude lysate was centrifuged at 290,000 x *g* for 25 min, and the resulting supernatant was incubated with IgG sepharose (Amersham Pharmacia) for 1 h at 4°C. The IgG beads were then washed twice with lysis buffer, and bound Halotag fusion proteins were labeled with 10 mM TMR-conjugated Halotag ligand (Promega). The beads were washed three times with TEV cleavage buffer [10 mM Tris (pH 8.0), 150 mM KCl, 10% glycerol, 0.1 mM ATP, 1 mM DTT, 1 mM Pefabloc], and the beads containing bound

dynein or dynactin were incubated with TEV protease for 1 hr at 16° C. The resulting solution of dynein or dynactin was aliquoted and frozen in liquid nitrogen.

**Motility assays**

For single molecule TIRF microscopy, Cy5-labeled sea urchin axonemes were added to a flow chamber, where they adhered tightly to glass, and the chamber was washed to remove free axonemes. Dynein, dynactin, or a mixture of the complexes were flowed into the chamber and incubated for 2 min. If dynein-dynactin complexes were to be observed, dynein and dynactin were first preincubated for 5 min. The chamber was washed again and motility buffer [30 mM HEPES (pH 7.4), 100 mM K-Acetate, 2 mM Mg-Acetate, 1 mM EGTA, 10% glycerol, 1 mM DTT, 1 mM MgATP, oxygen scavenger system (Reck-Peterson et al., 2006)] was added. TMR-labeled single molecules and Cy5-labeled axonemes were visualized with a custom built objective-type total internal reflection microscope equipped with a 100x 1.45 NA objective and a 1.6x optovar, using an argon laser with 514 nm illumination at 3 mW to image TMR and a helium-neon laser with 633 nm illumination to image Cy5. Images were acquired every 2 s for 8 m with a cooled, intensified CCD camera (Mega10 S30Z, Stanford Photonics), controlled with QED software. Velocities and run lengths of moving molecules were determined from kymograph analysis in ImageJ as previously described (Reck-Peterson et al., 2006). Mean velocity was determined by fitting a Gaussian function to a histogram of velocities using Origin software. Mean run length was calculated by evaluating the cumulative probability function of the measured run lengths, correcting for photobleaching and for the length of the axonemes, as previously described (Reck-Peterson et al., 2006). Every

construct was assayed from at least two separate cell growths and protein preparations.

Photobleaching experiments to determine dynein-dynactin stoichiometry were performed under the above described motility assay conditions, with the exception that the oxygen scavenger solution was not included when observing Nip100-TMR photobleaching. Samples were observed using an inverted microscope (TE2000U, Nikon) equipped for TIRF with a 100X, 1.45 NA objective. The sample was illuminated and imaged continuously with 491 nm (GFP photobleaching) or 561 nm (TMR photobleaching) diode lasers and images were captured every 100 msec (GFP photobleaching) or 200 msec (TMR photobleaching) using a back-thinned electron multiplying CCD camera (iXon$^{EM}$+, Andor). The system was controlled with μManager (http://www.micromanager.com) software. Fluorescence intensities of moving motors were determined from kymographs using ImageJ.

High spatial precision measurements of dynein-dynactin stepping were performed using the microscope setup described for photobleaching experiments. To prepare slides for these observations, flow chambers were coated with biotinylated BSA, incubated with strepavidin, and then incubated with biotinylated and Cy5-labeled microtubules. Dynein-dynactin complexes were introduced and exchanged into motility buffer as described above, except ATP was included at 300 nM with an ATP regenerating system (1% pyruvate kinase and 10 mM phosphoenolpyruvate). 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid was added to the motility buffer at 0.5 mg/ml to reduce TMR blinking. A 2D Gaussian fit to determine the centroid of dynactin-TMR spots was perfomed as described previously (Yildiz et al., 2003).

**Dynein-dynactin dissociation measurements**

A possible reason for an apparent shorter run length of ΔCC1-dynactin-dynein complexes might be a weakened association of ΔCC1-dynactin with dynein. This could cause dissociation of the fluorescently labeled dynactin from dynein before dissociation of dynein-dynactin from the MT track. To determine if CC1 truncation caused premature dissociation of dynactin, we observed dynein-dynactin complexes bound to axonemal MTs in rigor (no ATP). At the longest time of observation (8 min), >67% of wildtype and ΔCC1-dynactin remained associated with dynein, and for the mean duration of a dynein-dynactin run (~30 sec; mean run length/mean velocity), >90% of wildtype and ΔCC1-dynactin remained bound. From these measurements, we conclude that dissociation of dynactin from dynein does not contribute significantly to the observed change in run length.

**Nuclear segregation assay**

To assess the fidelity of nuclear segregation, cells were grown in YPD at 30°C to $OD_{600}$ = 0.3-1, diluted to $OD_{600}$ = 0.3, and grown for 16 h at 16°C. Cells were fixed and stained with DAPI, and wildtype and binucleate anaphase mother cells were counted.

**Live cell microscopy**

Dyn1 was C-terminally tagged with 3xGFP using a previously described plasmid (Lee et al., 2003). To observe Dyn1-3xGFP in living yeast cells with minimal background, cells grown overnight in minimal medium supplemented with 20 mg/l adenine were plated on agarose pads, as previously described (Waddle et al., 1996). The sample was imaged at

room temperature using a spinning disc confocal scanhead (Yokogawa Electric and Solamere) mounted on an inverted microscope (Axiovert 200M, Zeiss) with a 100x, 1.45 NA objective. Images were captured on a cooled CCD camera (C9100-13, Hamamatsu), and image acquisition was controlled with μManager (http://www.micromanager.com) software. Stacks of 0.2 μm slices were collected and collapsed into 2D projections using ImageJ.

**Dynein-dynactin coimmunoprecipitation**

Cultures of strains containing ZZ-TEV-tagged wildtype dynactin (VY597), ΔCC1-dynactin (VY671), and an untagged strain (VY1) were grown, lysed, cleared, bound to IgG beads, and washed as described for the purification of dynactin. The beads were subsequently incubated with unlabeled dynein (purified from VY263) in lysis buffer + 1 mg/ml casein for 1 h at 4°C. This solution was removed, and the beads were washed three times with lysis buffer. Bead samples were boiled to release bound proteins and separated on a 4-12% gel and transferred to nitrocellulose. Immunoblotting was performed to detect the HA epitope with HA.11 mouse monoclonal antibody (Covance).

**Immunoblotting**

Yeast cultures were grown in YPD to an $OD_{600}$ between 0.6 and 1. For protein level comparisons an equivalent number of cells/strain were pelleted and snap frozen in liquid nitrogen. Thawed pellets were lysed by alkaline lysis followed by TCA precipitation. Whole cell extracts were separated on a 4-12% gel and transferred to nitrocellulose. Immunoblotting was performed to detect the Myc epitope with 9E10 mouse monoclonal

antibody (Covance), and the FLAG epitope with mouse monocolonal anti-FLAG antibody (Sigma).

