Novel Regulatory Mechanisms of Cytoplasmic Dynein:  
A Role for the Complex Base  

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ABSTRACT

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Cytoplasmic dynein is unique among cellular motors not only in its size and complexity but also its diversity of functions. It is essential for many mitotic and interphase transport processes and its misregulation or malfunction results in devastating neurological disorders. Over 20 years of research in the field has identified many recruitment and regulatory factors, with dynactin and NudE/L-Lis1 being the most ubiquitous and well described. Additionally we have recently gained detailed, high-resolution structures of the dynein motor domain and models for dynein stepping and mechanochemistry based on single molecule studies. Despite this progress, little is known about the structure and coordination of functions at the base of the dynein complex, where nearly all interactions with regulatory and recruitment proteins occur. The studies herein examine two mechanisms of regulation that occur through dynein’s base. First we probe the contribution of the light chains to dynein function, structure and interaction with regulators. Second we identify a novel mechanism by which dynactin increases dynein run length solely via interactions with the intermediate chain. These findings represent the new frontier in the dynein field as investigators increasingly recognize the importance of long-range dynein regulatory mechanisms.
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Chapter 1
Introduction
Introduction to Cytoplasmic Dynein

Cytoplasmic dynein 1 (referred to as ‘dynein’ hereafter) is the principle minus-end directed microtubule (MT) motor in cells. Since its discovery 26 years ago (Paschal et al., 1987), we have learned that it performs an array of essential cellular functions. Dynein is responsible for minus end transport of membranous vesicles and organelles including Golgi and ER Golgi Intermediate Compartment (ERGIC) vesicles, lysosomes, endosomes, mitochondria and the nucleus (Akhmanova and Hammer, 2010; Murshid and Presley, 2004; van Spronsen et al., 2013; Vallee et al., 2012). Additionally, dynein has a repertoire of mitotic roles. It contributes to nuclear envelope breakdown (Salina et al., 2002), focuses spindle poles and orients the spindle by anchoring astral MTs at the plasma membrane (Radulescu and Cleveland, 2010), facilitates the formation of kinetochore-MT attachment, and upon proper MT attachment, removes checkpoint proteins from the kinetochore (Mao et al., 2010). Other dynein cargo include mRNAs whose polarized localization is essential during development (Bullock, 2011), centrosomal proteins whose delivery to the centrosome is required for centrosome maintenance (Zimmerman and Doxsey, 2000), and viruses, which hitch a ride to the nucleus with dynein (Dodding and Way, 2011). Unlike kinesins, which have evolved into a diverse group of specialized motors (Hirokawa et al., 2009), a single dynein must carry out these disparate, carefully regulated tasks. How dynein achieves this is an active area of investigation in the field, and studies have focused on dynein subunit diversity as well as interactions with regulators and recruitment factors.

Dynein Structure and Mechanochemistry

Dynein is a large, 1.2 MDa complex comprising dimers of heavy chains (HCs), intermediate chains (ICs), light intermediate chains (LICs), and three pairs of light chains (LCs)
The N terminal region of the 530 kDa HC mediates dimerization and binding to the other subunits while the C-terminus contains the motor domain. The salient features of the 380 kDa motor domain include the N-terminal linker region, a 13 nm ring formed by six concatenated AAA (ATPases Associated with various cellular Activities) domains, a protruding 10 nm stalk with a distal MT binding domain (MTBD), the so-called buttress or strut at the base of the stalk, and, in non-fungal dyneins, a C-terminal globular domain (Tynan et al., 2000a; Carter et al., 2011; Kon et al., 2011) (Fig. 1-1 B). The AAA domains are numbered 1-6 by their position within the HC with AAA1 being the most N-terminal. While AAA1-AAA4 contain nucleotide binding P-loops (Neuwald et al., 1999), AAA1 is the primary site of ATP hydrolysis and is absolutely required for dynein function (Gibbons et al., 1987; Kon et al., 2004). There is extensive evidence that nucleotide binding and/or hydrolysis occurs at the other domains and contributes to dynein activity, but how this is coordinated with AAA1 activity is unclear (Kon et al., 2005; 2004; Cho et al., 2008). Interestingly, the MTBD-containing stalk emerges as an extended coiled-coil from AAA4, across the ring from AAA1, separating the sites of MT binding and ATP hydrolysis by over 20 nm (Carter et al., 2011; Kon et al., 2011) (Fig. 1-1 B). This arrangement is distinct among cytoskeletal motors, where ATP hydrolysis and filament binding are typically coupled via structural proximity (Vale, 2003).

Impressive structural and biochemical work from the Sutoh, Burgess, Vale and Carter labs over the last several years provides insight into dynein’s unique structure and mechanochemistry. They have found that movement of the linker correlates with the ATP binding and hydrolysis cycle and generates force. EM, FRET, activity assays, and crystal structures show that in the apo (no nucleotide) state, dynein binds strongly to MTs and that the linker, arising from AAA1, crosses over the ring and contacts AAA5. ATP binding and
hydrolysis releases dynein from the MT and is thought to dislodge the linker, which bends and rotates the ring, moving the MTBD away from the linker and toward the minus end of the MT. In the ADP-P\textsubscript{i} state dynein interacts weakly with the MT and upon ADP-P\textsubscript{i} release the linker returns to the original conformation and the MTBD returns to a strong binding state, thereby translocating dynein along the MT (Roberts et al., 2009; Kon et al., 2005; Roberts et al., 2012; Kon et al., 2012; Schmidt et al., 2012) (Fig. 1-1 C). The MTBD’s affinity for the MT is altered by sliding of the stalk’s anti-parallel coiled-coil (Kon et al., 2009; Carter et al., 2008), which is thought to be influenced by the linker contacts with AAA5, and the buttress emerging from AAA5 and contacting the base of the stalk (Kon et al., 2012; Schmidt et al., 2012) (Fig 1-1 B).

Though tail-less artificially dimerized dynein motor domains have been used extensively to study dynein’s mechanochemistry and motor domain structure, the N-terminal tail region is essential for dynein function inside a cell. Mutations in the HC dimerization region destabilize the complex and decrease dynein run length (Ori-McKenney et al., 2010) resulting in neuronal migration defects in the developing brain and motor neuron disease (Harms et al., 2012; Ori-McKenney and Vallee, 2011). The HC tail interacts directly with the LICs (~50 kDa) and the ICs (~75 kDa) (Tynan et al., 2000a). The ICs bind the HC through C-terminal WD domains while their unstructured N-terminus interacts with three pairs of LCs (~10 kDa) (Fig 1-1 A). Except for the HC, in mammals there are two genes for each subunit and multiple splice variants of both IC genes, allowing for substantial variability in composition of the base (Pfister et al., 2005; Vaughan and Vallee, 1995; Kuta et al., 2010). A recent attempt to build recombinant dynein from the individually expressed subunits reveals that the base is required for dynein’s structural integrity. Recombinant HCs expressed in insect cells are highly insoluble unless both ICs and LICs are added, and EM images of HC-LIC-IC complexes show the motor domains
splayed away from each other, suggesting that LCs may stabilize the complex (Trocker et al., 2012). Given dynein’s size and complexity, in vitro data have mostly been gathered from mammalian dynein purified from brain and full dynein complexes or motor domain constructs expressed and purified in *D. discoideum* or *S. cerevisiae*.

**Figure 1-1. Dynein Structure and Mechanochemistry.** (A) The dimeric arrangement of dynein subunits (adapted from (Barbar, 2012)). The heavy chains (HCs) have a C-terminal motor domain and N-terminal tail, which interacts with the light intermediate chains (LICs) and intermediate chains (ICs). The IC C-terminal WD domain binds the HCs and the unstructured N-terminus interacts with three pairs of light chains (LCs) from the TcTex, LC8, and LC7/Roadblock families. Note that LICs form a dimer, which is not depicted here. (B) Crystal structure of the *D. discoideum* dynein motor domain (adapted from (Kon et al., 2012)). Six AAA domains are arranged in a ring with the stalk and strut/buttress extending from AAA4 and AAA5, respectively. The linker and C-terminal domain (C-sequence) lie on opposite faces of the ring. The MTBD at the tip of the stalk is not visible. Below is a color coded diagram of the HC domains. (C) The dynein power stroke cycle (adapted from (Roberts et al., 2012)). In the apo state, dynein binds the MT strongly and the linker contacts AAA5. ATP binding and hydrolysis trigger release from the MT and a series of conformational changes that dislodge and bend the linker, causing the MTBD to move along the MT. Upon ATP-Pi release the linker and MTBD return to their original conformation and dynein binds to the MT again.
Dynein Biophysical and Biochemical Properties

The biochemical and biophysical properties of dynein have been studied extensively and several groups have made significant advances in understanding dynein’s movement at the single molecule level. Mammalian dynein is purified in part via its ATP-dependent release from MTs (Paschal et al., 1991) and the kinetics of its MT-stimulated ATP hydrolysis under varying conditions have been characterized (Shpetner et al., 1988). Dynein’s activity and minus end specificity were originally confirmed by MT gliding assays, where dynein is immobilized in on a coverslip and powers the movement of MTs (Paschal and Vallee, 1987). Single molecule measurements of velocity, run length, step size and force became possible with the advent of optical trapping, by attaching motors non-specifically to carboxylated beads and measuring their movements on immobilized MTs (Wang et al., 1995; Svoboda and Block, 1994). More recent developments have made use of TIRF microscopy and various fluorescent moieties to label motors directly, though these methods clearly do not yield force measurements and are more easily applied to genetically tractable organisms, such as *S. cerevisiae*. Importantly, some of the biochemical and biophysical characteristics of *S. cerevisiae* dynein differ from those of mammalian dynein, potentially due to the loss of the C-terminal domain of the HC in most fungal dyneins (Hook, 2010). At saturating ATP concentrations mammalian dynein glides MTs at average speeds ranging from 0.6-1.25 μm/sec (Steffen et al., 1997; Paschal et al., 1987) and reported single molecule average speeds are similar at 0.7-1.0 μm/sec (King and Schroer, 2000; Mckenney et al., 2010; Ori-McKenney et al., 2010). Average mammalian dynein run lengths are 0.4-0.9 μm, and single molecules can produce up to 1.1-1.4 pN of force (King and Schroer, 2000; Mckenney et al., 2010; Wang et al., 1995; Ori-McKenney et al., 2010). In budding yeast values for MT gliding and single molecule velocities at saturating ATP concentrations are
similar to each other but approximately 10-fold slower than in mammals, averaging ~80 nm/sec. Average run length varies from 1-2 μm, and a single molecule can produce up to 7 pN of force (Kardon et al., 2009; Reck-Peterson et al., 2006; Gennerich et al., 2007). Both yeast and mammalian dynein take steps ranging from 8 nm to 32 nm and step size is shortest when the motor is under load (Mallik et al., 2004; Gennerich et al., 2007). Variable step size may confer multi-motor cooperative properties that are not observed with kinesin (Rai et al., 2013). Two recent studies used artificially dimerized yeast motor domains where each monomer was labeled with a different fluorophore, to show that dynein stepping is generally uncoordinated. They observe frequent back steps and repeated stepping by the same head and conclude that there is little communication between heads to prevent their simultaneous dissociation from the MT (DeWitt et al., 2011; Qiu et al., 2012). However, Qiu et al. note that as inter-head distance increases, the likelihood of more coordinated, alternate-head forward stepping also increases. Given there is much room for improvement, it is tempting to speculate that other dynein subunits or interaction partners may enhance inter-head coordination.

**Dynein Subunit Contributions to Function**

Soon after dynein’s discovery, researchers characterized dynein subunits and found that there are two genes for each (excluding the HC) and many isoforms of the IC (Pfister et al., 2006). Naturally theories began to develop that dynein is adapted for particular functions by incorporating different subunit genes or isoforms that interact solely with specific recruitment or regulatory factors. Indeed there is some evidence for this, however we still lack data about the specific biological role of many dynein subunit isoforms. Additionally, there is a growing body of structural information that underscores non-HC subunits contributions to dynein stability.
Intermediate Chains

The dynein intermediate chains have emerged as the nexus of dynein recruitment and regulation. In addition to interacting with dynactin and NudE/L, ubiquitous dynein regulation and recruitment factors, they can independently recruit a growing list of proteins (Fig. 1-2 A). Mammals contain two genes, IC1 and IC2, with six and eleven alternate splice variants, respectively. Except for one IC2 variant (IC-2C) all are found exclusively in the central nervous system or embryonic tissue (Kuta et al., 2010), suggesting specialized functions in these tissues. With the exception of two studies showing increased association of TrkB endosomes or mitochondria with different splice forms (Ha et al., 2008; Mitchell et al., 2012), the significance of different IC genes and alternative splice forms remains largely unknown. Note that in the following discussion specific residues will refer to rat IC-2C (Accession number NM_053880) numbering.