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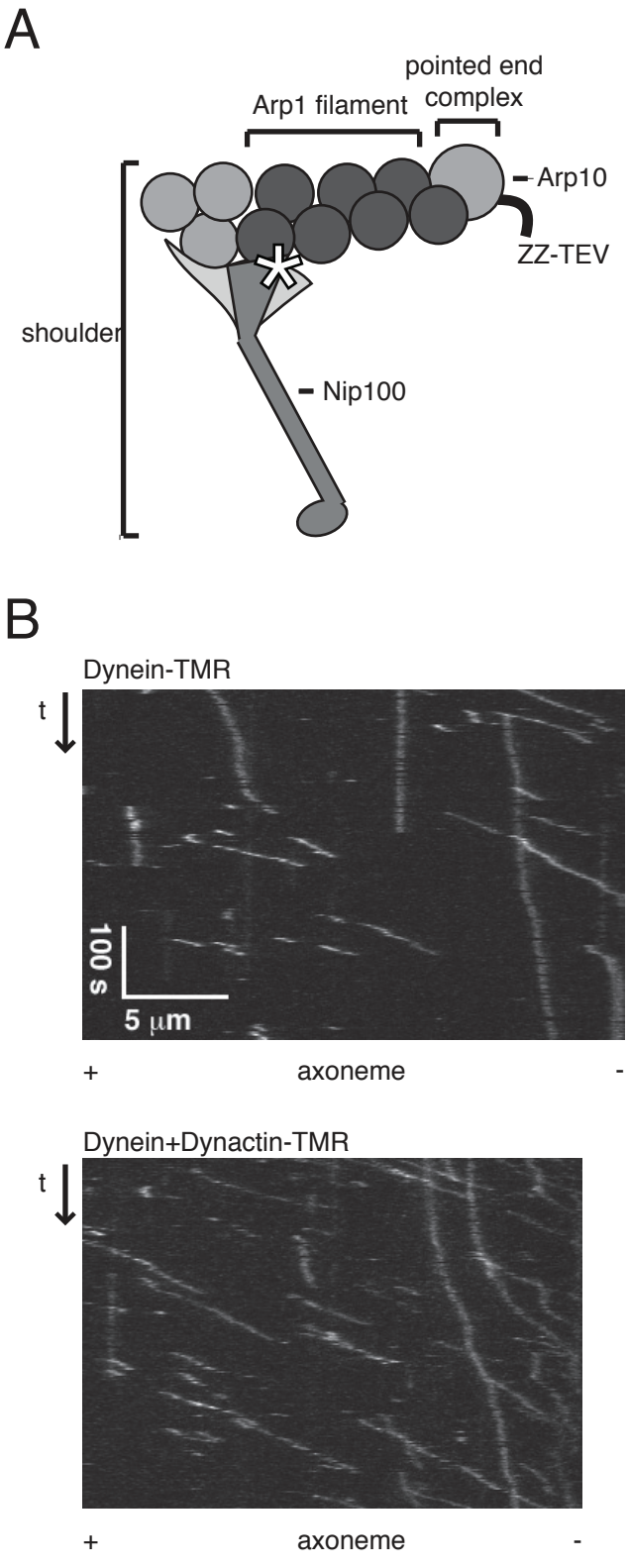
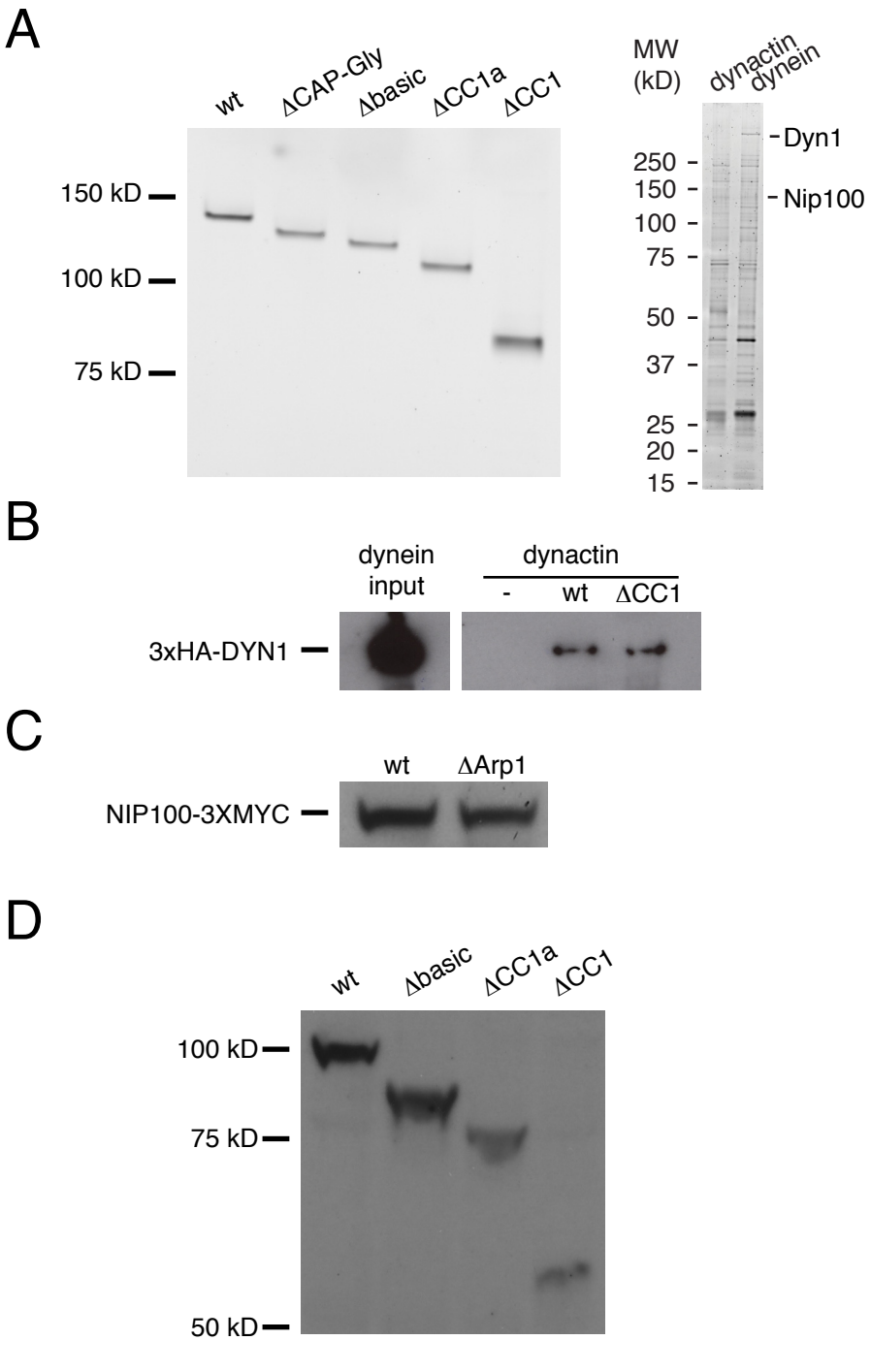
**Figure 1. Purification of dynactin from *S. cerevisiae*.** (A) Diagram of the dynactin complex. Intact dynactin complexes were affinity purified using a ZZ-TEV tag on the N-terminus of Arp10, and the Nip100 subunit was labeled with a TMR-conjugated C-terminal HaloTag (white asterix). (B) Reconstituted dynein-dynactin complexes move processively and unidirectionally along axonemes. A kymograph of unlabeled dynein and TMR-labeled dynactin (bottom) moving along axonemes is shown.



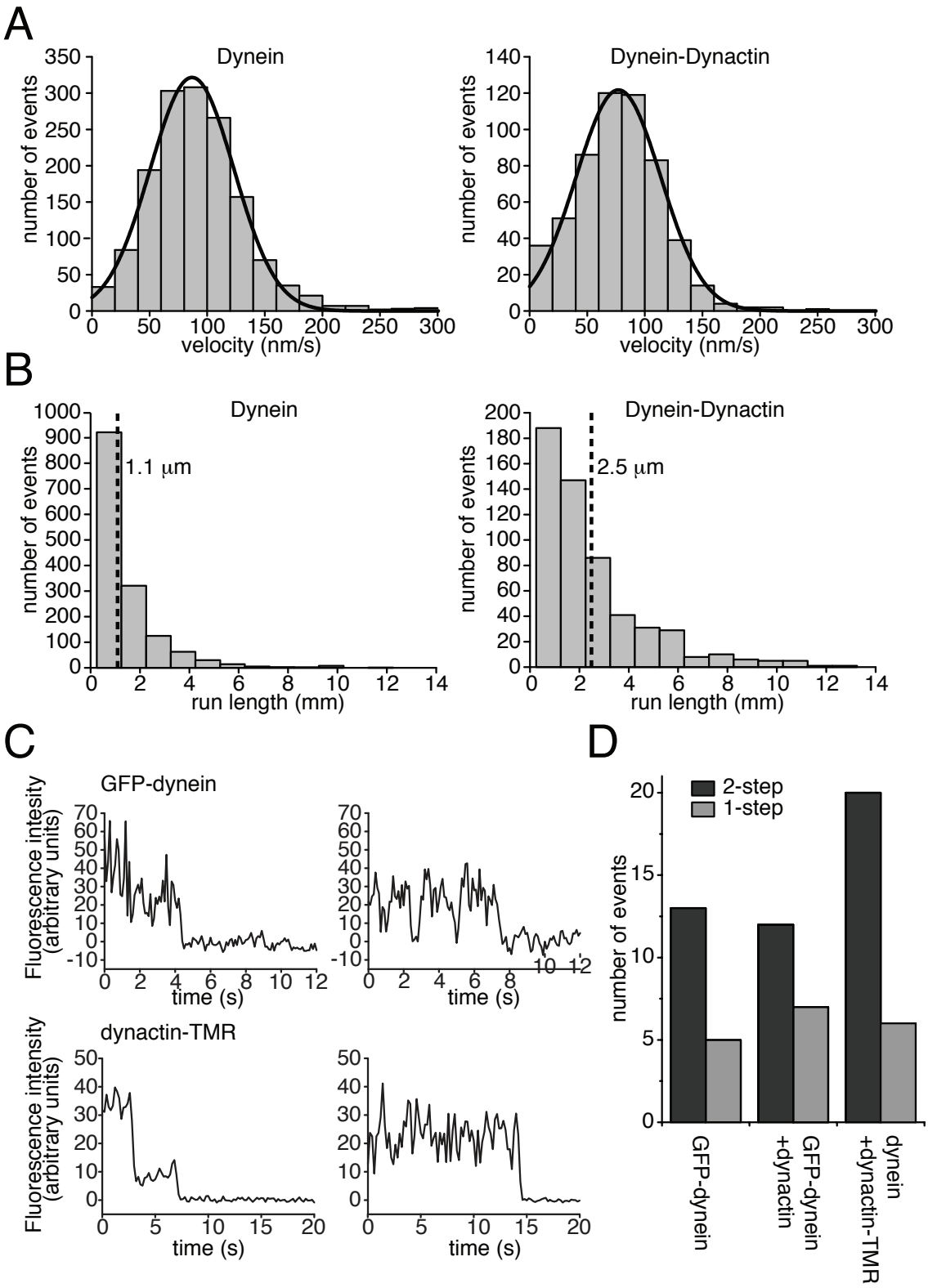

*Chapter 2, Figure 1*

**Figure 2. Effect of Dynactin Subunit Truncation and Deletion on Dynactin Complex Stability.** (A) All truncations of Nip100 copurified with Arp10, indicating that they still assembled into the dynactin complex. A gel of purified, TMR-labeled dynactin samples was scanned using 532 nm light on a Typhoon 9400 (GE Healthcare). The panel to the right shows a Sypro Ruby stained gel (Invitrogen) of purified samples of wt dynactin and dynein. The positions of the dynein heavy chain (Dyn1) and Nip100 are indicated to the right. The Nip100 band is largely obscured by background staining. (B) Dynein coimmunoprecipitates both with wildtype dynactin and with ΔCC1-dynactin. Lysate from strains containing ZZ-tagged Arp10 and an untagged strain (null) were bound to IgG beads. The beads were washed and subsequently incubated with purified 3xHA-tagged dynein. (C) Immunobloting of Nip100-3xFLAG in extracts from ARP1 and *arp1Δ* yeast indicated that Nip100 remained stable in the absence of Arp1. (D) Immunoblotting of truncations of Nip100 C-terminally tagged with 3xMyc indicated that truncation of CC1a or CC1 from Nip100 similarly reduced cellular levels of Nip100, while truncation of the N-terminal MT binding domains did not affect Nip100 levels.



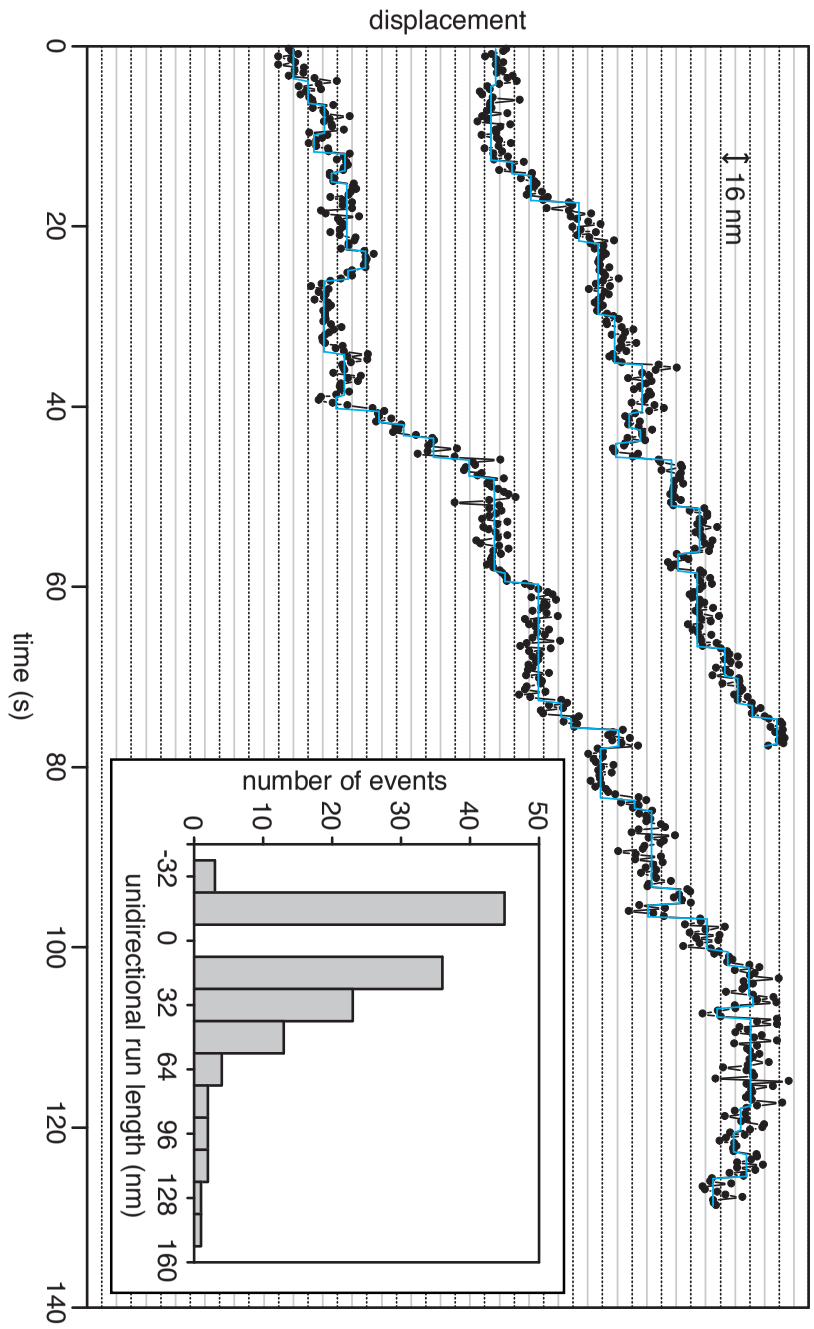

*Chapter 2, Figure 2*

**Figure 3. Single dynein-dynactin complexes exhibit robust minus-end-directed motility with enhanced processivity.** (A) Histograms of dynein and dynein-dynactin velocities. The velocity of dynein is 87 ± 36 nm/s (mean ± SD; n = 1499), and the velocity of dynein-dynactin is 77 ± 37 nm/s (n = 560). Red lines represent Gaussian fits of the data. A few outlying points are truncated from histograms of run lengths and velocity for display purposes. (B) Histograms of dynein and dynein-dynactin run lengths. The run length of dynein is 1.15 ± 0.04 μm (mean ± standard error), and the run length of dynein-dynactin is 2.54 ± 0.17 μm (determined from cumulative probability functions; Fig. 6). Red dashed lines indicate the mean. (C) Photobleaching of moving dynein-dynactin co-complexes. GFP-Dyn1 (top) exhibits two-step (left) and one-step (right) photobleaching alone and when bound to dynactin (traces shown are for dynein-dynactin). Nip100-TMR labeled dynactin (bottom) in complex with dynein also displays only two-step (left) and one-step (right) photobleaching. (D) Histogram of bleaching events. All bleaching events observed occurred in one or two steps. GFP-dynein, n = 18; GFP-dynein-dynactin, n = 19; dynein-dynactin-TMR, n = 26.



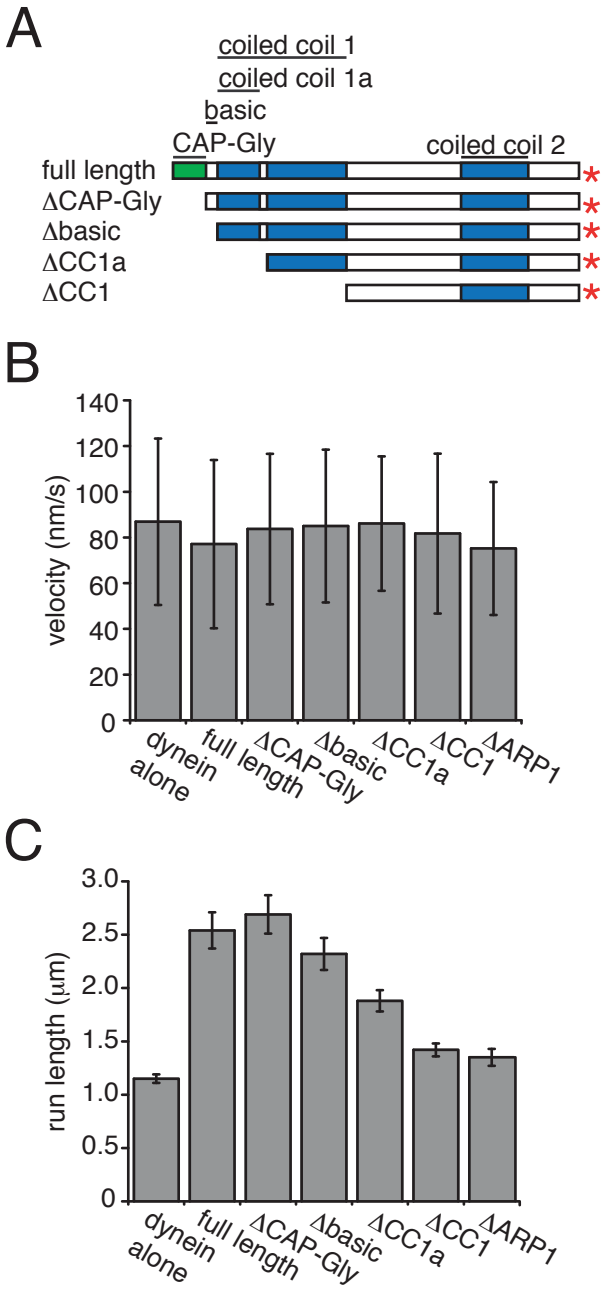

*Chapter 2, Figure 3*

**Figure 4. High spatial precision measurements of dynactin movement along MTs.** Gaussian fits of the position of two moving dynein-dynactin-TMR spots at each point in time are displayed as black circles connected by lines, and steps extracted from these fits are displayed as cyan lines. Horizontal lines are separated by 8 nm. Inset box displays a histogram of the length of unidirectional segments (distance traveled before a ≥ 8nm reversal) of dynein-dynactin movment.