All splice sites (Kuta et al., 2010), several phosphorylation sites (Vaughan et al., 2002; Whyte et al., 2008; Mitchell et al., 2012), the dimerization region and LC interaction sites (Nyarko and Barbar, 2010) and non-dynein protein binding sites (see Fig. 1-2 and below for references) are located in the N-terminal half of the ICs, while the C-terminal region binds the HCs and is predicted to contain seven WD domains (King, 2000; Paschal et al., 1992; Ma et al., 1999) (Fig. 1-2 A). Most of the IC N-terminus is unstructured, though the first 60 amino acids are predicted to form a coiled coil and there is increased order upon LC binding (Barbar, 2012). Importantly, NudE/L and dynactin p150 bind overlapping sites in the first 70 IC residues (Mckenney et al., 2011; Nyarko et al., 2012), the only example of mutually exclusive interactions with the dynein complex thus far, and which may have profound regulatory consequences. In addition the IC N-terminal region interacts with several proteins thought to
recruit dynein to specific locations (Fig. 1-2 A, Fig. 1-5 and Table 1-2). In particular, huntingtin and golgin 160 interact with the IC N-terminus and both are involved in recruiting dynein to membranes. Huntingtin is implicated in dynein mediated transport of Golgi, lysosomes, and endosomes and golgin160 interacts with ATP-Arf1 and can directly recruit dynein to the Golgi to maintain its pericentriolar organization (Caviston et al., 2011; 2007; Yadav et al., 2012). The neuronal SNARE binding protein snapin also interacts directly with IC1 in the same region as the LCs, and its interaction recruits dynein to late endosomes/lysosomes and TrkB signaling endosomes in neurons (Cai et al., 2010; Sheng et al., 2012). In blot overlay assays Zw10 binding to IC is increased by phosphorylation at tyrosine 89, suggesting Zw10 may interact directly with IC in this region and contribute to dynein recruitment to kinetochores (Whyte et al., 2008) or membranes (Varma et al., 2006; Civril et al., 2010).

Two other phosphorylation sites in this region have been identified, S81 and S84, and all three sites are present in both IC homologues (Pullikuth et al., 2013; Vaughan et al., 2001). T89 appears in all splice variants, but some IC1 splice forms lack S81 and S84 (Fig. 1-2 B). Importantly, the relative positions of these residues vary among splicing forms (Kuta et al., 2010) (Fig. 1-2). IC-2C phosphorylated at T89 and S84 individually decrease the interaction of dynactin p150 with dynein, though these residues are outside of the defined p150 binding region and in some splice variants may be more than 40 residues away (Whyte et al., 2008; Vaughan et al., 2001) (Fig. 1-2 A). Phosphorylation at S80 in one IC1 isoform (equivalent to S81 in IC-2C) was observed to increase association of dynein with Rab7 and TrkB vesicles in hippocampal neurons, though the underlying mechanism is not understood (Mitchell et al., 2012).
Figure 1-2. Dynein Intermediate Chain Map (modified from Kuta et al., 2010). (A) The longest IC1 (top) and IC2 (bottom) isoforms. Exons are in grey and the shortest splice variants lack the regions bracketed in yellow. DYNLT, DYNLL and DYNLRB specify Tctex, LC8, and Roadblock light chain family binding regions, respectively. DIM is the IC dimerization region. Note that a concurrent study found the dimerization and Roadblock binding regions overlap (Hall et al., 2010). Orange arrows indicate described phosphorylation sites (Vaughan et al., 2001; Whyte et al., 2008; Mitchell et al., 2012; Pullikuth et al., 2013) and black brackets delineate interaction regions with other proteins (Yadav et al., 2012; Caviston et al., 2007; Cai et al., 2010; Mckenney et al., 2011). The studies identifying snapin, huntingtin and golgin 160 interactions did not specify which IC isoforms were used, except that an IC1 interacted with snapin. Direct Zw10 interaction is questionable since it has not been observed with native proteins. (B) Magnification of IC regions involved in alternative splicing. Yellow regions are removed in shortest splice forms. The orange and black arrows indicate phosphorylation sites and alternative splicing sites, respectively.

It remains an open question if IC homologues or splice forms specify dynein interactions. The LCs are predicted to interact with both IC homologues and all splice forms and this has been verified with several IC isoforms for the Tctex and LC8 classes of LCs (Lo et al., 2007b). All IC isoforms tested so far interact with NudE and dynactin p150 (Mckenney et al., 2011). IC binding...
sites for other proteins have not been sufficiently defined to determine whether they have isoform-specific and/or mutually exclusive interactions. Furthermore several of these proteins are especially important in neuronal dynein function where all IC isoforms are expressed. A more detailed examination of isoform specific interactions may provide clues as to how dynein is adapted for specific functions.

**Light Chains**

The three families of dynein light chains are LC8, Tctex, and Roadblock. Dimers from each family have distinct binding locations in the N-terminal region of the IC (Fig. 1-1 A) (Nyarko and Barbar, 2010), allowing all three LC families to occupy the IC simultaneously (Fig. 1-3). The Tctex site is most N-terminal followed closely by the LC8 site, while the Roadblock site sits approximately 100 residues downstream and overlaps with the IC dimerization region (Hall et al., 2010). The identification of many non-dynein LC binding partners as well as the combinatorial variability provided by two mammalian genes from each of the three LC families (Pfister et al., 2006) propagated the hypothesis that LCs are cargo adaptors. However there are few examples where this has been demonstrated clearly. LCs are highly conserved and members of each family are found in axonemal and cytoplasmic dynein from most organisms (Pfister et al., 2006). LC8 and Roadblock family genes are essential and knockdown of many of the LC genes affect dynein activities (Varma et al., 2010; Raaijmakers et al., 2013; Palmer et al., 2009).

The two LC8 genes, LC8 1 and LC8 2 (a.k.a. DYNLL1 and DYNLL2), differ by only six amino acids and appear not to have distinct functions (Pfister et al., 2006). Most studies have focused on LC8 1 (henceforth referred to as LC8) so the following discussion will not include LC8 2. LC8 binds over 60 different proteins that participate in disparate cellular functions (Rapali et al., 2011a) (Table. 1-1) and a large amount of the cellular LC8 population is not
associated with dynein (Lo et al., 2007b) (Fig. 2-4). Sequence analysis of LC8 binding regions from over 40 proteins identified a loose consensus sequence (Rapali et al., 2011b) (Fig. 2-6 D), suggesting that interactions with different proteins occur through the same amino acids on LC8. This was confirmed by several NMR and crystal structures of LC8 bound to different proteins (Gallego et al., 2013; Fan et al., 2001; García-Mayoral et al., 2010; Lightcap et al., 2008), including dynein IC (Williams et al., 2007). All structures show dimeric LC8 binds to unstructured regions in dimeric binding partners, which interact with interdimer grooves formed between LC8 monomers. This tetrameric arrangement likely precludes the formation of ternary complexes between LC8 and two binding partners, implying LC8 does not link dynein to other proteins. In theory each binding site on an LC8 dimer could interact with different proteins but there are few examples of complexes formed between dynein IC, LC8 and another protein (Lee et al., 2006; Fejtova et al., 2009; Navarro et al., 2004), and for most cases the protein involved also interacts with other dynein subunits or adaptor proteins (Wilson and Holzbaur, 2010; Mckenney et al., 2011; Splinter et al., 2012). Much evidence suggests that LC8 functions instead to stabilize the dimeric state of its interacting partners (Barbar, 2008; Rapali et al., 2011a), and it increases the order in the IC dimerization region upon binding (Nyarko et al., 2004; Nyarko and Barbar, 2010). Furthermore, some LC8 binding partners have several tandem LC8 binding regions and interact with multiple LC8 dimers (Rapali et al., 2011a). For example, the *S. cerevisiae* nuclear pore protein, Nup159 has six LC8 binding sites in a disordered region and LC8 is required for Nup159 self-association and nuclear export of mRNA (Stelter et al., 2007; Nyarko et al., 2013). While LC8 inhibition has broad effects on dynein-mediated activities (Raaijmakers et al., 2013; Palmer et al., 2009; Varma et al., 2010), it is difficult to determine
how many of these are dynein specific, given LC8’s vast number of potentially dynein-independent interactions.

Table 1-1. LC8 Interacting Proteins (adapted from (Rapali et al., 2011a)). Only LC8 binding proteins where the LC8-interacting sequence is known are depicted. Highlighted proteins have multiple, tandem LC8 binding regions.

<table>
<thead>
<tr>
<th>Protein name</th>
<th>Organism</th>
<th>Uniprot</th>
<th>Paralog/ortholog</th>
<th>Sequence</th>
<th>First residue</th>
</tr>
</thead>
<tbody>
<tr>
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<td>Adenovirus</td>
<td>P11826</td>
<td>DYNLL1</td>
<td>C1TVKSQTV</td>
<td>104</td>
</tr>
<tr>
<td>ABC1 (BCAS1)</td>
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<td>DYNLL1/Q57898</td>
<td>KRLMQQYTD</td>
<td>563</td>
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<tr>
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<td>O43313</td>
<td>DYNLL1/Q57898</td>
<td>L6SDKM8TD</td>
<td>665</td>
</tr>
<tr>
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<td>ASF virus</td>
<td>Q4TWM2</td>
<td>DYNLL1/Q57898</td>
<td>VTTQNTAQM</td>
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<tr>
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<td>DYNLL1/2</td>
<td>PTTANIQTE</td>
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The Tctex genes, Tctex1 and RP3 (a.k.a. DYNLT1 and DYNLT3), are closely related to each other and structurally homologous to LC8 (King et al., 1998; Williams, 2005). Tctex1 and RP3 can heterodimerize but only homodimers interact with dynein IC (Lo et al., 2007b). Their expression patterns are similar, though RP3 is expressed at higher levels in brain and liver tissue (King et al., 1998). Tctex1 and RP3 are found predominately in the dynein complex (Lo et al., 2007b) and their interaction with the IC is mutually exclusive (Tai et al., 2001) indicating they may adapt dynein for specific functions. Accordingly, Tctex1 specifically recruits rhodopsin containing vesicles to dynein (Tai et al., 1999; 2001) and inhibits the RhoGEF, Lfc, in a dynein-dependent manner (Meiri et al., 2012). Other Tctex1 interacting proteins have been identified, but their association with the dynein complex has not been demonstrated (Bauch et al., 1998; Campbell et al., 1998; Nagano et al., 1998) and some studies have shown dynein independent functions for Tctex1 and RP3 (Sachdev et al., 2007; Chuang et al., 2005; Yeh et al., 2005; Li et al., 2011). Tctex1 but not RP3 is needed for dynein kinetochore localization, ER to Golgi transport, and normal Golgi and recycling endosome distribution, though there is disagreement in the literature about the requirement of each protein for mitotic progression (Lo et al., 2007a; Raaijmakers et al., 2013; Palmer et al., 2009; Varma et al., 2010). It is unclear what role Tctex1 plays in these processes and why RP3 is not required. Tctex1 may be structurally important as binding of either LC8 or Tctex1 to the IC enhances the interaction of the other LC (Hall et al., 2009) and potentially contributes to stability of the IC dimer. Several proteins have multiple adjacent LC8 binding regions (Table 1-1), including Pac11 (Stuchell-Brereton et al., 2011), the dynein IC homologue in *S. cerevisiae*, which contains two LC8 binding sites and lacks genes for the Tctex and Roadblock LCs. Recently dynein purified from yeast with the LC8 gene deleted was shown to have reduced run lengths and decreased association between the ICs and HCs (Rao
et al., 2013). Given the structural homology of the Tctex and LC8 families, it is tempting to speculate that Tctex1 might also stabilize the dimeric state of IC or other proteins, but again, it is not obvious why Tctex1 and RP3 would have different effects on IC structure. To date, no structural studies have been performed with RP3 and though it competes with Tctex1 for binding to the IC, the homologues are only 53% identical and there may be important uncharacterized structural differences.

Of the LCs the Roadblock family, RB1 and RB2 (a.k.a. DYNLRB1/DYNLC2A/LC7/km-23 and DYNLRB2/DYNLC2B) is the least studied, despite having the most aliases. TGFβ and Rab6 interact with RB1, but is it unclear if they recruit the dynein complex (Wanschers et al., 2008; Tang et al., 2002). The only characterized Roadblock gene in lower eukaryotes is required for axonal transport of synaptic cargo and mitotic progression in *D. melanogaster* (Bowman et al., 1999) and for spindle orientation and organelle transport in *C. elegans* (Kimura and Kimura, 2011; Couwenbergs et al., 2007). In HeLa cells RB1 is necessary for almost all dynein functions (Raaijmakers et al., 2013; Palmer et al., 2009) whereas RB2 had no significant functions in one study (Raaijmakers et al., 2013) and was not detectable in another (Palmer et al., 2009). For most of these processes it is unclear if RB1 is a recruitment factor or has a structural role, however Raaijmakers and coworkers show that RB1 knockdown prevents dynein mediated anchoring of the centrosome near the nucleus in prophase, despite proper dynein localization at the nuclear envelope. Structural data suggest that LC8 and *D. melanogaster* Roadblock have opposing effects on the IC region between their binding sites (Nyarko and Barbar, 2010), and they may modulate IC dimer stability or accessibility of this region to binding partners (Fig. 1-3).
Long heralded as dynein adaptors, there is surprisingly little evidence that light chains can commit dynein to specific functions. Where there are apparent functional differences between light chain homologues, the structural basis of these differences is not clear. Despite observations that phosphorylation can regulate LC8 and Tctex1 interactions with dynein (Song et al., 2007; Chuang et al., 2005), it is not known whether specific populations of dynein lack LCs and what physiological role LC-less dynein would play. The structural data imply that LCs modulate IC dimerization and/or accessibility but further studies of the whole dynein complex with specific LC combinations are required to determine LC recruitment and structural functions. The research in Chapter 2 will address LC effects on dynein activities, possible mechanisms for these effects, and a dynein independent interaction between LC8 and the dynein co-factor NudE.

It should also be noted that many viruses interact with LCs, particularly LC8, but almost none have been conclusively linked to dynein via the LCs (Dodding and Way, 2011; Merino-Gracia et al., 2011). Work from our lab has shown that LIC1 recruits Adenovirus to dynein during infection and finds no role for the LCs (Bremner et al., 2009). If LCs do not recruit...
dynein to viral particles, it will be important and interesting to determine what advantage, if any, they gain through LC interactions.

*Light Intermediate Chains*

In vertebrates there are two LIC genes encoding LIC1 and LIC2, which are 75% homologous yet they do not heterodimerize and cannot simultaneously interact with dyenin HCs (Tynan et al., 2000b) indicating there are distinct LIC1 and LIC2 containing dynein complexes. Pericentrin interacts only with LIC1 (Tynan et al., 2000b), while Par3 is LIC2 specific (Schmoranzer et al., 2009). Thus LIC1-dynein may transport pericentrin to the centrosome (though this is also dynactin dependent) (Young et al., 2000), while LIC2-dynein is anchored at the plasma membrane by Par3 where it pulls on the MT array to move the centrosome in migrating fibroblasts (Schmoranzer et al., 2009). LICs have also been differentially implicated in membranous cargo transport, though results are not consistent among labs. One study found that LIC1 RNAi disrupts ERGIC and Golgi distribution as well as ER to Golgi transport, while LIC2 RNAi specifically disrupts recycling endosomes (Palmer et al., 2009). However, our lab showed that knockdown of LIC1 and 2 does not affect the Golgi or early endosomes, but both LICs were required for normal lysosome distribution and LIC1 can recruit dynein to lysosomes in a dynactin independent fashion (Tan et al., 2011). Importantly in both studies LIC knockdown did not affect dynein sedimentation on a sucrose gradient suggesting the effects are not due to structural changes in the dynein complex, though LICs are necessary for the solubility of recombinant dynein complex (Troktor et al., 2012). Other studies imply that the LICs are redundant since both interact with FIP3 to recruit dynein to recycling endosomes via Rab11 (Horgan et al., 2010b; a). Furthermore, in mitosis inhibition of spindle pole focusing, dynein kinetochore localization, chromosome alignment, and progression through mitosis were only
seen when both genes were silenced (Raaijmakers et al., 2013). LIC redundancy may be a relic from earlier forms of dynein that contain only one LIC gene as observed in non-vertebrate organisms (Pfister et al., 2006). *C. elegans* LIC recruits dynein to the nuclear envelope through its interaction with the KASH-domain containing ZYG-12 (Malone et al., 2003) (Fig. 1-5 A and Table 1-2), however LIC interacting proteins that may contribute to LIC-dependent mitotic functions have yet to be identified, leaving open the possibility that loss of LICs perturbs dynein function through an undetected structural or mechanochemical alteration.

**Dynein Recruitment and Regulation by Dynactin and NudE/L-Lis1**

The previous sections described how the ICs and LICs interact directly with various proteins that recruit dynein to specific locations or organelles. However, the majority of dynein functions require the recruitment and regulatory complexes NudE/L-Lis1 and/or dynactin. Though recruitment is a form of regulation, here regulation will refer specifically to alterations in dynein’s mechanochemical output. Importantly, NudE and the p150 subunit of dynactin compete for binding to dynein’s IC (Mckenney et al., 2011; Nyarko et al., 2012), suggesting that they may adapt dynein for specific functions. The following discussion will first describe dynactin’s structure and involvement in dynein activity, which is also the subject of research conducted in Chapter 3, followed by a brief overview of NudE-Lis1 roles in dynein functions.

**Dynactin Structure**

Like dynein, dynactin is a large 1.2 MDa complex comprising 11 different subunits visible in EM images as a 40 nm filament-like base with a 20 nm projecting arm (Fig. 1-4 A). The base contains an estimated seven actin related protein 1 (Arp1) subunits and one bona fide β actin subunit, capped by the conventional actin capping protein dimer CapZ αβ on one end, and by a heterotetrameric complex of actin related protein 11 (Arp 11), p62, and p25/p27 on the other
(Schroer, 2004). Unlike Arp11 and p62, p25 and p27 are dispensable for dynactin complex stability (Yeh et al., 2012). Dynactin’s projecting arm is formed by the N-terminal region of dimeric p150, which is a predicted 50nm long coiled-coil terminating in a region enriched in basic residues and a CAP-Gly (Cytoskeleton Associated Protein-Glycine rich) domain (Fig. 1-4 B). The C-terminal half of this coiled-coil region is highly conserved and interacts with the dynein IC (Gill et al., 1991; Mckenney et al., 2011) (Fig. 3-1 C). A second predicted coiled-coil in the p150 C-terminal region is thought to mediate the interaction with the Arp1 filament (Waterman-Storer et al., 1995) and mutants lacking this region are not incorporated into the complex (McGrail et al., 1995). The base of the projecting arm contains a tetramer of p50 and dimer of p24, which form a stable complex with p150. Overexpression of p50 releases p150 and p24 from the Arp1 complex, suggesting it mediates the interaction of the sidearm and the base (Echeverri, 1996). The role of p24 is least well understood, but studies in S. cerevisiae and C. elegans, indicate that p24 mediates the interaction between p50 and p150 (Amaro et al., 2008; Terasawa et al., 2010), though its overexpression has no effect on dynactin complex stability (Quintyne et al., 1999).

Though several dynactin subunits are alternatively spliced, p150 variants have received the most attention since they alter dynactin’s interaction with MTs (Hammesfahr and Kollmar, 2012). The most notable variant, p135, was initially noticed in SDS gels of dynactin preparations from brain and lacks the entire CAP-Gly region and half of the basic region. It is neuron specific and does not heterodimerize with p150 (Tokito et al., 1996). Splicing also occurs in the basic domain by altering inclusion of three exons. Full-length p150 binds MTs in vitro (Dixit et al., 2008) and decorates MTs or MT plus ends upon overexpression in cells (Waterman-Storer et al., 1995; Dixit et al., 2008; Vaughan et al., 2002) while p150 lacking exons
in the basic domain has a decreased affinity for MTs and exclusively tracks MT plus ends \textit{in vivo} (Dixit et al., 2008). Though tyrosinated \(\alpha\)-tubulin and the MT plus end proteins EB1 and CLIP-170 contain the CAP-Gly binding EEY/F motif (Weisbrich et al., 2007), this finding suggests that the basic domain mediates the interaction with tubulin while the CAP-Gly domain is required for plus tip binding. Furthermore C-terminally truncated p150 adsorbed non-specifically to beads diffuses along MTs independently of the CAP-Gly domain (Culver-Hanlon et al., 2006). The cell can also control p150’s association with MTs via phosphorylation at serine 19, N-terminal to the CAP-Gly domain. \textit{In vitro} S19 can be phosphorylated by PKA and Aurora A, and in mammals the latter is required for removal of p150 from spindle poles during anaphase and for central spindle formation (Vaughan et al., 2002; Reboutier et al., 2013). The exact contribution of dynactin’s MT-interacting domains to dynein’s function is of considerable controversy and will be addressed in the following section and in Chapter 3.

![Dynactin Structure and p150 Domain Map](image)

\textbf{Figure 1-4. Dynactin Structure and p150 Domain Map.} (A) Model of the dynactin complex based on negative stain EM images (B) (Adapted from (Schroer, 2004)). (C) Dynactin p150 functional domains. The N-terminus contains MT-binding CAP-Gly and basic domains (+). The first coiled-coil mediates dynein interaction and C-terminal regions are involved in incorporation into the dynactin complex and binding to membranous cargo.
**Dynactin Effects on Dynein Mechanochemistry**

Dynactin first emerged as a contaminant in dynein preparations from calf and chick brains (Paschal et al., 1993; Gill et al., 1991). Initial studies showed it was necessary for dynein-based vesicular movement *in vitro*, thus it was named dynactin (*dynein activator*) (Gill et al., 1991; Schroer and Sheetz, 1991). Though these studies showed that dynactin was not required to recruit dynein to purified vesicles, dynactin does link dynein to many subcellular structures (discussed in detail below). Additionally, there is extensive evidence that dynactin can modulate dynein’s activity *in vitro* and *in vivo* by increasing dynein run lengths (also termed processivity). The first studies to carefully examine dynactin effects on dynein’s single molecule activity found that dynactin could increase dynein’s processivity two-fold, an effect that was dependent specifically on the basic domain in the MT binding region of p150. Dynactin did not alter dynein velocity, or curiously, its MT dependent ATPase activity (King and Schroer, 2000; Culver-Hanlon et al., 2006). Subsequent studies, however, suggested that dynactin increases dynein processivity independently of the p150 MT interacting domain (Kardon et al., 2009; Kim et al., 2007). It has also been observed that dynein-dynactin complexes can move processively towards plus and minus ends of microtubules (Ross et al., 2006) but that force production of the co-complex was similar to dynein alone (Schroeder et al., 2010). The ambiguity of these data is the subject of the research described in Chapter 3.

**Cellular Functions of Dynactin**

In addition to altering dynein’s activity, dynactin recruits dynein to various subcellular locations, most prominently to membranous organelles (Fig. 1-5 A and B). Overexpression of dynactin p50, which displaces p150 from the dynactin complex, causes massive disruption of the Golgi apparatus, early endosomes and lysosomes (Echeverri, 1996; Burkhardt et al., 1997).
Interactions between Golgi associated spectrin and Arp1 recruit dynactin and dynein to membranes (Muresan et al., 2001; Holleran et al., 2001; Holleran, 1996) so that p50 overexpression separates the dynein binding region of dynactin from the cargo binding region. Dynein and dynactin are also recruited to the Golgi-associated small GTPase Rab6, which interacts directly with p150 and with BicD1 and BicD2, which is in turn thought to bind p50 as well as dynein (Short et al., 2002; Matanis et al., 2002; Hoogenraad et al., 2001; 2003; Splinter et al., 2012). Interactions with ER to Golgi vesicular cargo occur via the p150 C-terminus binding to the COPII protein, Sec23 and ERGIC Transit Particle Protein (TRAPP) and overexpression of the p150 C-terminus sequesters cargo in the ER (Zong et al., 2012; Watson et al., 2004) (Fig. 1-4 C and 1-5 A).

The SNARE interacting NRZ complex (NAG/Rint1/Zw10) is also implicated in dynein-based vesicle transit between the ER and Golgi (Civril et al., 2010; Hirose et al., 2004) (Fig. 1-5 A). Zw10 RNAi decreases dynein signal at the Golgi, disperses the Golgi, endosomes and lysosomes (Varma et al., 2006) and prevents exit of ER cargos (Hirose et al., 2004). Since p50 binds Zw10 (Starr et al., 1998), dynactin was originally thought to link membrane-associated Zw10 to dynein, however Rint1 and p50 compete for the same binding site on Zw10 (Inoue et al., 2008) suggesting that Zw10 may recruit dynein directly through dynein’s IC, as has been observed at kinetochores (Whyte et al., 2008) or possibly through NudE/L (Stehman et al., 2007). How Zw10 participates in lysosome or endosome transport has not been determined.

On lysosomes the Rab7 Interacting Lysosomal Protein, RILP recruits dynactin and dynein and interacts directly with p150’s C-terminus (Johansson et al., 2007; Jordens et al., 2001) (Fig. 1-4 C and 1-5 A). However, dynein localization on RILP positive lysosomes is dependent on LIC1 (Tan et al., 2011), which also interacts directly with RILP (Julian Scherer,
unpublished data). Another dynactin mediated link to vesicles of varying types may also occur through huntingtin (Htt), the mutated protein in Huntington’s Disease, which interacts directly with dynein IC and indirectly with p150 through HAP1 (Huntingtin Associated Protein 1) (Li et al., 1998; Caviston et al., 2007; Caviston and Holzbaur, 2009) (Fig. 1-4 C) and mediates transport of lysosomes, endosomes, and Golgi vesicles (Caviston et al., 2011; 2007) (Fig. 1-5 A).

Dynactin also interacts with other membranes for dynein localization to mitochondria, the nuclear envelope (NE), and plasma membrane (Fig. 1-5 A-C). Mitochondrial associated TRAK proteins bind p150 and recruit dynein in neurons (van Spronsen et al., 2013). Various developmental processes that involve nuclear movement have led to the discovery of several pathways for dynein NE recruitment that involve dynactin. In *C. elegans* embryonic development the nesprin Unc83 may recruit dynein-dynactin through BicD1 (Fridolfsson et al., 2010), which is implicated in dynein-dynactin recruitment in Golgi-ER transport (Matanis et al., 2002). Nesprin-2 immunoprecipitates dynein and dynactin and is required for nuclear migration in the developing mouse retina (Yu et al., 2011) while Par6 and Par3 localize dynein and dynactin to the NE of migrating nuclei in myoblasts (Cadot et al., 2012), though the later may be mediated by LIC2 (Schmoranzer et al., 2009). In G2 and early prophase, dynein is employed at the NE for nuclear envelope breakdown (Salina et al., 2002). The nuclear pore complex protein RanBP2, which binds BicD2 in the same region as Rab6 localizes BicD2 and dynein-dynactin to the NE in G2 (Splinter et al., 2010).

During mitosis, dynein localization at the plasma membrane is essential for correct mitotic spindle orientation, and dynactin has been implicated in dynein recruitment to the cortex through the NuMA-LGN complex (Kiyomitsu and Cheeseman, 2012; Kotak et al., 2012). Interestingly huntingtin localizes to spindle poles and its depletion reduces the dynein and
dynactin signal at poles, causes misoriented spindles and alters cell fate in the developing mouse brain (Godin et al., 2010). How huntingtin’s interaction with dynein and dynactin contributes to spindle orientation is not completely understood. Huntingtin may function similarly to NuMA, which orients the spindle through actions at the cortex but also localizes to spindle poles and focuses MT minus ends. Depletion of NuMA, or disruption of the dynein-dynactin interaction causes unfocused spindle poles (Merdes et al., 1996; 2000; Gaglio et al., 1997). Furthermore, overexpressed Arp1 causes multiple spindle poles to form. NuMA, p150, p50, and dynein IC associate with overexpressed Arp1 and presumably contribute to ectopic focusing and pole formation (Clark and Meyer, 1999). The N-terminal MT binding region of p150 is also required to focus spindle poles as rescue of p150 knockdown with endogenous levels of ΔN-p150 (lacking the MT binding region) causes multipolar spindles (Kim et al., 2007).