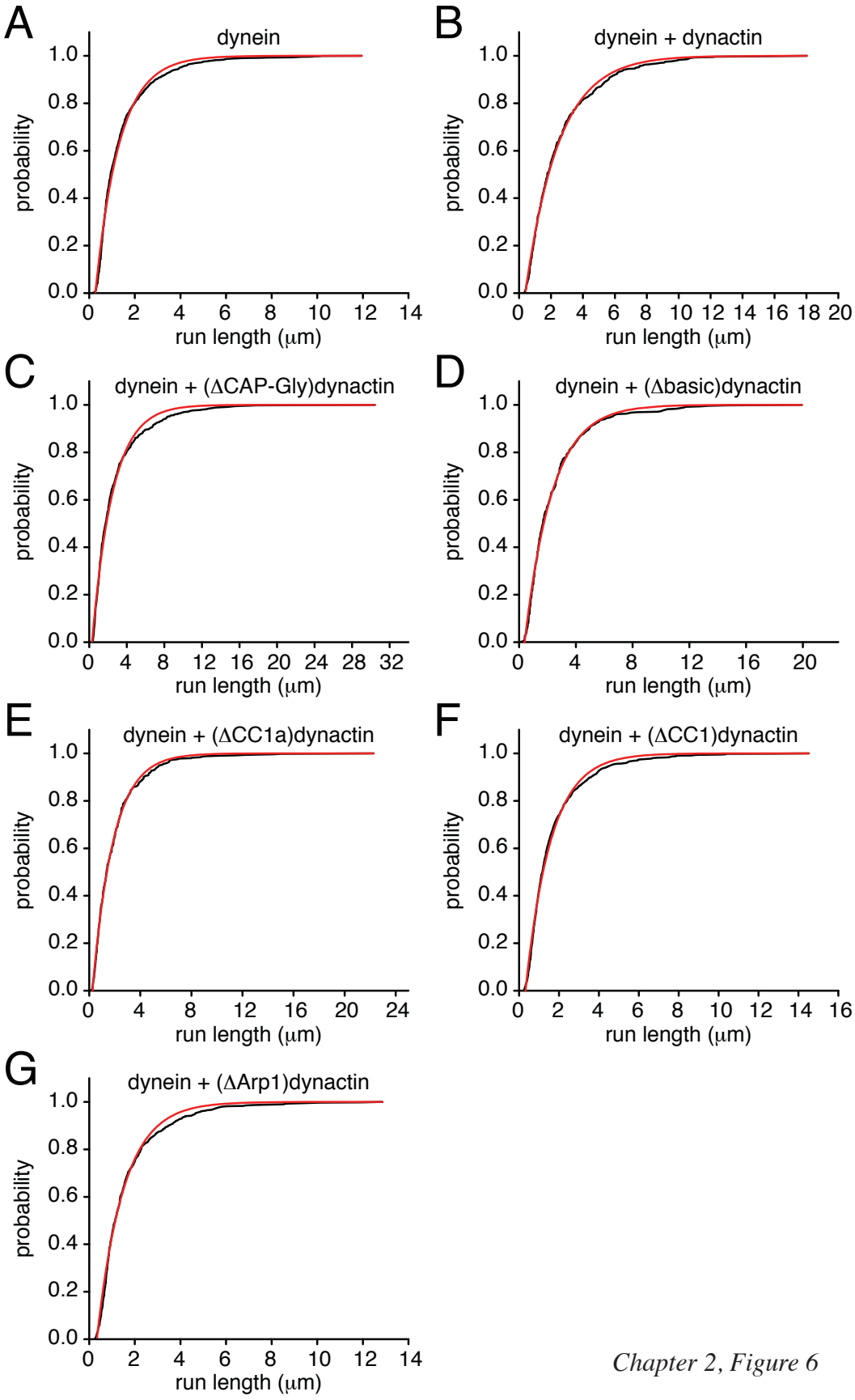

*Chapter 2, Figure 4*

**Figure 5. The Nip100 coiled-coil 1, but not the N-terminal microtubule binding domains, is required for dynein processivity enhancement.** (A) Diagram of truncations of the Nip100 subunit of dynactin. The N-terminally truncated Nip100 subunits lack the following residues: ΔCAP-Gly, 1-68; Δbasic, 1-102, ΔCC1a, 1-182; ΔCC1, 1-377. (B) Mean velocities of dynein-dynactin containing truncations in Nip100 or lacking Arp1, determined from a Gaussian fit. Error bars show SD. Dynein, n = 1499; dynein + dynactin, n = 560; dynein + ΔCAP-Gly-dynactin, n = 899; dynein + Δbasic-dynactin, n = 487; dynein + ΔCC1A-dynactin, n = 496; dynein + ΔCC1-dynactin, n = 939; dynein + ΔArp1-dynactin, n = 599. (C) Mean run lengths of dynein-dynactin variants, determined from cumulative probability distributions (Fig. 6). Error bars show standard error.



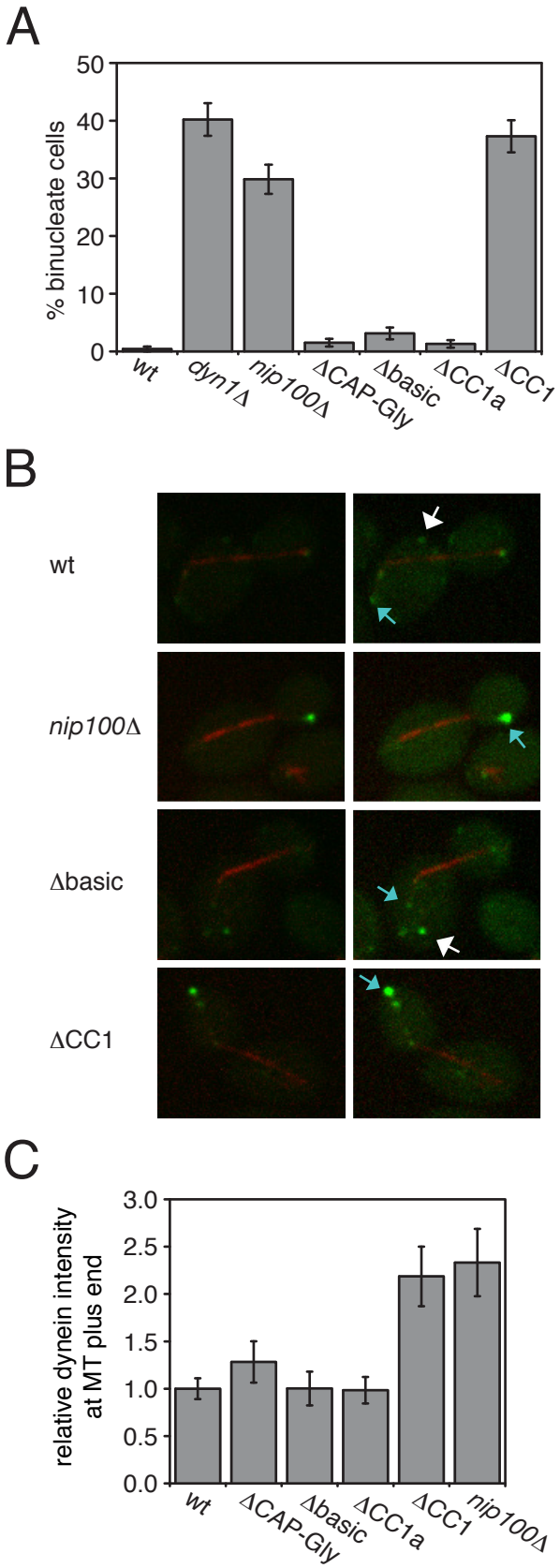

*Chapter 2, Figure 5*

**Figure 6. Cumulative probability distribution functions used to determine mean run lengths of motors.** Experimental cumulative probability functions are drawn in black, and theoretical functions are drawn in red. The mean run length ($\lambda$) and the lower limit for run length detection ($\chi_0$) were determined from the fit, and velocity ($\nu$), mean axoneme length ($l$) and the photobleaching rate of the TMR label ($k_{bleach}$, 0.00255/s) were fixed. (A) Dynein alone. Fitted parameters: $\lambda = 1.15 \pm 0.04$ μm, $\chi_0 = 0.27$ μm. Fixed parameters: $\nu = 86.9$ nm/s, $l = 21.7$ μm. (B) Dynein + dynactin. Fitted parameters: $\lambda = 2.54 \pm 0.17$ μm, $\chi_0 = 0.40$ μm. Fixed parameters: $\nu = 77.1$ nm/s, $l = 22.6$ μm. (C) Dynein + (ΔCAP-Gly)dynactin. Fitted parameters: $\lambda = 2.69 \pm 0.18$ μm, $\chi_0 = 0.30$ μm. Fixed parameters: $\nu = 83.7$ nm/s, $l = 22.6$ μm, $k_{bleach} = 0.00255$/s. (D) Dynein + (Δbasic)dynactin. Fitted parameters: $\lambda = 2.32 \pm 0.15$ μm, $\chi_0 = 0.39$ μm. Fixed parameters: $\nu = 85.0$ nm/s, $l = 21.7$ μm. (E) Dynein + (ΔCC1a)dynactin. Fitted parameters: $\lambda = 1.88 \pm 0.10$ μm, $\chi_0 = 0.25$ μm. Fixed parameters: $\nu = 86.1$ nm/s, $l = 25.2$ μm. (F) Dynein + (ΔCC1)dynactin. Fitted parameters: $\lambda = 1.42 \pm 0.06$ μm, $\chi_0 = 0.32$ μm. Fixed parameters: $\nu = 81.7$ nm/s, $l = 21.0$ μm. G) Dynein + (ΔArp1)dynactin. Fitted parameters: $\lambda = 1.35 \pm 0.08$ μm, $\chi_0 = 0.34$ μm. Fixed parameters: $\nu = 75.2$ nm/s, $l = 19.7$ μm.




*Chapter 2, Figure 6*

**Figure 7. Effect of Nip100 truncations on dynactin function in yeast cells.** (A) The fidelity of nuclear segregation in cells containing truncated *Nip100*, as indicated by the percent of anaphase cells with binucleate mothers. Error bars show SEM; n > 200 for each strain. (B) Localization of dynein in cells containing truncated *Nip100*, displayed as Z-projections of confocal stacks. Images on the right are duplicates of those to their left, scaled such that wildtype localization is easily visible. Examples of astral MT plus-end and cortical localization are indicated with blue and white arrows, respectively. Bar = 5 (m. (C) Quantification of Dyn1-3xGFP intensity at astral MT plus-ends. Intensities are normalized to intensity in wt cells. Error bars show SEM. wt, n = 22; *ΔCAP-Gly-NIP100*, n = 8; *Δbasic-NIP100*, n = 6; *ΔCC1A-NIP100*, n = 12; *ΔCC1-NIP100*, n = 11; *ΔNIP100*, n = 11.




*Chapter 2, Figure 7*

**Chapter 2, Table 1.** All strains were made in the VY1 background.

| Strain | Genotype | Source |
|---|---|---|
| VY1 | *MATa his3-11,5 ura3-52 leu2-3,112 ade2-1 trp-1* | *Eshel et al., 1993* |
| VY2 | *dyn1Δ::HIS3* | *Eshel et al., 1993* |
| VY167 | *pep4::HIS3 ZZ-TEV-GFP-3XHA-DYN1 PAC11-13MYC::TRP prb1D NIP100-3XFLAG-KAN* | *Reck-Peterson et al., 2006* |
| VY263 | *pep4::HIS3 prb1Δ PAC11-13XMYC::TRP ZZ-TEV-GFP-3XHA-DYN1-GS-HALOTAG::KAN nip100Δ* | this study |
| VY596 | *pep4::HIS3 prb1Δ PAC11-13XMYC::TRP ZZ-TEV-3XHA-DYN1-GS-HALOTAG::KAN nip100Δ* | this study |
| VY597 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1Δ ZZ-TEV-ARP10 NIP100-GS-HALOTAG::KAN* | this study |
| VY630 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1Δ ZZ-TEV-ARP10 (ΔCAP-Gly)NIP100-GS-HALOTAG::KAN* | this study |
| VY631 | *pep4::HIS3 ZZ-TEV-GFP-3XHA-DYN1 PAC11-13MYC-TRP prb1D NIP100-3XFLAG-KAN arp1::URA3* | this study |
| VY649 | *DYN1-3xGFP::TRP1* | this study |
| VY652 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1Δ ZZ-TEV-ARP10 (Δbasic)NIP100-GS-HALOTAG::KAN* | this study |
| VY670 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1Δ ZZ-TEV-ARP10* | this study |

| | | |
|---|---|---|
| | *(ΔCC1a)NIP100-GS-HALOTAG::KAN* | |
| VY671 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1ΔZZ-TEV-ARP10* | this study |
| | *(ΔCC1)NIP100-GS-HALOTAG::KAN* | |
| VY685 | *(ΔCAP-Gly)NIP100* | this study |
| VY686 | *(Δbasic)NIP100* | this study |
| VY687 | *(ΔCC1a)NIP100* | this study |
| VY688 | *(ΔCC1)NIP100* | this study |
| VY689 | *(ΔCAP-Gly)NIP100 DYN1-3xGFP::TRP1* | this study |
| VY690 | *(Δbasic)NIP100 DYN1-3xGFP::TRP1* | this study |
| VY691 | *(ΔCC1a)NIP100 DYN1-3xGFP::TRP1* | this study |
| VY692 | *(ΔCC1)NIP100 DYN1-3xGFP::TRP1* | this study |
| VY693 | *nip100Δ* | this study |
| VY694 | *nip100Δ DYN1-3xGFP::TRP1* | this study |
| VY706 | *pep4::HIS3 prb1Δ pac11::LEU2 dyn1Δ 3xHA-ARP10* | this study |
| | *NIP100-GS-HALOTAG-TEV-ZZ::KAN* | |
| VY753 | *Nip100-3xMYC::TRP1* | this study |
| VY754 | *(Δbasic)Nip100-3xMYC::TRP1* | this study |
| VY755 | *(ΔCC1)Nip100-3xMYC::TRP1* | this study |
| VY758 | *(ΔCC1a)Nip100-3xMYC::TRP1* | this study |

# APPENDIX 1

Dynein light chain isoform function

Chapter 1 of this dissertation consists of a review of the adaptation of cytoplasmic dynein to its cellular functions through the action of several associated proteins and complexes. Only briefly touched on there was the role of the dynein light chains in this cellular adaptation, which was the focus of my earlier work in the Vale lab.