Maintenance of centrosomal MTs arrays is also dynactin-dependent. p50 overexpression disrupts the MT array and displaces Arp1, and the centriolar proteins pericentrin, centrin and ninein from the centrosome. Unfocused MT arrays are correlated with multiple γ-tubulin foci and seem to result from a failure to maintain clustered pericentriolar proteins, which are likely transported to the centrosome by dynein (Dammermann, 2002; Burkhardt et al., 1997; Quintyne et al., 1999; Young et al., 2000). GSK-3β can phosphorylate BicD1 promoting its interaction with dynein and knockdown of GSK-3β prevents ninein localization at centrosomes and results in unfocused MT arrays (Fumoto et al., 2006) suggesting a role for GSK-3β in dynein-dynactin mediated centrosome maintenance. While dynactin localizes to the centrosome continuously, centrosomal dynein occurs only in S phase, G2 and mitosis, suggesting dynactin might have a dynein independent function at centrosomes (Quintyne and Schroer, 2002).
Different roles have been proposed for dynactin at the kinetochore, where it localizes with dynein in prometaphase (Echeverri, 1996; Steuer et al., 1990). Overexpression of p50 and knockdown of several dynactin subunits decreased dynein signal at the kinetochore (Echeverri, 1996; Raaijmakers et al., 2013). However other studies show that Spindly and Zw10 can recruit dynein independently from dynactin (Whyte et al., 2008; Griffis et al., 2007). Whyte et al. propose that phosphorylated dynein IC interacts with Zw10 at the kinetochore and upon proper chromosome alignment it is dephosphorylated allowing recruitment of dynactin, thereby activating poleward transport of checkpoint proteins.

Finally, dynactin likely plays a role in dynein recruitment to the plus ends of MTs, through CAP-Gly interactions with EB1 and CLIP-170 (Vaughan et al., 1999), though the extent to which this is required for many dynein activities is unknown. Plus tip binding activity is not required for membranous cargo trafficking in general (Watson and Stephens, 2006; Moughamian and Holzbaur, 2012), but has a role in initiating transport at axon tips and in normal spindle positioning and formation (Moore et al., 2008; Moughamian and Holzbaur, 2012; Kim et al., 2007).

Determining dynactin’s role in dynein activities is complicated by multiple recruitment pathways and difficulty differentiating between dynactin mediated activation versus localization. Though dynactin is implicated in all dynein functions studied to date, there are dynactin independent pathways for dynein recruitment and regulation involving dynactin subunits (discussed previously) or NudE/L (discussed below).
**Figure 1-5. Proteins Involved in Dynein Cellular Functions.** (A) Proteins required for dynein association with membranous organelles or the plasma membrane and (B) mitochondria. (C) Factors involved in dynein localization during mitosis. Proteins either interact directly with dynein (ZYG-12, Snapin, Golgin 160, Huntingtin, FIP3, RILP, Par3), with dynactin (BicD1/2, Spectrin IIIβ, TRAK1/2, Sec23, TRAPP, Rab6), or NudE/L (CENP-F). Details of NuMA, Spindly and Nesprin2 interactions with dynein and dynactin are unknown. Zw10 has been reported to interact directly with dynein, dynactin, and NudE (See text for references, Images adapted from Molecular Biology of the Cell, 4th ed).
The Elusive Dynein-Dynactin Complex: An Emerging Role for Scaffolding Proteins

As discussed, dynactin is involved in nearly all dynein activities and p150 and dynein IC interact strongly in biochemical assays. Despite this, purified dynein and dynactin do not form a stable complex. Recently, the first-ever purified co-complex was formed but it required a third protein, namely the N-terminal region of BicD2. Curiously, BicD2 did not interact with either dynein or dynactin alone. Additionally, their recruitment to membranes via BicD2 is interdependent such that knockdown of HC prevents localization of p150 and vice versa (Splinter et al., 2012). While BicD2 is the only protein conclusively shown to link the two complexes, several other proteins might serve as dynein-dynactin scaffolds (Fig. 1-6). For example, NuMA very cleanly co-precipitates both complexes and the NuMA-dynein-dynactin complex can focus spindle poles in vitro (Merdes et al., 1996). RILP also interacts directly with dynein and dynactin subunits (Johansson et al., 2007)(and Julian Scherer, unpublished data) as do the huntingtin-HAP1 (Li et al., 1998; Caviston et al., 2007) complex and possibly Zw10 (Starr et al., 1998; Whyte et al., 2008).

Perhaps the large size of both dynein and dynactin complexes precludes their stable interaction, and it is tempting to speculate that scaffolding proteins may facilitate their interaction and specify their location or function. At this point however, whether proteins other than BicD2 can function as scaffolds remains an open and interesting question.
NudE/L-Lis1 Structure and Effects on Dynein Mechanochemistry

Other than dynactin, NudE (and its homologue NudEL) in conjunction with Lis1, is the only dynein interacting complex studied to date that affects dynein’s mechanochemical properties. Lis1 and NudE/L both have N-terminal coiled-coil domains that mediate dimerization. Lis1 C-terminal WD domains mediate interactions with the NudE/L coiled coil and dynein HC (Vallee et al., 2012) (Fig. 1-6 and 2-8 A). Lis1 interacts with AAA3 and 4 of the dynein motor domain but efficient recruitment requires NudE, which binds dynein IC, forming a triple complex (Mckenney et al., 2010; Huang et al., 2012). In vitro NudE alone inhibits dynein’s interaction with MTs, however when bound to NudE-Lis1, dynein can maintain its maximal force producing state for long periods of time and displays slightly longer run lengths.
under no load compared to dynein alone. Lis1 decreases the single molecule velocity of purified
*S. cerevisiae* dynein and interacts specifically with mammalian motor domain locked in the pre-
power stroke ADP-Pi state (mimicked by adding ATP + vanadate (Burgess et al., 2003)) (Fig. 1-1 C). The current model is Lis1 stabilizes dynein’s interaction with MTs during a point in the
counter stroke when it is normally weakly attached to MTs, enabling dynein to withstand high
force for extended periods of time (Mckenney et al., 2010; Huang et al., 2012).

Table 1-2. Dynein, Dynactin, and NudE/L-Lis1 Binding Proteins. Proteins that have demonstrated biochemical
interactions with dynein or dynein regulators are listed below. A direct interaction has been shown for BicD2 C-
terminus and p50 (Hoogenraad et al., 2001) but BicD2 N-terminus is sufficient to recruit dynein and dynactin
(Hoogenraad et al., 2003), so the significance of the p50 interaction is unknown. NuMA coprecipitates dynein and
dynactin (Meredes et al., 1996) but specific interactions with subunits are unidentified. Interactions between dynein,
dynactin, and NudE/Lis-1 are not included. See text for references.

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**Cellular Functions of NudE/L-Lis1**

The in vitro properties NudE-Lis1 confers on dynein fit well with their role in brain
development. Knockdown of Lis1 inhibits interkinetic nuclear migration of neuronal precursors
and subsequent migration of differentiated neurons (Tsai et al., 2007; 2005), both of which
depend on dynein to move one of its largest cargos, the nucleus, through a crowded environment. In cultured neurons, NudE/L or Lis1 inhibition more severely affects the retrograde transport of large lysosomes compared to small ones (Yi et al., 2011), consistent with a role in high load transport. However, like dynactin, NudE/L and Lis1 participate in almost all dynein functions, including those that are not overtly high load. Perhaps this indicates that regulation by NudE/L-Lis1 is controlled on a small spatial and temporal scale. The situation is further complicated by the roles of NudE/L and Lis1 in recruitment, and reported NudE/L-independent Lis1 functions.

NudE/L and Lis1 have mitotic roles in both dynein recruitment and regulation. NudE and NudEL are recruited to the kinetochore by CENP-F, and NudE is required to recruit dynein while NudEL is needed for proper chromosome alignment (Vergnolle and Taylor, 2007; Stehman et al., 2007). Dynein-mediated spindle pole focusing requires NudE/L and Lis1 (Raaijmakers et al., 2013; Wang and Zheng, 2011; Zylkiewicz et al., 2011). NudEL also localizes to interphase centrosomes where it can recruit dynactin, Lis1, and the centriolar proteins pericentrin and PCM-1 and knockdown of NudEL prevents both nucleation and anchoring of MTs at the centrosome (Guo et al., 2006).

Other interphase roles of NudE/L and Lis1 include the transport of membranous cargo in non-neuronal cells. Knockdown of Lis1 or NudEL, or overexpression of Lis1 or dynein binding deficient NudEL, disrupts Golgi, lysosome and endosome distribution (Lam et al., 2010; Liang et al., 2004). NudE/L double knockdown, and to a lesser extent, Lis1 knockdown decreased dynein association with membranes in vitro implicating NudE/L-Lis1 in dynein recruitment (Lam et al., 2010). It is not clear how NudE/L or Lis1 might be associated with vesicular membranes but their reported interactions with Zw10 (Stehman et al., 2007) and p50 (Tai et al., 2002), respectively, may play a role (Fig. 1-6).
NudE/L-Lis1 are also active at the nuclear envelope (NE). NudE/L is recruited there in G2 through CENP-F, which binds the nuclear pore protein Nup133 (Bolhy et al., 2011). Inhibition of Lis1, NudE/L together, CENP-F, or Nup133 prevent dynein mediated tethering of the centrosome near the nucleus (Bolhy et al., 2011; Raaijmakers et al., 2013). Interestingly, one study showed that Lis1 but not NudE/L, is necessary for dynein and dynactin localization at the NE (Raaijmakers et al., 2013), while another showed that NudE/L was required for dynactin recruitment to the NE (dynein was not examined) (Bolhy et al., 2011). Furthermore Lis1 is necessary for BicD2 mediated dynein and dynactin recruitment to Golgi and nuclear membranes (Splinter et al., 2012). These data likely reflect multiple dynein and dynactin recruitment pathways acting at the NE (Fig. 1-5 A) and a potentially NudE/L-independent role for Lis1. Though NudE and dynactin p150 interact with dynein in a mutually exclusive manner, the three complexes could be linked via Zw10 or p50 (Fig. 1-6). Finally, Lis1 can directly bind CLIP-170 (Coquelle et al., 2002; Tai et al., 2002), possibly linking it to growing MT tips and/or dynein and dynactin in the absence of NudE/L. Plus tip binding of Lis1 is consistent with its function in A. nidulans, where it initiates retrograde movement of membranous organelles but seems not to be necessary for sustained motion (Egan et al., 2012).

Similar to dynactin, NudE/L and Lis1 are involved most dynein processes and have both recruitment and regulatory roles. Additionally it is not clear if they always function together, as some NudE/L independent roles have been observed for Lis1. Interestingly some of these roles may involve dynactin and suggest that the model of a binary dynein regulation system comprised of NudE/L-Lis1 and dynactin may require modification.
Dynein Associated Diseases

Dynein and its associated proteins are involved in several brain development disorders and neurodegenerative diseases, underscoring the physiological importance of this motor complex. One of the most well studied dynein-related disorders is Type I Lissencephaly, meaning ‘smooth brain’, which is characterized by cortical lamination defects and lack of cortical convolutions, and results in severe cognitive deficiencies at a young age. Mutations in Lis1 or NudE/L cause Lissencephaly in humans (Walsh, 1999; Alkuraya et al., 2011). Work from our lab shows that developmental defects likely arise from disruption of interkinetic nuclear migration required for division of neuronal precursors and failed neuronal migration, activities that require the sustained force of the dynein-NudE/L-Lis1 complex (Tsai et al., 2007; Mckenney et al., 2010).

Several human mutations in the dynein HC dimerization region have been identified in patients with spinal muscular atrophy (SMA), a degenerative motor neuron disease (Tsurusaki et al., 2012; Harms et al., 2012). Mice with mutations in the same region have motor neuron degeneration and loss of lower limb strength. The brains of these mice show cortical lamination and neuronal migration defects, cultured neurons show defective axonal transport and axonal elongation, and dynein purified from these animals is less stable leading to a loss of processivity (Ori-McKenney et al., 2010; Ori-McKenney and Vallee, 2011). Recently several mutations in BicD2 were identified in SMA patients and shown to affect BicD2’s association with dynein and cellular localization as well as Golgi structure (Peeters et al., 2013; Oates et al., 2013).

Mutations in the p150 subunit of dynactin are associated with the degenerative diseases amyotrophic lateral sclerosis (ALS), distal hereditary motor neuropathy 7B (HMN7B), and Perry Syndrome. The ALS mutations occur in the C-terminal region of p150 and so far have not
yielded cellular phenotypes (Dixit et al., 2008). The HMN7B mutation and Perry syndrome mutations are in the CAP-Gly domain and have pronounced effects on dynein cellular functions. The former reduces p150 association with MTs, induces protein aggregation, and prevents timely recovery of Golgi structure following nocodazole washout. Additionally this mutation prevented retrograde and anterograde motion of lysosomes in neurons (Levy et al., 2006; Moughamian and Holzbaur, 2012). The Perry Syndrome mutations, on the other hand don’t disrupt axonal transport but prevent initiation of transport at the axon tip (Moughamian and Holzbaur, 2012). The authors speculate that the different effects on transport may explain the different neuronal populations affected in each disease.

Finally, dynein and dynactin association with huntingtin and huntingin associated protein 1, respectively, and the role of all of these proteins in membranous cargo transport suggest their involvement in the pathology of Huntington’s Disease (HD). Indeed, the disease causing poly-glutamine expansion in huntingtin results in decreased retrograde trafficking of TrkB signaling endosomes (Liot et al., 2013).

Interestingly, all dynein associated diseases result from defects in brain or neuronal tissue, perhaps highlighting the importance of dynein based transport in elongated neuronal processes. The observation that dynein IC isoform diversity is greatest in embryonic and nervous tissues suggests significant regulatory versatility in these tissues and it will be important to continue to study dynein’s regulation with this in mind.

Conclusions

From both a structural and functional point of view, dynein is an impressive motor complex. With each step the energy from ATP hydrolysis is translated into conformational changes that are propagated over tens of nanometers. While the field has made great progress in
understanding the structure and mechanochemistry of dynein’s motor domain, there is no high-resolution structural information for dynein’s base and to a large extent, the significance of isoform diversity is not understood. Though dynactin and NudE/L-Lis1 have emerged as mutually exclusive regulatory and recruitment systems, they are involved in many of the same dynein functions suggesting that alternation between the two systems may occur on small temporal and spatial scales. Still we have much to learn about the interplay of these systems with each other and with other recruitment mechanisms.

The studies presented here focus on two aspects of dynein regulation, both mediated through the base of the dynein complex. In Chapter 2 we ask if the LCs affect dynein function and address the questions of how they may alter dynein function either directly or, in the case of LC8, indirectly through interactions with NudE/L. In Chapter 3 we carefully analyze dynein’s interaction with dynactin and identify a new regulatory mechanism through dynactin’s interaction with the dynein IC.
Abbreviations
AAA: ATPases Associated with various cellular Activities
CAP-Gly: Cytoskeleton Associated Protein-Glycine rich
EM: Electron Microscopy
ERGIC: ER Golgi Intermediate Compartment
FRET: Fluorescence Resonance Energy Transfer
HAP1: Huntingtin Associated Protein 1
HC: Heavy Chain
IC: Intermediate Chain
LC: Light Chain
LIC: Light Intermediate Chain
MT: Microtubule
MTBD: Microtubule Binding Domain
NE: Nuclear Envelope
RILP: Rab7 Interacting Lysosomal Protein
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Chapter 2
Dynein Light Chains Have Roles in Dynein Transport and Regulation
Introduction

The dynein light chains were introduced in Chapter 1 as the smallest dynein subunits comprising three families with two homologues each (Fig. 1-1 A and Fig. 1-3). Though they are ubiquitously associated with dynein and highly conserved, we know little about how they participate in dynein’s many functions. Furthermore, studies have been complicated by non-dynein interactions of LC8, and possibly Tctex/RP3. These interactions initially lead investigators to conclude that LCs could recruit dynein to specific cargo and in some cases this seems to be the case (Tai et al., 1999; Lee et al., 2006; Meiri et al., 2012), though generally evidence for this is lacking.

The studies described here address the following questions regarding LC functions: (1) whether the LCs contribute to dynein-driven transport of membranous cargo, (2) how LCs might affect dynein function and (3) the nature of the interaction between LC8 and the dynein regulator NudE/L. We found that indeed LC8 and Tctex LC families affect dynein mediated movement of membranous cargo and validated a novel tool to acutely sequester LCs from dynein or other binding partners. Preliminary data suggest that LC8 or Tctex/RP3 may influence the interaction of dynein with two mutually exclusive regulatory systems, NudeE/L-Lis1 and dynactin. Finally, we define the LC8 binding region on NudE, and show that this interaction is independent from the NudeE-dynein interaction. Note that the Roadblock family of LCs was not included in these studies due to the lack of structural information and antibodies at that time. Additionally given the high degree of similarity between LC8 1 and LC8 2 and the focus on LC8 1 in the literature, only LC8 1 (henceforth referred to as LC8) was considered in these studies.
Results

Acute Inhibition of LC8 and Tctex Families of Light Chains

In order to understand LC contributions to dynein function, our collaborator John Williams (now at City of Hope in Duarte, CA) developed inducible, transfectable light chain traps, which acutely sequester the LC8 and Tctex LC families. Trap design is based on the crystal structure of dynein IC in complex with LC8 and Tctex (Williams et al., 2007), and a dimerization system developed by Ariad™, in which dimerization of modified FKBP is induced upon addition of a cell-permeable, biologically inert rapamycin analogue (AP20187) (Fig. 2-1). Williams and colleagues demonstrated that in vitro the LC8 or Tctex binding region of IC fused to FKBP binds LC only when the AP20187 ligand is added and that the IC regions are specific for each LC (Varma et al., 2010).

![Figure 2-1. Light Chain Trap Design.](image)

(A) Crystal structure of LC8 and Tctex in complex with IC (adapted from Williams et al., 2007)). The unstructured IC binds grooves on the two LCs, which are structurally homologous.
(B) Model of the LC8 trap. FKBP linked to IC forms a dimer capable of binding LC8 upon addition of AP20187.
(C) Model of LC sequestration by the LC trap (B and C are adapted from Varma et al., 2010).
Our lab used transfectable LC8 and Tctex traps to assess the effect of acute LC inhibition on lysosomes, endosomes and the Golgi. All organelles we examined were re-distributed by activation of either trap, and a subsequent study using LC RNAi confirmed these results (Palmer...
et al., 2009). The trapping system allowed us to observe the kinetics of this aberrant organelle transport, which interestingly, are much slower for the Golgi. Lysosomes and endosomes were fully dispersed at one hour after addition of AP20187, but a full effect on the Golgi required 4 hours (Fig. 2-2 A, data collected by Dileep Varma). Because the effects of activated traps were not fully penetrant we observed lysosomes after co-expression of both traps, but did not see a more severe phenotype (Fig. 2-2 B and C). We did not observe any effects on mitotic progression (Varma et al., 2010), as was recently observed after LC knockdown (Raaijmakers et al., 2013).

**Figure 2-3. The LC8 Trap Binds LC8 *In Vivo* and Sequesters LC8 from Dynein** (Adapted from (Varma et al., 2010)). (A) GFP-LC8 trap co-precipitates LC8. Anti-GFP was used to pull down GFP-LC8 trap in lysates of trap-expressing Cos7 cells treated with AP20187 or ethanol, as a control, for 8 hrs. Western blotting shows LC8 only co-precipitates with the trap when AP20187 was added. (B) The LC8 GFP-trap sequesters LC8 from dynein. GFP LC8-trap expressing Cos7 cells were treated with AP20187 or EtOH for 8 hr. Western blotting shows anti-dynein IC immunoprecipitation from lystates of sorted cells has less LC8 associated with dynein when cells are treated with AP20187 compared to controls. (C) Quantification of LC8 in dynein IP relative to IC band. Error bars are S.D. for three independent experiments. (in = input, sup = supernatant, pel = pellet)
Our observations imply that the traps work as expected, however LC8 and Tctex also interact with other proteins in potentially a dynein independent manner. We used the LC8 trap to confirm that expressed traps bind LCs and sequester them from dynein in our cellular assays. Over-expressed GFP-LC8 trap co-precipitated LC8 only when AP20187 was added. Additionally, 60% less LC8 co-precipitated with dynein from sorted, GFP-LC8 trap-expressing cells when AP20187 was added (Fig. 2-3). While we cannot rule out off target effects from sequestering LC8 from other proteins, it is plausible that the phenotypes are dynein-related, as the organelles examined are well-know dynein cargo (Burkhardt et al., 1997).

The Potential Effects of LC Inhibition on Dynein

Varma et al. and other studies show a role for the LCs in several dynein functions, but mounting structural data argue against LCs as cargo adaptors. In particular LC8 is known to interact with several other proteins (Table 1-1) and in some cases to stabilize their dimeric state (Barbar, 2008). Furthermore, LC8 increases the helical content of the IC dimerization region upon binding (Nyarko and Barbar, 2010; Nyarko et al., 2004). Thus loss of LC8 from the dynein complex may affect dynein function by destabilizing the IC-IC interaction, which could in turn destabilize the entire complex. However, sucrose gradient sedimentation of lysates treated with LC8 or control RNAi showed identical dynein sedimentation patterns, with dynein peaking at 20S (Fig. 2-4). If LC8 does alter IC-IC interaction it may be difficult to detect and/or may not affect the whole complex. Alternatively, multiple LCs might participate in complex stabilization, though our previous observations indicate that inhibiting one LC is sufficient to disrupt dynein function and that co-inhibition does not exacerbate the phenotype, at least in the case of lysosomes (Fig. 2-2 B and C).
**Figure 2-4. LC8 Knockdown Does Not Alter Dynein’s Sedimentation on a Sucrose Density Gradient.** Lysates from Cos7 cells treated with LC8 or control RNAi for 48 hrs were centrifuged through to a 5-20% sucrose gradient. Gradient fractions were Western blotted for dynein HC, IC, LC8 and dynacin p150. Sedimentation profiles of HC, and IC were similar under both conditions. Note that in the control lysates much LC8 does not co-sediment with dynein.

Another way LCs might affect dynein activity is by altering their association with regulatory proteins, namely NudE or dynactin. In the most ubiquitous IC isoform (IC2C), Tctex and LC8 interact with the IC approximately 30 residues C-terminal to the NudE and dynactin binding sites. Dynactin p150 and NudE compete for overlapping sites in the first 70 amino acids of IC, and we hypothesized that LCs might regulate this competition. To assess this, recombinant IC 1-250, which contains LC8 and Tctex/RP3 binding sites was pre-incubated with one or both LCs followed by addition of NudE or the dynein binding region of p150, CC1. Interestingly, the LCs did not affect the CC1-IC interaction but together they increased IC binding to NudE nearly 5-fold and LC8 alone more than doubled the amount of IC co-precipitating with NudE (Fig. 2-5). When both NudE and CC1 are present, dynein or
recombinant IC N-terminus bind preferentially to CC1 (Mckenney et al., 2011; Nyarko et al., 2012). Our data indicate LCs may shift the binding toward NudE, though the experiments in Mckenney et al. used purified dynein, which is at least partially occupied by LCs. Additionally, LC8 binds directly to NudE (Stehman et al., 2007) and it is possible that LC8 recruits NudE to the IC. As such, we decided to examine the NudE-LC8 interaction in more detail.

**Figure 2-5. LCs Increase NudE Association with IC 1-250.** (A) Purified recombinant LC8 and/or RP3 were mixed with purified recombinant IC 1-250 and co-precipitated with beads loaded with anti-HA and HA-NudE. Note the antibody heavy chain reacts with the secondary antibody and runs just above NudE. (B) The same experiment as (A) but with Flag-CC1 instead of HA-NudE. (C) Quantification of Western blot IC signals normalized to NudE or CC1 signal. Error bars are S.D. of three independent experiments.
Characterization of the NudE-LC8 Interaction

We first set out to define the LC8 binding region on NudE. Having determined that LC8 does not bind the NudE N-terminal coiled-coil (residues 1-191) (Fig. 2-6 A and B), we used the pepscan technique, which was successfully employed to determine LC8 binding sequences in several other proteins (Navarro-Lérida et al., 2004; Rodríguez-Crespo et al., 2001). Recombinant LC8 was incubated with a membrane spotted with overlapping dodecapeptides covering the C-terminus of NudE, followed by blotting with primary LC8 and secondary antibodies. The strongest reacting spots share the sequence, KRTDMAVQATGS, which spans residues 200-209 (Fig. 2-6 C and E). We further confirmed binding to this region using recombinant NudE 1-218 (Fig. 2-6 A and B). Shortly after this work was published, Rapali and colleagues determined the LC8 binding consensus sequence from more than 40 confirmed LC8 binding sequences (Rapali et al., 2011b). The sequence we identified in NudE fits this consensus and is highly conserved across species in both NudE and NudEL (Fig. 2-6 D and E).