One possible mechanism by which the single cytoplasmic dynein motor could be adapted to its different transport functions is by differential assembly of dynein light chains on to the heavy chain, producing functionally distinct dynein complexes. For most of the five light chains that co-assemble with the metazoan dynein heavy chain to form the dynein holoenzyme, multiple genomic and splicing-generated isoforms exist. While there is some evidence that particular isoforms of dynein light chains might specify the cytoplasmic dynein complex for particular functions, this hypothesis had not been tested. I decided to use RNAi in *Drosophila* S2 cells to individually deplete isoforms of dynein light chains, and then assay the depleted cells for defects in different dynein functions. A list of the genomic dynein subunit isoforms in *D. melanogaster* is depicted in Table 1.

I was able to establish two robust assays for dynein function in S2 cells, by which I could test the requirement for each of the light chain isoforms. Gohta Goshima, while a postdoc in the Vale lab, had established that dynein depletion by RNAi in S2 cells caused an increase in the mitotic index of the cell population (the percentage of cells in mitosis); largely due to loss of dynein transport of spindle assembly checkpoint proteins away from the kinetochore and thus a strong delay in progression into anaphase (Goshima and

Vale, 2003)(Figure 1A). I used this phenotype to assay for dynein light chain isoforms required for dynein kinetochore function, using conditions established by Nico Stuurman and Uschi Wiedemann for high-throughput screening for mitotic factors.

I was also able to develop an assay for an interphase function of dynein, transport of Rab5-labeled endosomal compartments. S2 cells do not spontaneously spread on glass, but can be induced to spread by plating on glass adsorbed with the lectin Concanavilin A (ConA). This causes a flat "fried egg" morphology that is advantageous for microscopy, but the exact pathway by which the ConA signal is transduced into cell spreading is not known. I noticed that Rab5-GFP S2 cells plated on ConA over time shifted the Rab5 compartments from an even dispersal throughout the cell to a strong perinuclear accumulation (Figure 1B). This is similar to the phenomenon of "frustrated phagocytosis" that is observed in macrophages presented with a surface-immobilized ligand, or a phagocytosis-stimulatory particle too large to phagocytose (Takemura et al., 1986). As might be expected for centripetal transport, this perinuclear accumulation was dependent on dynein and did not occur in cells treated with dynein RNAi (Figure 1B).

Developing assays for dynein function in S2 cells was challenging; depletion of the dynein heavy chain in most cases did not cause a peripheral redistribution of organellar cargos (as has been observed upon disruptions of dynein function in some cell types), perhaps due to the absence of a clear radial array of microtubules in these cells, or due to interdependence between dynein and kinesin transport. I observed the distribution of Golgi (labeled with KDEL receptor-GFP), endoplasmic reticulum (KDEL-GFP), mitochondria (MITO-GFP), lysosomes (LAMP1-GFP), and peroxisomes (SKL-GFP) in living cells on a spinning disc and a widefield microscope, and formaldehyde-fixed S2

cells on a widefield microscope; their distribution did not change upon dynein depletion by RNAi. Dynein depletion sometimes induced a reduction or even complete loss of motility of these organelles, again indicating the interdependence of dynein- and kinesin-driven transport, but this reduction in motility was not sufficiently reproducible for use in even a small-scale, focused screen. An interesting phenomenon that I observed was that the motility of organelles increased dramatically as a function of plating time. Motility was very low soon after plating (within 1 h), and had still not fully recovered after 1.5 h. 2-4 h post-plating was the best time for observation of organelle motility. I and many others in the lab have observed the depolymerization of microtubules after plating cells in fresh medium, and Nico Stuurman traced this effect to a breakdown product in the medium, that presumably has been metabolized in conditioned medium. Microtubule repolymerization seems to be complete after about 1.5 h, however, suggesting that the defect in motility was not the simple result of the lack of microtubules. There are some suggestions in the literature that posttranslational modifications of tubulin (such as acetylation and detyrosination) that accumulate on long-lived microtubules are important for the motility of microtubule-based motors, so it is possible that at earlier time points post-plating, the microtubule cytoskeleton may be insuffiently modified for normal transport.

Using these two assays, one for mitotic dynein function and one for dynein-driven organelle transport, I tested the requirement for each of the dynein light chain isoforms. I was able to identify an isoform of the light chain roadblock, robl1, as the isoform required both for dynein endosome transport (Figure 2A) and kinetochore function (Figure 2B, C). Depletion of light chain 8 isoform 4 caused a small but reproducible

increase in endosome dispersal (Figure 2D), but no light chain 8 isoform produced a reproducible mitotic phenotype, nor did any tctex isoform. In *D. melanogaster*, only one light intermediate chain exists. The *D. melanogaster* intermediate chains were too similar to separate by RNAi, as discussed below.

A major challenge to depleting individual isoforms of dynein subunits by RNAi was presented by the high sequence similarity of many isoforms. In particular, the intermediate chains in *D. melanogaster* underwent a recent gene duplication event, and exhibit particularly high similarity in their nucleotide sequence. Revisions of the *D. melanogaster* genome sequence assembly after I had begun working on this project revealed that the intermediate chains were even more similar than had initially been indicated, and are not separable by RNAi probes (using the guideline of no 21 bp sequences with greater than 19 identical positions, and at least 500 bp of sequence in the RNAi probe). Other dynein light chains, while exhibiting less sequence similarity, were still sufficiently similar that mutiple shorter probes were required to avoid cross-reacting sequences. Whether the efficiency of RNAi in these cases was reduced could not be determined, as antibodies to individual isoforms do not exist (and would be prohibitive to generate).

These results indicated that differential assembly of the dynein holoenzyme may not be a major mechanism for adapting dynein to its multiple functions. This specification instead may be conferred by phosphorylation and by extrinsic regulators present on different dynein cargos. However, the assay I developed for dynein transport of Rab5 endosomal compartments is a robust assay for dynein interphase function that could be useful for other studies.

## MATERIALS AND METHODS

### S2 cell culture, transformation, and RNAi

Drosophila S2-I cells (original stock from Invitrogen) were cultured in Schnieder's complete medium supplemented with 10% heat-inactivated fetal bovine serum, and Invitrogen antimycotic-antibiotic cocktail (catalog number 15240). A stable cell line of S2-I cells expressing Rab5-GFP under the control of the actin 5C promoter was generated as previously described(Rogers et al., 2004) RNAi was performed as follows: Cells were pelleted in a clinical centrifuge and resuspened in serum- and antibiotic/antimycotic-free medium to 1.5 x $10^6$ cells/ml. 30 ul of cells were aliquoted per well of a 96 well plate. 1 ul (at least 2.5 ug) of dsRNA was added (produced by Invitrogen Megascript kit or homemade kit) per well, and cells were incubated for 50 minutes in a 23°C incubator. 30 uL 20% serum-containing medium was added per well. The plate was sealed with parafilm, and incubated at 23°C for 5 days.

### Immunofluorescence

Cells were plated on ConA-coated glass coverslips or glass-bottomed 96 well plates, prepared as follows: The glass surface was incubated for 6 hour with a 0.5 mg/ml solution of Concanavilin A, after which the solution was aspirated, and the glass was briefly washed with water and allowed to dry. After cell plating, cells were incubated at 23°C for 2h for observation of mitosis or 3h for observation of Rab5 compartment

perinuclear clustering. Culture medium was aspirated, and the cells were fixed in 6.4% paraformaldehyde/HL3 for 15 minutes. The fixing solution was replaced with a permeabilization solution (0.5% SDS/PBS or 0.5% Triton X-100/PBS) and incubated for 10 minutes. Cells were washed twice with 0.1% Triton X-100/PBS (PBST), and incubated for 1 h in 3% BSA/PBST. For observation of mitosis, cells were incubated with rabbit anti-phospho-histone H3 antibody (from Upstate Biotech) at 1:1000 dilution and mouse anti-tubulin antibody at 1:1000 dilution in 3% BSA/PBST, and incubated overnight at 4°C. Cells were washed three times, with a five minute incubation each time, in 3% BSA/PBST, and incubated for 1h at room temperature with 0.3 μg/ml DAPI, rhodamine-conjugated anti-mouse antibody (1:300), and Alexa 647 conjugated anti-rabbit antibody (1:300). Cells were washed three times with 3% BSA/PBST, and covered in DAKO mounting medium.

**REFERENCES**

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Rogers, S. L., Wiedemann, U., Hacker, U., Turck, C., and Vale, R. D. (2004). Drosophila RhoGEF2 associates with microtubule plus ends in an EB1-dependent manner. Curr Biol *14*, 1827-1833.