Because dynein IC binds to the interdimer grooves on an LC8 dimer, as do all other proteins for which there are structures with LC8 (Rapali et al., 2011a), we wanted to know if the same LC8 dimer could bind dynein and NudE simultaneously. Recombinant GST-IC 70-154, which binds LC8 but not NudE, could compete LC8 from preformed LC8-NudE complexes (Fig. 2-7 A and B), demonstrating that LC8 does not participate in the interaction between NudE and IC and that NudE must bind the same location on the LC8 dimer as IC. Furthermore, we could not compete dynein complex from NudE using excess LC8 (Fig. 2-7 C, data collected by Richard McKenney), again supporting that LC8 binds NudE in a dynein independent fashion. These data are consistent with the fact that the defined dynein interacting regions on NudE (Fig. 2-8 A) do no overlap with the LC8 binding site that we discovered.
Having established a dynein independent interaction between NudE and LC8, we performed several preliminary experiments to investigate its biological significance. LC8 binds NudE just
C-terminal to its coiled-coil region. The NudE C-terminus is disordered and also contains one of two dynein interacting regions (Liang et al., 2004; Alkuraya et al., 2011; Zylkiewicz et al., 2011; Wang and Zheng, 2011)(Fig. 2-8 A). Given LC8’s ability to induce structure in disordered regions we hypothesized that LC8 might affect dynein’s interaction with NudE’s C-terminal region. However, co-precipitation of NudE pre-incubated with LC8 bound purified dynein to the same extent as NudE alone (Fig. 2-8 B, data collected by Richard McKenney). Recently LC8 was reported to induce oligomerization of Nek9, a NIMA kinase involved in early mitosis (Regue et al., 2011). LC8 interacts with Nek9 in a region adjacent to a coiled-coil, analogous the LC8 interaction site on NudE and crystal structures of NudEL’s coiled-coil suggest that it may form tetramers (Derewenda et al., 2007). To assess if LC8 alters NudE’s oligomeric state, we sedimented NudE alone or with LC8 on sucrose density gradients, however there was no difference in the sedimentation patterns (Fig. 2-8 C). Finally, based on the finding that LC8’s interaction with Nek9 is regulated by phosphorylation at a site near the LC8 interaction region (Regue et al., 2011), we examined the effect of NudE phosphorylation on LC8 binding. NudE/L contains several conserved CDK5 and Erk1/2 phosphorylation sites in the C-terminal unstructured region (Fig. 2-8 A), which have been shown to affect interaction with Lis1 and dynein and are involved in dynein’s mitotic and axonal transport functions (Niethammer et al., 2000; Hebbar et al., 2008; Yan et al., 2003; Pandey and Smith, 2011; Zylkiewicz et al., 2011). Two of these phosphorylation sites are immediately adjacent to the LC8 binding region in NudE (Fig. 2-6 E) so we made recombinant phosphomimetic NudE constructs for these sites. Co-precipitation revealed that neither phosphorylation site affected the interaction. Since both sites can be phosphorylated by CDK5, a change in binding may only be observed in the double phosphomimetic, which we have not tested.
Figure 2-7. **LC8 Binds NudE Independently from Dynein.** (A) Western blot showing IC 70-154 competes LC8 from NudE. HA-NudE was preincubated with LC8 followed by addition of increasing amounts of IC 70-154, which does not bind NudE alone. The Western blot on the right shows that IC 70-154 binds LC8. Diagram below shows full length IC and the domains in IC 70-154. (B) Quantification of LC8 bound to NudE in the presence of increasing concentrations of IC 70-154. Error bars are S.D. for three independent experiments. (C) Western blot showing LC8 cannot compete dynein from NudE. 0X (lane 1), 1X (lane 2), 5X (lane 3), or 10X (lane 4) LC8 was added to pre-formed NudE dynein complexes. LC8 coprecipitates without displacing dynein. Control beads were incubated with LC8 alone (lane 5) (C is Adapted from Mckenney et al., 2011), and data were collected by Richard McKenney.)
Figure 2-8. The Significance of the LC8-Nude Interaction Remains Unclear. (A) Diagram of Nude domains, interaction regions, and phosphoroylation sites (green lollipops) (Courtesy of Shahnaz Kemal). Nude’s N-terminal region is a coiled-coil, which mediates dimerization, and its C-terminus is unstructured. (B) Western blot showing pre-binding LC8 to Nude does not change its affinity for dynein. HA-Nude was preincubated with LC8 (lanes 1 and 2) and combined with purified dynein (lane 2) or HA- Nude was bound to dynein in the absence of LC8 (lane 3). Recombinant LC8 runs slightly slower on the gel and does not affect Nude’s ability to bind dynein. The control shows dynein and LC8 don’t bind beads without Nude (lane 4)(Adapted from Mckenney et al., 2011, data collected by Richard Mckenney). (C) Western blot showing Nude with or without LC8 has the same sedimentation pattern on a sucrose density gradient. (D) Western blot showing S196E and T215E (mouse Nude numbering) phosphomimetic Nude constructs bind LC8 as well as wild type Nude. Nude 1-191 does not bind LC8, as expected.
Discussion

Acute Inhibition of LC8 and Tctex Families of Light Chains

The LC trapping experiments demonstrate both the efficacy of the traps and the contribution of LCs to dynein mediated transport of lysosomes, endosomes, and Golgi vesicles. Unfortunately, their specificity is limited by the specificity of the LCs themselves and in the case of LC8, which has over 60 binding partners, it will always be difficult to prove that dynein is the only protein affected. Until we can mutate the dynein IC to prevent LC binding in cells, we will not be able to clearly distinguish the biological role of LCs in dynein-specific functions. This has been done in *S. cerevisiae*, where dynein function is limited to spindle pole positioning and the phenotype is similar to deleting the LC8 gene (Stuchell-Brereton et al., 2011), despite a well-described non-dynein role for LC8 in yeast (Stelter et al., 2007). For now, to study mammalian LCs we have to suffice with depleting LCs broadly. Thus far the data indicate there are important roles for LCs in dynein function.

The Potential Effects of LC Inhibition on Dynein

If we relinquish the idea of LCs as cargo adaptors, the most obvious alternative is that they serve a structural role. EM images of recombinant LC-less dynein from the Surrey lab support this, as this dynein has a more splayed appearance compared to the full complex (Trokter et al., 2012). Dynein purified from an *S. cerevisiae* LC8 deletion strain show reduced association of HC and IC and decreased run lengths in single molecule studies (Rao et al., 2013). Interestingly, budding yeast has only one LC8 gene and no other LCs and there are two adjacent LC8 binding sites in the dynein IC. This may imply that in mammalian dynein, LCs contribute redundantly to dynein structure and explain why depletion of LC8 did not alter dynein S-value in our hands. Furthermore it suggests that LC inhibition phenotypes originate from reduced single
molecule dynein run lengths. However, our data also show that interactions with regulators may be affected by LC8 or Tctex/RP3, though this remains to be confirmed in the context of the entire dynein complex.

**Characterization of the NudE-LC8 Interaction**

When we first discovered the LC8-NudE interaction we naturally assumed it was related to the NudE-dynein interaction. Our results demonstrate this is not the case and that NudE binds LC8 independently. Furthermore we define the LC8 binding region on NudE and show that it is highly conserved and closely related to the defined LC8 binding consensus sequence. We were unable to determine any biochemical function of the NudE-LC8 interaction, but many possibilities remain. The location of LC8 binding within NudE may influence phosphorylation in this region or interactions with CENP-F, which recruits NudE/L to the nuclear envelope and kinetochores (Bolhy et al., 2011; Vergnolle and Taylor, 2007), and/or with 14-3-3-ε, which maintains NudEL phosphorylation and is required for normal brain development (Toyo-oka et al., 2003) (Fig. 2-8 A). EM and crosslinking data indicate that the C-terminus of NudE folds back to interact with the coiled-coil. Whether LC8 affects this interaction is an open question (Soares et al., 2012). Notably, *in vivo* LC8 may be involved in NudE regulation of primary cilia length and cell cycle (Kim et al., 2011) or may affect dynein regulation or recruitment by NudE and determining the biochemical basis of this will be important.

**Conclusions and Future Studies**

There appear to be important roles for LC8 and Tctex families of LCs in dynein function, however more specific manipulation of the mammalian dynein complex is required to fully understand LC contributions to dynein’s activities. Furthermore, the Roadblock family of LCs remains understudied. Interestingly Roadblock 1 is necessary for dynein mediated anchoring of
the centrosome near the nuclear envelope, but not for dynein localization there, implying Roadblock LCs may have regulatory and/or structural importance. We identified a potential role for LC8 and RP3 in mediating the IC-NudE interaction. Several other proteins bind the dynein IC in the same region as the LCs, and it is possible that LCs regulate these interactions as well (Fig 1-2 A). Notably, Roadblock and LC8 have opposite effects on the IC region between their binding sites (Nyarko and Barbar, 2010), which could affect the accessibility of the IC.

Lastly, the dynein-independent interaction between LC8 and NudE is unexpected and determining its significance remains important. Though it is not obvious why this single protein has become so widely used in the cell, continued characterization of its roles in specific cellular functions will hopefully reveal the evolutionary story of this fascinating protein.
Materials and Methods

Construction of LC trap expression plasmids (carried out by the Williams Lab)

The FKBP-LC8<sub>TRAP</sub> was generated using PCR to isolate a cDNA fragment containing the LC8 binding region of rat dynein intermediate chain (IC) (isoform 2C) residues 125-138 (REIVTYTKETQTP), and subcloning this segment into PC<sub>7</sub>F<sub>1</sub>E mammalian expression vector (generous gift from Ariad Pharmaceuticals, Cambridge, MA) using the Spe<sub>1</sub> and BamH<sub>1</sub> restriction sites (NEB, Beverly MA). An equivalent construct for bacterial expression was generated by isolating the cDNA for the FKBP-LC8<sub>TRAP</sub> and subcloning this fragment into the Nco<sub>1</sub> and Hind<sub>III</sub> sites of the pet21D vector (Novagen).

The FKBP-TcTex<sub>1</sub><sub>TRAP</sub> constructs for both mammalian and bacterial expression vectors were generated using PCR by isolating the cDNA fragment containing the TcTex-1 binding region of the rat DIC (residues 107-125, GRGPIKLGMAKITQVDFPPR) and following the same protocol for the FKBP-LC8<sub>TRAP</sub>. Enhanced green fluorescence protein fusions or GFP tagged FKBP-LC8<sub>TRAP</sub> and FKBP-TcTex<sub>1</sub><sub>TRAP</sub> were generated by inserting specific cDNA fragments into the Sac<sub>1</sub> and EcoR<sub>1</sub> restriction sites of the pEGFP-C1 vector (Clontech). The sequencing of each construct was confirmed by automated DNA sequencing (Kimmel Cancer Center DNA core facility).

Cloning for Protein Expression

S. Weil cloning: Human LC8 (Accession Number NM_003746) and human RP3 (NM_006520) were cloned from pCMVβ into pGEX 6P-1 (Amersham Biosciences) using the EcoRI and XhoI restriction sites. NudE truncated proteins 1-191 and 1-218 were cloned from full length mouse NudE (Accession Number Q9CZA6) into pGEX 6P-1 using the BamHI and EcoR<sub>1</sub> restriction sites and primers were designed to include N-terminal HA tag and C-terminal 6X-His tag.
Phospho-mutant NudE constructs were generated using Quickchange (Agilent Technologies) in full length HA-NudE.

**R. Mckenney cloning:** Dynein IC fragments from rat (NM_053880) were cloned into pGEX6P-1 with a Myc tag at the C terminus, or into pCDNA 3.1 (IC2C 1–260 and 123–280) or pEGFP (IC2C 1–100) for mammalian cell expression. Full-length mouse HA-NudE-6X His, untagged NudE truncations 10-191 and 10-165, and flag-p150-CC1 (224-555) were cloned into pGEX6P-1 p150 Glued fragments were cloned from a full-length rat (EDL91133.1)

**Protein Expression and Purification**

BL21-CodonPlus RIPL competent cells (Agilent Technologies, #230280) were transformed with pGEX 6P-1 containing the gene of interest. Overnight cultures were used to inoculate 0.5-1 L of LB or Terrific Broth (Sigma, T5574). All cultures were grown at 37°C to an OD (λ600) of 0.5-0.7 after which, LC8 and RP3 expression were induced with 0.5mM IPTG for 4-6 hours at 37°C while NudE, p150, and IC construct expression were induced with 0.5mM IPTG for 4-6 hours at 20°C after 10 minutes cold shock. Following induction cultures were pelleted and frozen at -80°C until purification. For purification, pellets were resuspended in cold PBS with 1mM DTT and 1:500 protease inhibitor cocktail (Sigma, P8340), sonicated on ice for 10 minutes, and centrifuged at 4°C for 30 min at 150,000g with a final concentration of 1% Triton-X. The supernatant was incubated with glutathione beads (GE, 17-0756-01) for 1 hour at 4°C, collected on a column and washed. Protein was eluted from beads with 10mM reduced glutathione in Tris HCl pH 8.0 followed by buffer exchange into storage buffer (50mM Tris-HCl pH 7.0, 150mM NaCl, 1mM EDTA, 5% glycerol, 1mM DTT) and flash frozen and stored at -80°C. Alternatively, proteins on glutathione beads were washed into cleavage buffer (50mM Tris-HCl...
pH 7.0, 150mM NaCl, 1mM EDTA) supplemented with 1mM DTT and incubated overnight at 4°C with Precission Protease (GE, 27-0843-01). The supernatant was collected and supplemented with a final concentration of 5% glycerol before aliquoting, flash freezing and storage at -80°C.

*Trap Transfections*

All transfections were performed with Effectene Transfection Reagent (Qiagen) according to manufacturers instructions, for 24 hrs.

*FACs Sorting*

Cell sorting was performed on a BD FACS Aria and the 30% of the cells were determined to give a strong GFP signal and collected for the dynein immunoprecipitations. AP20187 was kept at a concentration of 100 nM during sorting and added to the IP buffer at a concentration of 1 µM.

*Immunoprecipitations*

IPs from trap-expressing cells were performed in RIPA buffer (100 mM NaCl, 50 mM Tris, 1mM EGTA, 1% NP-40, pH 7.4) with 1mM DTT and 1:100 protease inhibitors (Sigma, P8340) at 4°C for 2hrs. AP20187, or the equivalent amount of ethanol, was kept at 1µM during IP and wash steps. IPs with purified proteins were done in HEPES-NaCl buffer (50 mM HEPES, 50-100 mM NaCl, 1mM EDTA, .1% Tween, 1 mM DTT, .05 mg/ml BSA, pH 7.4) for 1 hr at 4°C with 200nM protein unless excess is indicated in the figure. All IPs were performed with protein A agarose beads (Invitrogen). IP and blotting antibodies are: anti-GFP (Abcam, ab1218), anti-dynein intermediate chain 74.1 (Millipore, MAB1618), anti-LC8 (Santa Cruz, PIN-FL89), anti-FKBP (Abcam, ab2918), anti-HA (Covance, 16B12), anti-GST (Santa Cruz, sc-53909), anti-LC8 (Abcam, ab51603), anti-myc (Sigma, M4439), anti-Flag (Sigma, F1804 M2) and anti-RP3 (generous gift from Kevin Pfister).
**GST-NudE Construct Pull Downs**

800 nM of GST-NudE full length, 1-191, or 1-218 were incubated with glutathione beads (USB) and a 3X molar excess of purified, recombinant LC8 in buffer (50 mM HEPES, 50 mM NaCL, 1 mM EDTA, .1% Tween) supplemented with 0.05 µg/ul BSA and 1mM DTT for 1 hour at 4˚C. The beads were washed 4 times, resuspended in buffer, and supernatants and pellets were analyzed by SDS-PAGE electrophoresis and Coomassie staining.