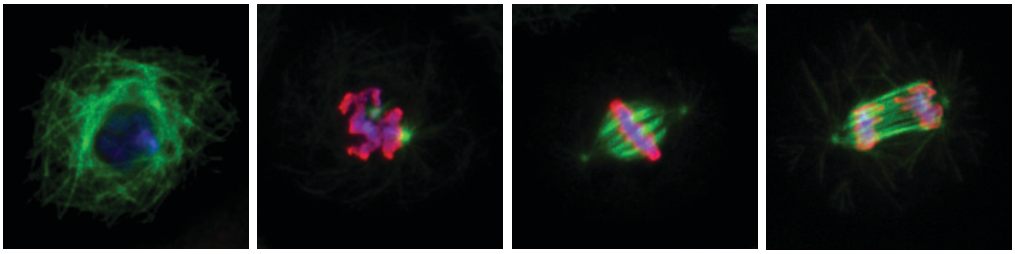
***Appendix 1, Table 1***

| | isoform | Flybase ID | Affymetrix expression | alternative name |
|---|---|---|---|---|
| dynein heavy chain | Dhc64C | CG7507 | 8.7 | |
| dynein light chains | | | | |
| light intermediate chain | LIC | CG1938 | 6.5 | Dlic2 |
| intermediate chain | IC 1 | CG18000 | | short wing, Cdic |
| | IC 2 | CG32823 | | |
| | IC 3 | CG9580 | 5.4 | Sdic |
| | IC 4 | CG10859 | 4.4 | |
| | IC 5 | CG9313 | | |
| | IC 6 | CG6053 | 4.2 | |
| | IC 7 | CG1571 | 4.1 | |
| | IC 8 | CG13930 | 6 | |
| | IC 9 | CG7051 | 4.9 | |
| | IC 10 | CG14838 | 4.7 | |
| light chain 8 | LC8 1 | CG6998 | 8.8 | cut up |
| | LC8 2 | CG5450 | 4.6 | CdLC82 |
| | LC8 3 | CG8407 | 4.7 | |
| | LC8 4 | CG6971 | 3.4 | |
| | LC8 5 | CG10839 | 4.2 | |
| | LC8 6 | CG8800 | 3.9 | |
| Tctex | tctex-1 | CG12363 | 9 | Dlc90F |
| | tctex-2 | CG7276 | 4.3 | |
| | tctex 3 | CG5359 | 5.4 | |
| | tctex 4 | CG14763 | 4.5 | |
| | tctex 5 | CG12838 | 4.7 | Tsp42Eo |
| roadblock | roadblock 1 | CG10751 | 8.2 | robl |
| | roadblock 2 | CG10838 | 6.9 | robl22E |
| | roadblock 3 | CG10834 | 3.6 | |
| | roadblock 4 | CG10822 | 5.1 | |
| | roadblock 5 | CG16837 | 5.7 | |
| | roadblock 6 | CG15171 | 5.6 | robl37BC |
| | roadblock 7 | CG31275 | 4.2 | bxd |
| | roadblock 8 | CG1014 | 4.8 | robl62A |

*Julie Hollien and Gohta Goshima, personal communication

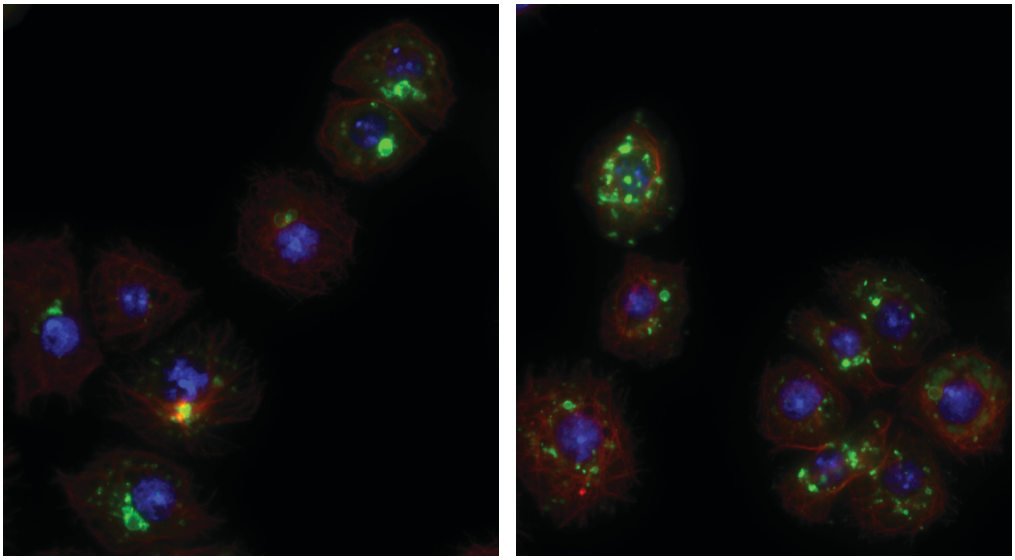
**Figure 1. Assays for dynein function in S2 cells.** (A) The most prominent phenotype resulting from dynein depletion in S2 cells is a delay of cells in metaphase and thus an overall increase in the percentage of cells in mitosis. Mitotic cells were detected by phosphohistone staining, which is not apparent in interphase cells. Cells with phosphohistone staining, without aligned chromosomes and a bioriented spindle (with microtubule arrays oriented in opposite directions from the chromatin; multipolar or unfocused spindles are common in wildtype S2 cells) were classified as prophase cells. Cells with phosphoshistone-stained and aligned chromosomes with a bioriented spinle were classifed as metaphase cells, and cells with phosphohistone staining in which separation of the chromosomes had initiated, but cytokinesis had not begun, were classified as anaphase cells. Red, phosphohistone; green, tubulin; blue, DAPI. (B) Rab5-labeled compartments form perinuclear clusters in cells plated on a Concanavlin A coated surface, while this clustering is disrupted in dynein-depleted cells. Green, Rab5-GFP; blue, DAPI. dhc: dynein heavy chain.

A



interphase prophase metaphase anaphase

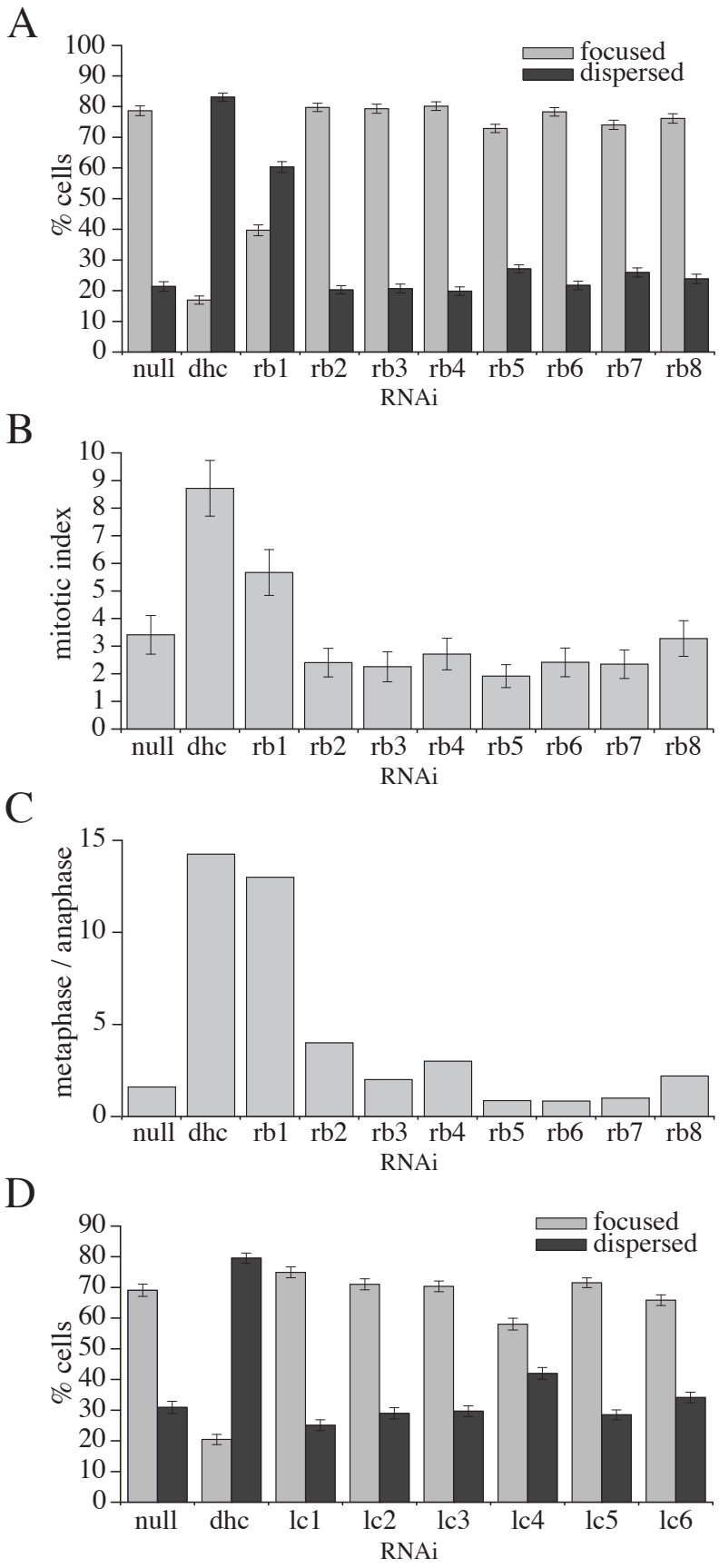
B



untreated dhc RNAi

*Appendix 1, Figure 1*

**Figure 2. The requirement for dynein light chain isoforms in dynein function in endosome transport and during mitosis.** (A) Roadblock 1 is required for dynein-mediated endosome transport. The percentage of cells with perinuclear Rab5 foci was determined using the assay described in Figure 1B. Rb: roadblock; dhc: dynein heavy chain (B) Roadblock 1 is required for dynein function in mitosis. The mitotic index of cells was determined using the assay described in Figure 1A. (C) Roadblock 1 is required for dynein function during metaphase. The percentage of mitotic cells in metaphase was determined, using the assay described in Figure 1A. (D) Light chain 4 is involved in dynein function during endosome transport. Error bars represent standard error of proportion.