**Live Cell Imaging of Lysosomes in Cos7 Cells**

Cells were co-transfected with mcherry-LC8 trap and GFP-Tctex trap for 24hrs and treated with Lysotracker (Invitrogen) for 1 hr. Lysotracker was washed out and images were collected in a temperature controlled environmental chamber every minute for 1 hr following addition of 5 µM AP20187 using a Leica DMRB microscope outfitted with Photometrics Coolsnap EM-CCD camera.

**Determination of LC8 Bidning Region on NudE with Pepscan Membrane**

A membrane array spotted with overlapping dodecapeptides was generated based on mouse NudE (Accession Number Q9CZA6) C-terminus residues 192-344 (JPT Peptide Technologies, Berlin). Each spot contains approximately 5 nmol of a 12 amino acid-long peptide that is covalently linked to a cellulose-βalanine membrane. The sequence of peptides in adjacent spots are shifted C-terminally by two residues such that two neighboring spots overlap by ten residues. Before use, the membrane was reconstituted at room temperature in methanol for 5 min, followed by three ten minute washes with TBS. Blocking was performed for 1 hr with 5% milk in TBS-T. Non-specific interaction of antibodies with the membrane was determined first by 1 hr incubation simultaneously with primary rabbit monoclonal LC8 antibody (Abcam, ab51603) at a dilution of 1:2500 and secondary anti-rabbit Alexa Fluor 680 (Invitrogen, A10043) at a
dilution of 1:10,000 in 5% milk in TBS-T at room temperature. The membrane was then scanned using Odessy Imaging System (LI-COR). 200 nM of recombinant purified human LC8 in 5% milk in TBS-T was incubated with the membrane overnight at 4°C and for 1 hour at room temperature the following day, followed by three 10 minute washes with TBS-T, sequential probing with primary and secondary antibodies, and scanning as before. Scans of the membrane before and after incubation with LC8 were compared to identify the residues in the C-terminus of NudE that are involved in binding LC8.

**LC8 RNAi**

Cos7 cells were treated with smart pool of RNAi oligonucleotides (Dharmacon) for 24 hrs. Oligos were against sequences: GUUCAAAUCUGGUUAAAAGUU, GAAGGACAUUGCGGCUCAUUU, GUACUAGUUUGUCGUGGUUUU, CAGCCUAUAUUCAAAUAUU.

**Sucrose Gradients**

Gradients were made by overlaying 4 layers of 20,15,10, and 5% sucrose in Tris-KCl (20 mM Tris, 50 mM KCl, 1 mM MgSO₄,1 mM EDTA, pH 7.4), which were allowed to linearize for 1 hr. Purified proteins or soluble portion of lysates were applied to gradients, which were centrifuged 3 hrs at 20,000 x g. Fractions were analysed by Western blot with HA and LC8 antibodies (listed above).
References


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Chapter 3
A Novel Mechanism for Dynactin Regulation of Dynein
Introduction

As discussed in Chapter 1, dynactin has emerged as a ubiquitous regulatory and recruitment factor for dynein. This dual role for dynactin, as well as the lack of a well-defined stable dynein-dynactin complex has complicated study of this important transport system and many questions remain regarding the interaction of dynein and dynactin, the extent to which dynactin participates in recruitment versus direct modulation of dynein activity and the nature and mechanism of this modulation.

The most recognized regulatory role for dynactin is as a processivity factor, an activity originally thought to arise from microtubule interacting domains on the p150 subunit of dynactin, which contains N-terminal CAP-Gly and basic domains (Fig. 3-1 B). In early in vitro single-molecule studies performed with purified mammalian dynein and dynactin bound non-specifically to carboxylated beads, dynein could drive minus end movement of beads along MTs and the average run length was doubled upon addition of dynactin. Including a monoclonal antibody to the N-terminal region of p150, which presumably disrupts its interaction with MTs, abolished the effect (King and Schroer, 2000). However, a direct interaction between purified dynein and dynactin was never demonstrated, and it was recently shown that these two complexes do not interact in vitro on their own. Instead an N-terminal coiled-coil region of BicD2, a factor known to recruit dynein and dynactin to nuclear and vesicular membranes, is required to bridge the interaction (Schroer and Sheetz, 1991; Splinter et al., 2012; King and Schroer, 2000).

Furthermore, some studies have called into question the involvement of p150 MT-interacting regions in increasing dynein’s processivity. Dynactin complexes purified from Saccharomyces cerevisiae and labeled with HaloTag® show processive motion only in the
presence of unlabeled dynein, indicating their direct interaction in this system. The run length of these co-complexes is approximately 2.5 times greater than dynein alone, an effect that is maintained when the endogenous p150 gene is N-terminally truncated (Kardon et al., 2009). Furthermore, several studies have demonstrated that the CAP-Gly and basic domains are not required for transport of various dynein cargos \textit{in vivo}. Disruption of the Golgi by p150 knockdown in HeLa cells is rescued by p150 or p135, a neuronal isoform lacking the CAP-Gly domain and half of the basic domain (Dixit et al., 2008). In neurons \(\Delta N\)-p150 and full length protein rescue lysosomal movement along axons following p150 RNAi, however lysosome recruitment to axon tips and initiation of retrograde transport requires full-length p150 (Quintyne et al., 1999; Moughamian and Holzbaur, 2012; Flores-Rodriguez et al., 2011). p150 is localized to MT plus ends via EB1 and CLIP170, however knockdown of either protein shows no effect on membranous organelle localization or transport (Johansson et al., 2007; Watson and Stephens, 2006). These studies show that dynactin’s MT-binding region does not have a general role in recruiting or activating dynein transport of membranous cargo, and may instead be necessary in neurons to localize dynein to MT plus ends and initiate retrograde transport. Importantly, run lengths of vesicles and mRNA particles \textit{in vivo} are not affected by the loss of p150’s MT-binding domain. (Bremner et al., 2009; Kim et al., 2007).

In light of these disparate claims the role of p150’s MT-binding region in dynein-dynactin functions, we initiated a study to test whether a minimal dynein-interacting region of dynactin could recapitulate reported activities. We thoroughly characterized this protein, and several other p150 constructs and tested their effects on a range of dynein behaviors in single and multiple motor assays. We find that the minimal binding region (denoted herein as CC1B’) increases dynein run length in single molecule assays, but in the context of larger fragments of
p150, the effects on dynein activity are mixed. Furthermore we find that none of the p150 fragments tested affect dynein’s force production, suggesting that unlike other dynein regulators, dynactin does not modulate dynein’s response to high loads (Splinter et al., 2012; Mckenney et al., 2010).

Figure 3-1. Dynactin p150 Domain Map and Dynactin-Dynein Interactions. (A) Hypothetical model of dynein-dynactin co-complex (Adapted from (Kardon et al., 2009; Vallee et al., 2012; Kim et al., 2007)). The projecting p150 arm is 24 nm and likely contains most of CC1A' (see B). A predicted break in the first coiled-coil may correspond to the shoulder region. Dynein N-terminal projects out from the dynein base (Siglin et al., 2013; Watanabe et al., 2011) and interacts with CC1B' of p150 (see B). (B) Map of p150 domains and constructs used in this study. Numbering corresponds to the rat protein (EMBL ID: EDL91132.1). (C) Alignment of CC1 region of p150. CC1 is highlighted in purple with the predicted break in the coiled-coil indicated. The first residue of CC1B' is highlighted in yellow.
Results

Structural Characterization of p150 Fragments

p150 Interactions with Dynein

Early biochemical studies identified the subunits of dynein and dynactin that appear to mediate their interaction, namely dynein IC N-terminus and a region including the first predicted coiled-coil (CC1) in dynactin p150 (Mckenney et al., 2011; Vaughan and Vallee, 1995; Karki and Holzbaur, 1995). These regions were subsequently narrowed to the first 70 amino acids of the IC and the C-terminal half of p150 CC1 (CC1B) (Kardon et al., 2009; Mckenney et al., 2011; King and Schroer, 2000) and during the course of this study, were further refined to amino acids 1-44 of IC and 415-530 of p150 (Qiu et al., 2012; Siglin et al., 2013). For this study, CC1B (a.a. 381-530) was modified from that used in McKenney et al. to more closely correspond to a predicted, conserved break in the coiled-coil and is denoted CC1B’ (a.a. 358-555) (Fig. 3-1 B and C). This region of p150 along with the N-terminal half of CC1 (CC1A’ a.a. 224-357) and CC1 were expressed in E. coli along with p135-CC1, an N-terminal extension of CC1 corresponding to the neuronally expressed 135 kDa splice variant of p150 (Fig. 3-1 B). p135 lacks the CAP-Gly domain and half of the basic domain and does not to heterodimerize with p150 (Ross et al., 2006; Tokito et al., 1996). Its function in the cell remains unknown. Interaction of p150 with purified bovine dynein through CC1B’ was confirmed by immunoprecipitation (IP) using C-terminal flag tags on the p150 fragments (Fig. 3-2 A). We previously showed that a monoclonal antibody to the dynein IC N-terminus (Culver-Hanlon et al., 2006; Siglin et al., 2013), 74.1, blocks dynactin binding (Wang and Sheetz, 1999; Mckenney et al., 2011). To confirm that all p150 constructs interact with the IC N-terminal region, purified dynein was immunoprecipitated with 74.1 in the presence of the p150 fragments. The antibody
blocked all interactions with the fragments confirming that the IC N-terminus is the principle site of interaction (Fig. 3-2 B). Additionally, CC1, CC1B, nor CC1A interacted with baculovirus-expressed GST dynein motor domain (Fig. 3-2 C).

![Figure 3-2. Recombinant p150 Fragments Interact with Dynein IC N-Terminus via CC1B'.](image)

(A) Western blot showing all p150 fragments except CC1A’ immunoprecipitate purified bovine dynein. Control is dynein and flag antibody only. (B) Western blot showing that monoclonal dynein antibody 74.1 IP of dynein in the presence of p150 fragments. The interaction of p150 fragments with dynein is blocked by the 74.1 antibody. (C) Coomassie stain of pull down of recombinant dynein GST-motor domain with p150 fragments. None of the fragments tested interact with the motor domain. p150 fragments were used at 40X molar excess to dynein or motor domain. (Ab HC / LC = antibody heavy chain / light chain, DIC = dynein IC, MD = motor domain, sup = supernatant)

**p150 Fragments Effects on Dynein Structure**

Our lab recently showed that mutations in the N-terminal dynein heavy chain (HC) tail region destabilize the dynein complex, indicated by a slight shift to a lower S-value following
sedimentation on a sucrose density gradient (Cooper and Wordeman, 2009; Ori-McKenney et al., 2010; Harms et al., 2012). These mutations occur in the HC dimerization region and presumably slightly destabilize the HC dimer and thus the entire complex. To test whether the p150 fragments affected dynein’s inter-subunit interactions, purified bovine brain dynein was sedimented on sucrose gradients in the presence of a 10-fold molar excess of each p150 fragment. While association of CC1B’ with dynein on the gradient was weak (Fig. 3-3 B), CC1 and p135 clearly co-sedimented with dynein (Fig. 3-3 A and D). As expected, CC1A’ did not co-sediment with dynein (Fig. 3-3 C). Importantly, dynein’s sedimentation profile was not altered in the presence of the p150 fragments, indicating that there is no gross perturbation of the dynein complex stability. Over-expression of CC1-GFP and CC1B’-GFP in Cos7 cells also showed co-sedimentation of the p150 fragments with dynein but had no effect on dynein’s S-value (Fig. 3-3 E).
A

Dynein IC Quantification

CC1-Flag Quantification

B

Dynein IC Quantification

CC1B'-Flag Quantification
**Figure 3-3. p150 Fragments Do Not Grossly Affect Dynein’s Structure.** (A-D) Purified bovine dynein with 10X molar excess of p150 fragment sedimentation on sucrose density gradients. Western blots of gradient fractions show dynein sedimented alone, p150 fragment sedimented alone, or dynein and fragment sedimented together. Dynein IC and p150-flag signals are quantified in the graphs. Error bars are S.D. of 2 independent experiments. (E) Sucrose gradient sedimentation of Cos7 lysates over-expressing GFP, CC1-GFP, or CC1B’-GFP. Western blots for dynactin p150 and dynein IC show no change in dynein or dynactin sedimentation.

**Analysis of Secondary Structure and Absolute Molecular Weights**

CC1 is a strongly predicted, highly conserved α-helical coiled-coil (Fig. 3-1 C). To confirm that our p150 proteins were forming the predicted secondary structure we carried out circular dichroism (CD) over a range of temperatures and calculated the corresponding molar ellipticities as a function of wavelength. Alpha-helical proteins have a characteristic CD profile with a maximum at 190 nm and minima at 208 and 222 nm and a ratio of molar ellipticities at 222 nm and 208 nm greater than one is consistent with a coiled-coil (Mckenney et al., 2010; Lau et al., 1984). All proteins tested (CC1, CC1B’, CC1A’, and p135-CC1) were predominately α-helical coiled-coils at 4°C and 25°C but began to unfold at higher temperatures (Fig. 3-4 A and
Table 3-1). Melting curves obtained for CC1 and CC1B’ at 222 nm indicate that CC1B’ is substantially less stable than CC1 (Fig. 3-4 B).

**Figure 3-4. Circular Dichroism Analysis of p150 Fragments.** (A) CD spectra of p150 fragments at different temperatures. CC1A’ and CC1B’ unfold at 37˚C but can refold upon returning to 4˚C. (B) Melting curves for CC1 and CC1B’ at 222 nm show that both proteins can refold after exposure to high temperatures and CC1B’ has a melting temperature approximately 10˚C lower than CC1.
Table 3-1. Secondary Structure Predictions of p150 Fragments. Predicted proportion of alpha helices, beta strands, and disordered regions at different temperatures for each of the p150 fragments. The ratio of the molar ellipticity at 222 to 208 is given in the right column and when this is greater than 1.0 it indicates coiled-coil.