*Appendix 1, Figure 2*

# Appendix 2

Dynein light intermediate chain phosphorylation is required for dynein function during prophase

In addition to the alternative assemblies of light chains discussed in Appendix 1, another mechanism by which dynein might be specified for different functions is by posttranslational modification of the dynein complex. One dynein accessory protein, the light intermediate chain (LIC), is heavily phosphorylated during mitosis (Niclas et al., 1996). The phosphorylation site has been mapped to a conserved serine, and CDK1 has been identified as the probable kinase (Addinall et al., 2001; Dell et al., 2000). It has not been determined whether LIC phosphorylation is required for dynein function during mitosis, or what its specific role might be. Mitotic phosphorylation of dynein by CDK1 induces release of dynein from cellular membranes, but it is not knownwhether this is due to LIC phosphorylation (Addinall et al., 2001; Dell et al., 2000; Niclas et al., 1996). I found that LIC phosphorylation is required for dynein function in mitosis, specifically during prometaphase.

To test the requirement for LIC phosphorylation in mitosis, I made S2 cell lines stably transfected for either wildtype 9S3, phosphomutant, or phosphomimic I depleted the endogenous LIC protein by RNAi, and induced expression of either wildtype or phophomutant LIC-GFP. I found that LIC depletion substantially reduced the levels of dynein heavy chain. Because the LIC-GFP expressing cell lines were quite heterogeneous, with some cells not expressing any transgene and some heavily overexpressing it, inducing expression of wildtype or mutant LIC-GFP did not substantially alter dynein levels in the population of cells. (Figure 1A). Therefore, to avoid characterizing a nonspecific dynein destabilization phenotype, I only included cells in my analysis within a fixed range of LIC-GFP fluorescence.

To detect defects in mitosis, I determined the mitotic index of each population, which I defined as the percentage of cells staining for phosphorylated histone 3, a mitosis-specific phosphorylation. After depletion of LIC, expression of wildtype LIC-GFP reduced the mitotic index by over two fold. However, expression of phosphomutant LIC (LIC-GFP S377A) after LIC RNAi only slightly lowered the mitotic index, indicating that LIC S377 phosphorylation is required for dynein function in mitosis (Figure 1B, C). The slight reduction in mitotic index induced by LIC S377A expression, and the incomplete rescue by wildtype LIC, are probably both due to partial rescue of the defect caused by reduction in dynein level. The mitotic index was highly variable between populations of wildtype cells, and the noise from this fluctuation made differences between conditions difficult to establish. The level of rescue obtained by expressing wildtype LIC was consistently larger than that obtained by expressing phosphomutant LIC ($p = 0.03$). A phosphomimic construct (LIC-GFP S377E) was not sufficient to rescue LIC depletion above the level observed for the phosphomutant; this was not surprising, as simple amino acid substitutions often do not successfully mimic phosphorylation (data not shown).

To determine the stage at mitosis at which LIC S377 phosphorylation is required, I measured the number of cells at different stages in mitosis (prometaphase, metaphase, and anaphase) after LIC RNAi and rescue with either wildtype or S377A LIC-GFP. This assay was considerably more robust and less noisy than a simple measurement of mitotic index. In contrast to the metaphase block observed after dynein depletion, cells rescued with LIC-GFP S377A accumulated in prophase, suggesting that LIC phosphorylation

could be important for dynein function in spindle assembly or centrosome remodeling for mitosis (Figure 1D).

## MATERIALS AND METHODS

S2 cell culture, transfection, RNAi, and immunofluorescence were performed according to the procedures described in Appendix 3A. A wildtype LIC-GFP plasmid with inducible expression was prepared by ligation of wildtype LIC cDNA into a pMT/V5-His plasmid already containing GFP. LIC-GFP S377A and S377E plasmids were prepared by primer-mediated point mutation of the wildtype plasmid. Cell lines stably transfected with LIC-GFP and mutants were induced to express their transgenes by addition of 100 μm $CuSO_4$ into the medium 24 h before observation.

## REFERENCES

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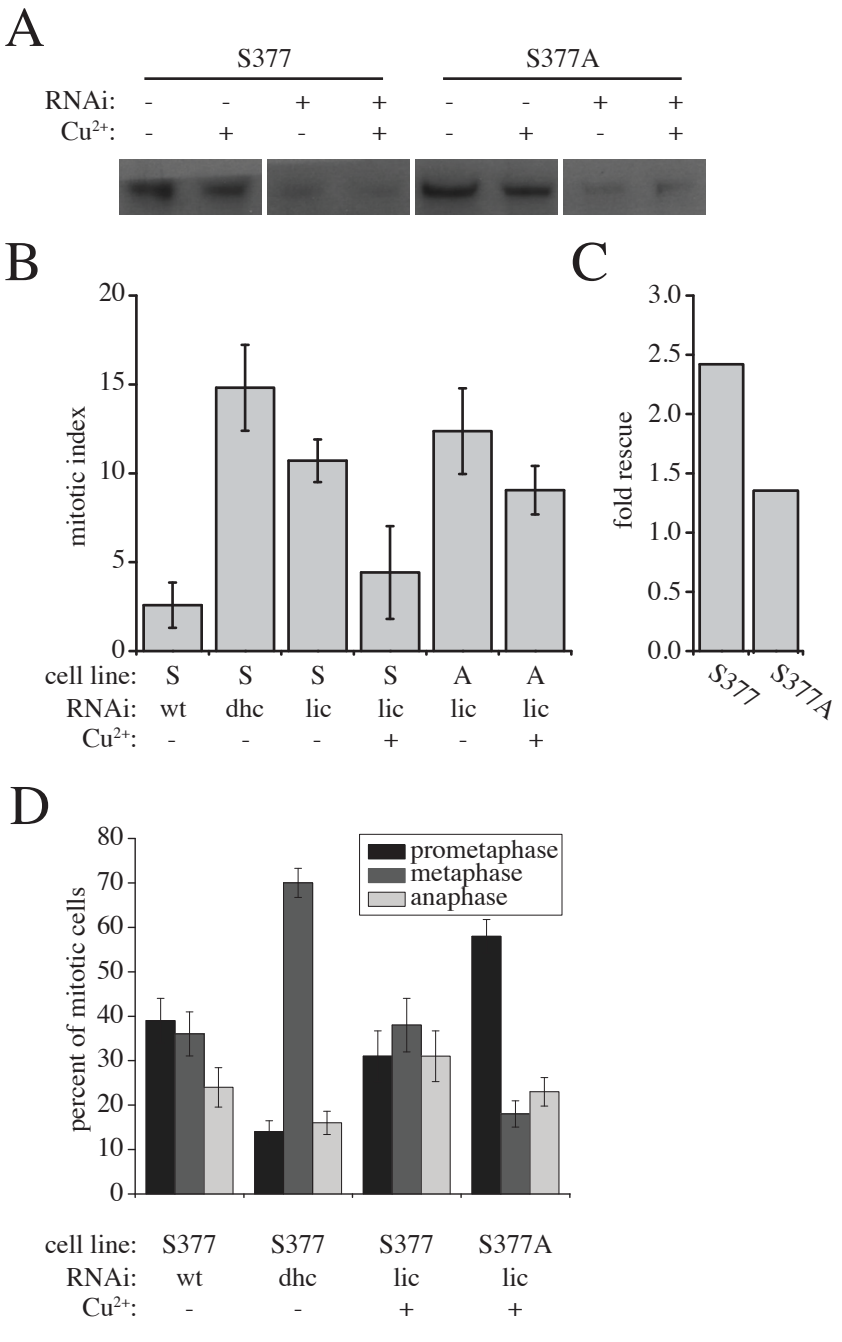
Dell, K. R., Turck, C. W., and Vale, R. D. (2000). Mitotic phosphorylation of the dynein light intermediate chain is mediated by cdc2 kinase. Traffic *1*, 38-44.

Niclas, J., Allan, V. J., and Vale, R. D. (1996). Cell cycle regulation of dynein association with membranes modulates microtubule-based organelle transport. J Cell Biol *133*, 585-593.

**Figure 1. The requirement for light intermediate chain phosphorylation in dynein mitotic function.** (A) Western blot of S2 cell lysate. LIC depletion causes a large decrease in dynein levels, and exogenous expression of light intermediate chain is not sufficient to restore normal dynein levels in the entire population of cells. (B) Mitotic index (percent mitotic cells) of cells after RNAi and expression of wt (S) and phosphomutant (A) LIC-GFP transgenes. (C) Fold rescue (reduction in mitotic index) induced in LIC-depleted cells by expression of LIC-GFP transgenes, expressed as a ratio of the mitotic indix observed in uninduced cells to the mitotic index observed in induced cells, as indicated in (B). (D) Percent of mitotic cells in prometaphase, metaphase, or anaphase of untreated, dynein-depleted, or LIC-depleted and LIC-GFP expressing cells. dhc: dynein heavy chain. Error bars represent standard error of proportion.




*Appendix 2, Figure 1*

# Appendix 3

High precision measurements of dynein-dynactin movement

The work described in Chapter 2 demonstrated that dynactin acts as a processivity factor for dynein, and that its activity is not dependent on dynein multimerization or on microtubule binding by dynactin; these findings leave the mechanism by which dynactin does act to increase dynein processivity as a mystery. Since dynactin enhances dynein's processivity without providing an extrinsic tether, it may instead influence dynein's catalytic cycle such that it steps with higher fidelity along the microtubule. To detect any change in dynein stepping behavior in the presence of dynactin, high precision tracking of dynein-dynactin complexes is required. Although I did not have time to complete these experiments, some of the methods I developed and observations I made could be useful in the future.

Many superresolution microscopic techniques have been developed; these techniques allow the position of fluorescent labels to be determined with nanometer-scale precision. This is accomplished by fitting the distribution of photons emitted from the molecule of interest to a Gaussian function to determine the center of mass. The precision of this determination is inversely related to the square root of the number of photons detected, so brightly fluorescing labels, such as Qdots, are important to increase the precision of observation sufficiently to separate 8 nm steps of the dynein motor. I tried several strategies for labeling the dynein-dynactin complex with a Qdot, but none of them produced active, labeled dynein-dynactin co-complexes. Dynein and dynactin complexes that I had engineered for Halotag-based labeling contained Dyn1 and Nip100 C-terminal HaloTag fusions, respectively (yeast strains VY596 and VY597). I labeled these complexes using a biotin-HaloTag ligand during purification, and then once the motors were bound to microtubules in a flow chamber, sequentially added streptavidin,

and then biotin conjugated Qdots. When I conjugated a Qdot to the C-terminus of Dyn1, I never observed labeled dynactin co-localizing and moving with the Qdot-labeled dynein, regardless of whether dynein and dynactin were preincubated, or whether dynactin was added to the flow chamber after dynein-Qdot conjugation. Similarly, after conjugating a Qdot to the C-terminal Nip100 HaloTag, I did not observe motile dynactin. A similar labeling strategy, using Cy5-labeled streptavidin, was also unsuccessful. These C-terminal labels are located on the AAA ring of dynein and the shoulder of dynactin, which may be in close apposition to each other in the co-complex and preclude function or co-complex formation after the addition of a large label such as a Qdot or the 200 kD streptavidin tetramer.

To avoid this possible steric clash, I prepared a yeast strain containing an N-terminal HaloTag-dynein fusion. Although I was able to purify dynein from this strain, it exhibited almost no motility. This was surprising, as N-terminal fusions of GFP with dynein (such as in VY263) are fully functional. I suspect that the single Gly-Ser linker between the HaloTag and dynein may not have been sufficient; the C-terminus of the HaloTag is tightly folded and could distort the native structure of Dyn1. With more time to test other linkers, I think that this labeling strategy could be successful.

Because of these difficulties, I performed some high-precision tracking of dynein-dynactin co-complexes using a TMR-Halotag label on the C-terminus of Nip100, which are discussed in Chapter 2. Although the organic dye TMR is much less bright and photostable than Qdots, I was able to collect enough photons from some particles to discern individual steps. Future observations with this label or with a Qdot at the N-

terminus of dynein, or with two-color observation of both labels, could yield important insight into the mechanism by which dynactin modulates the processivity of dynein.

# Appendix 4

The effects of dynein light chains on dynein processivity

An observation that intrigued me when I first began working with yeast dynein and dynactin in single molecule motility assays was that dynein subjected a final purification step of microtubule bind and release changed in both its biochemical composition and the quality of its motility. Specifically, Sam Reck-Peterson had observed that full length dynein after release by TEV cleavage from IgG sepharose beads still is bound to Nip100, Pac1, Pac11 (intermediate chain), and Dyn3 (light intermediate chain), while after a subsequent microtubule bind and release, Nip100 and Pac1 are lost, and the amount of copurifying Pac11 may be reduced (Reck-Peterson et al., 2006). Also, Sam observed that while the run length of full length dynein ($Dyn1_{471kD}$)after a microtubule bind and release was very similar to the run length of truncated, GST-dimerized dynein (GST-$Dyn1_{331kD}$), full length dynein before this step had a considerably greater run length. Since the dynein intermediate chain interacts with dynactin p150/Nip100, I wanted to use the more intact dynein complex obtained without a bind and release purification step. I observed that the dynein I used, purified from a *nip100Δ* strain (VY263), still exhibited a longer run length than wildtype dynein subjected to a microtubule bind and release (VY218), indicating that this difference in run length was not attributable to loss of dynactin, and that another processivity determinant existed within the dynein complex. I decided to quantify this difference, and to determine what other dynein subunits might affect its run length.

Using the single molecule TIRF motility assay described in Chapter 2, with the exception that the salt concentration in the assay buffer was kept at 50 mM KAc (instead of 100), I measured the run length of VY263 dynein before and after a microtubule bind and release (Figure 1A and B). This confirmed that the population of motors released

with high ATP concentrations from a microtubule affinity purification is less processive (by one third) than the population of motors before this step.

Next, I wanted to address what the source of this decrease in processivity was. It seemed likely that the coincedent loss of a dynein light chain could account for a decrease in processivity; these light chains could be lost due to the physical rigors of the microtubule bind and release treatment, or because the higher processivity of motors retaining these light chains disfavored their release from microtubules, even under very high ATP concentrations. I made deletions of each dynein light chain in the VY263 strain background, and observed the motility of dynein complexes purified from these deletion strains. I found that all light chain deficient dynein complexes exhibited reduced run length, some below that of dynein after microtubule bind and release (Figure 2C-H). The loss of multiple light chains observed after microtubule bind and release treatment could thus easily account for the reduced run length of dynein complexes so treated. What this loss of processivity represents in terms of changed mechanics of the dynein motor is unclear; because this effect was not confined to any specific light chain, but seemed to be general to loss of any light chain, I did not pursue it further.

## MATERIALS AND METHODS

Microtubule bind and release procedure was performed as described previously (Reck-Peterson et al., 2006). Yeast strain creation, protein preparations and single molecule TIRF motility assays were performed as described in Chapter 2. Strains used for protein preparation were as follows: wildtype dynein, VY263; dynein ΔIC (*pac11Δ*),

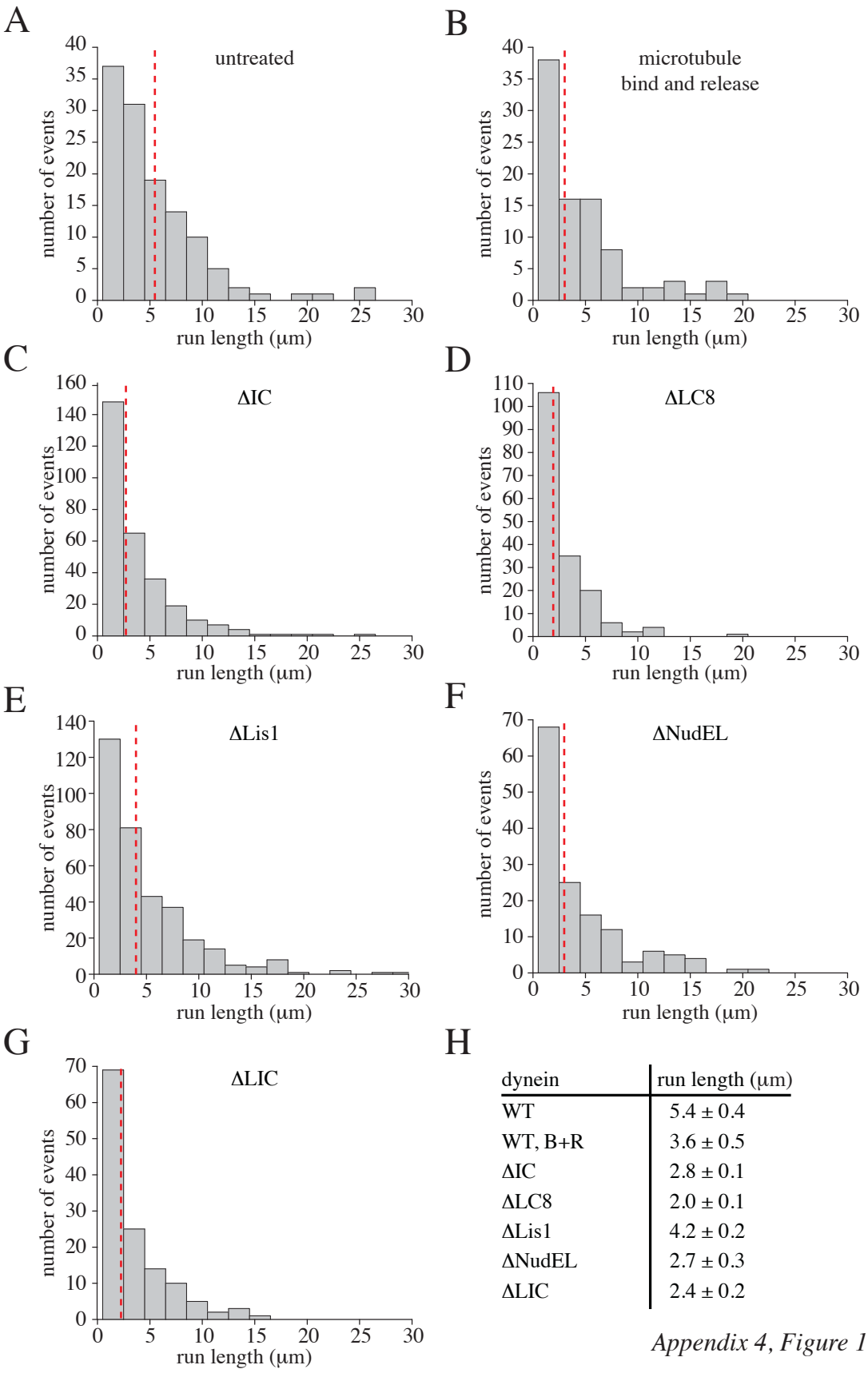
VY559; dynein ΔLIC (*dyn3Δ*), VY561; dynein ΔLis1, VY562; dynein ΔNudEL (*ndl1Δ*),

VY563; dynein ΔLC8 (*dyn2Δ*), VY564.

## REFERENCES

Reck-Peterson, S. L., Yildiz, A., Carter, A. P., Gennerich, A., Zhang, N., and Vale, R. D. (2006). Single-molecule analysis of dynein processivity and stepping behavior. Cell *126*, 335-348.

**Figure 1. The effects of microtubule bind and release treatment and dynein light chain deletion on dynein processivity.** Distribution of measured run lengths is shown for (A) Dyn1-TMR, (B) Dyn1-TMR, microtuble bind and release treated, (C) Dyn1-TMR ΔIC (*pac11Δ*) (D) Dyn1-TMR ΔLC8 (*dyn2Δ*) (E) Dyn1-TMR ΔLis1 (*pac1Δ*) (F) Dyn1-TMR ΔNudEL (*ndl1Δ*) (G) Dyn1-TMR ΔLIC (*dyn3Δ*). Red dashed lines indicate the mean. (G) Characteristic run lengths ± standard error, determined from cumulative probability functions; see Chapter 2, Methods and Fig. 6.




H

| dynein | run length (μm) |
|---|---|
| WT | 5.4 ± 0.4 |
| WT, B+R | 3.6 ± 0.5 |
| ΔIC | 2.8 ± 0.1 |
| ΔLC8 | 2.0 ± 0.1 |
| ΔLis1 | 4.2 ± 0.2 |
| ΔNudEL | 2.7 ± 0.3 |
| ΔLIC | 2.4 ± 0.2 |

*Appendix 4, Figure 1*

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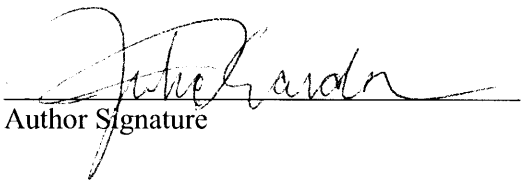

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