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<tr>
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<th>Beta Strands</th>
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<td>4°C</td>
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<tr>
<td>CC1</td>
<td>0.984</td>
<td>0.016</td>
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<td>1.090</td>
</tr>
<tr>
<td>p135 CC1</td>
<td>0.944</td>
<td>0.022</td>
<td>0.034</td>
<td>1.027</td>
</tr>
<tr>
<td>25°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC1</td>
<td>0.975</td>
<td>0.015</td>
<td>0.01</td>
<td>1.075</td>
</tr>
<tr>
<td>CC1B'</td>
<td>0.975</td>
<td>0.015</td>
<td>0.01</td>
<td>1.050</td>
</tr>
<tr>
<td>CC1A'</td>
<td>0.974</td>
<td>0.012</td>
<td>0.013</td>
<td>1.016</td>
</tr>
<tr>
<td>p135 CC1</td>
<td>0.94</td>
<td>0.024</td>
<td>0.035</td>
<td>1.008</td>
</tr>
<tr>
<td>37°C</td>
<td></td>
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<tr>
<td>CC1</td>
<td>0.665</td>
<td>0.031</td>
<td>0.304</td>
<td>0.964</td>
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<tr>
<td>CC1B'</td>
<td>0.359</td>
<td>0.05</td>
<td>0.591</td>
<td>0.650</td>
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<tr>
<td>CC1A'</td>
<td>0.579</td>
<td>0.026</td>
<td>0.394</td>
<td>0.814</td>
</tr>
<tr>
<td>p135 CC1</td>
<td>0.944</td>
<td>0.022</td>
<td>0.034</td>
<td>0.956</td>
</tr>
</tbody>
</table>

To confirm that the coiled-coils formed were dimeric, we performed multi-angle light scattering at 4°C to obtain an absolute molecular weight for CC1 and CC1B’. In both cases, analyses indicated that the vast majority of the protein population is dimeric at this temperature, consistent with the formation of dimeric coiled-coils (Table 3-2).

Table 3-2. Molecular Weights of CC1 and CC1B’ from Multi-Angle Light Scattering Measurements. The majority of CC1 (94%) and CC1B’ (95%) have a molecular weight similar to the predicted MW of a dimer. A small percentage of each protein population formed higher order oligomers.

<table>
<thead>
<tr>
<th></th>
<th>Dimer MW (kDa)</th>
<th>Oligomer MW (kDa)</th>
<th>predicted dimer (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC1</td>
<td>88.9 ± 6.2 (94%)</td>
<td>514.3 ± 36 (6%)</td>
<td>82.2</td>
</tr>
<tr>
<td>CC1B'</td>
<td>53.3 ± 5.9 (95%)</td>
<td>5807 ± 116 (4%)</td>
<td>50.2</td>
</tr>
</tbody>
</table>
Analysis of p150 Fragment Effects on Single-Molecule Dynein Behavior

All single-molecule studies were performed by Suvranta Tripathy in the Gross Lab at UC Irvine Department of Developmental and Cell Biology using purified bovine dynein and the p150 fragments described and characterized above. The data presented below is not shown due to pending submission for publication. Based on studies that implied the p150 MT binding regions are not necessary for dynactin to increase dynein’s processivity (Johansson et al., 2007; Kardon et al., 2009; Kim et al., 2007), we hypothesized that only the dynein-interacting region of dynactin was required to increase dynein run length through some unknown mechanism. We began by assessing the effects of CC1 and CC1B’ on dynein’s single-molecule velocity, MT-binding, run length and force production. Dynein was non-specifically attached to carboxylated beads, which were manipulated with an optical laser trap used to bring the bead to a MT and to measure the force generated by the attached motor. All single molecule experiments were performed at 23°C with an MT binding fraction at or below 30%, such that predominately one dynein molecule interacted with the MT (Yadav et al., 2012; Svoboda and Block, 1994). Velocity, force, and run lengths for dynein alone were similar to previously described measurements for mammalian dynein (Muresan et al., 2001; Mckenney et al., 2010; King and Schroer, 2000). Interestingly, addition of 20-fold molar excess of CC1B’ increased the average run length of dynein 2.3-fold, in line with reported run lengths for dynein associated with the entire dynactin complex (Hoogenraad et al., 2003; King and Schroer, 2000; Matanis et al., 2002). CC1B’ had no other effects on dynein’s single molecule behavior in these assays. Surprisingly, though CC1 contains CC1B’, it induced inhibition of several dynein activities, including a 65% reduction in beads capable of binding a MT and a 50% reduction in velocity. Additionally, 70% of these beads showed no processive directional motion and could not escape from the weakest
trap, compared to 10-25% for controls. We have termed these molecules ‘diffusive’. Processivity and force production for non-diffusive dyneins were unchanged in the presence of CC1.

We hypothesized that the inhibitory effect of CC1 may be steric, since it is predicted to be a nearly 50 nm coiled-coil (at 1.5 Å/a.a. (Caviston et al., 2007; Whitby et al., 1992)) with the N-terminal 20 nm CC1A’ unbound to the IC, and potentially obstructing motor activity. We were interested in whether this represented a real function of dynactin and decided to test a p150 fragment with a biologically relevant N-terminus, namely p135-CC1 (Fig 3-1 B), which has the same C terminal boundary as CC1 and an N-terminus corresponding to a naturally occurring neuronal splice variant that lacks the CAP-Gly domain and part of the basic domain. This fragment induced a mixture of effects on dynein, causing a 2-fold increase in average run length in 50% of beads while the remaining beads were diffusive. Non-diffusive beads were also slightly slower. Importantly, p135-CC1 alone on beads did not interact with MTs, suggesting that increased run length is mediated by CC1B’. Furthermore, these results imply that there may be some inhibitory function of the N-terminal portion of p150.

To further assess the mechanism of CC1B’-induced increased run length, we observed stepping both parallel and perpendicular to the MT. Studies of dynein’s stepping behavior have shown that dynein can take different sized steps and has a relatively high rate of backstepping (Paschal et al., 1991; Qiu et al., 2012; DeWitt et al., 2011; Mallik et al., 2004). We also observed this behavior for dynein alone. Strikingly, in the presence of CC1B’ dynein took fewer backsteps, larger forward steps and showed less lateral motion and fewer shifts between MT protofilaments. Combined, these stepping changes can account for the 2.4-fold increase in dynein processivity observed with CC1B’.
Analysis of p150 Fragment Effects on Ensemble Dynein Behavior

p150 Fragment Effects on Dynein-Mediated MT Gliding

MT gliding assays are a classical method to determine activity and direction of MT motors, whereby motors that are non-specifically immobilized on a glass coverslip power the movement of MTs (Fig. 3-5 A). Average velocities of the MTs are dependent on temperature, motor density and ATP and salt concentrations (Böhm et al., 2000). A recent study showed that the dynein regulator Lis1 decreases the MT gliding velocity of single headed dynein motors, corresponding to decreased single molecule velocities and increased association with MTs that likely explains dynein’s increased persistence under high loads when associated with Lis1 (Mckenney et al., 2010; Huang et al., 2012). To test for effects that might correspond to our single molecule data, we assayed dynein-driven MT gliding with the various p150 fragments (Fig. 3-5 B and Table 3-3). Interestingly, p135-CC1 slowed MT gliding velocity, perhaps consistent with increased association of motors with MTs required for longer run lengths. Conversely, CC1 increased gliding speed, which may be caused by the decreased MT binding and increased diffusive molecules observed in the bead assays. This inhibition may serve to reduce drag on the MT as it is moved by several motors. However, it is unclear how the increased velocity occurs despite lower single molecule velocities. CC1A’ had very little effect on gliding velocity, as expected. CC1B’ slightly increased MT speed, but due to the set up of our imaging system, gliding assays were performed at 26-27°C, where the majority of CC1B’ would be unfolded (Fig. 3-4 B).
Figure 3-5. The Effect of p150 Fragments on Dynein-Driven MT Gliding. (A) Experimental setup for gliding experiments. Dynein is non-specifically adsorbed to a coverslip and fluorescent MTs, ATP, p150 fragments, and an oxygen scavenging system are flown in. (B) Plots of gliding velocities for individual MTs for dynein with 10-fold molar excess of p150 fragments. Horizontal black lines indicate average velocities. Data were collected from at least 3 independent experiments. Control is dynein alone.

Table 3-3. MT Gliding Velocities for Dynein with p150 Fragments. Average MT gliding velocities for dynein alone (control) or dynein with 10-fold molar excess of p150 fragments as shown in Fig 3-5 B. N indicates the number of MTs observed in at least three independent experiments. P values from a 2-tailed T test are shown in the right hand column.

<table>
<thead>
<tr>
<th></th>
<th>Mean Velocity (um/sec)</th>
<th>Standard Deviation</th>
<th>N</th>
<th>p (2-tailed T test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.4</td>
<td>0.14</td>
<td>185</td>
<td></td>
</tr>
<tr>
<td>10X CC1</td>
<td>0.49</td>
<td>0.12</td>
<td>158</td>
<td>4.14E-11</td>
</tr>
<tr>
<td>10X CC1B'</td>
<td>0.45</td>
<td>0.16</td>
<td>226</td>
<td>1.29E-04</td>
</tr>
<tr>
<td>10X CC1A'</td>
<td>0.37</td>
<td>0.12</td>
<td>124</td>
<td>4.77E-02</td>
</tr>
<tr>
<td>10X p135-CC1</td>
<td>0.31</td>
<td>0.1</td>
<td>147</td>
<td>1.13E-09</td>
</tr>
</tbody>
</table>
**p150 Fragment Effects on Dynein Affinity for MTs**

To assess for changes in association of dynein with MTs, we performed cosedimentation experiments with or without ATP in the presence of a 10-fold excess of p150 fragments (Fig. 3-6). Dynein will bind tightly to MTs in the apo (no nucleotide) state but will release due to stepping in the presence of ATP (Paschal et al., 1991). The only p150 fragment that affected dynein’s cosedimentation with MTs was p135-CC1, which caused an increase in MT bound dynein in the presence of ATP and is consistent with slowed MT gliding velocity. Given that CC1 reduces dynein binding in single molecule assays, it is unclear why that behavior is not detected in MT sedimentation. The effect may be sufficiently subtle as to be undetectable by this method. None of the p150 proteins interacted with MTs in the absence of dynein (data not shown).

**Figure 3-6. MT Sedimentation of Dynein with p150 Fragments at 25˚C.** Dynein was mixed with 10-fold molar excess of p150 protein and MTs with or without 10mM ATP. Samples were sedimented and supernatants and pellets were analyzed by Sypro staining of SDS gels. BSA was included to prevent non-specific interactions and appears in the gels. (S = supernatant, P = pellet) Quantification of the dynein HC band is shown in the graph. Error bars are S.D. for three independent experiments (**p<.005, two-tailed T-test).
To address this question more directly, we performed kinetic assays using malachite green phosphate detection to determine dynein’s ATPase activity at increasing MT concentrations (Fig. 3-7). At 37°C these assays revealed that CC1 decreases dynein’s affinity for MTs ($K_{MT}$ ~20 uM vs ~2 uM for dynein alone). There was no effect of CC1B’ on dynein’s ATPase activity, likely do to loss of secondary structure at this temperature (Fig. 3-4, Table 3-1). Attempts to repeat these experiments at 25°C were unsuccessful due to decreased sensitivity of the assay.

**Figure 3-7. ATPase Kinetics for Dynein with CC1B’ and CC1 at 37°C.** ATPase activities at increasing MT concentrations were recorded and fit with Michaelis-Menten kinetics. $V_{\text{max}}$ and $K_{MT}$ were calculated from the curves, $R^2$ represents goodness of fit. Error bars are S.D. from three replicates in one experiment.
In Vivo Studies with p150-GFP Over-Expression Constructs

Given the effects of p150 fragments on dynein’s in vitro behavior, we examined different dynein cargos following over-expression of CC1 and CC1B’-GFP in Cos7 cells. Historically, CC1 overexpression has consistently disrupted dynein-based transport and is often used as a proxy for direct dynein inhibition (Quintyne et al., 1999), as has overexpression of the dynactin subunit p50, also known as dynamitin because its excess dissociates p150 from the dynactin complex (Echeverri, 1996; Burkhardt et al., 1997). The hypothesized mechanism is that a surplus of CC1 or p50 dissociates dynein from dynactin and prevents dynein recruitment to cargo and/or its activation, however our biophysical data indicate that CC1 may have additional inhibitory activity. We were curious if in cases where dynactin is only required for activation and not recruitment, CC1 may be inhibitory while CC1B’ is activating.

Before examining specific cargo, we assayed for any effects of CC1 or CC1B’-GFP on dynein or dynactin complex integrity. We found that both fragments cosedimented with dynein without altering IC or p150 sedimentation patterns, suggesting that they interact with dynein in vivo, are not incorporated into dynactin (since p150 is not displaced), and do not disrupt either complex (Fig. 3-3 E).

We next examined effects on the interphase MT network because CC1 or p50 overexpression are reported to increase the incidence of unfocused MT arrays (Quintyne et al., 1999; Burkhardt et al., 1997). Quintyne and colleagues proposed that dynactin anchors MTs at the interphase centrosome and requires dynein for transport there. This model predicts that CC1B’ will have a similar effect on MT organization, and indeed CC1 and CC1B’ overexpression caused similar two-fold increases in unfocused MT networks relative to controls.
(Fig. 3-8). We therefore excluded cells with abnormal MTs in all further analyses.

Figure 3-8. CC1 and CC1B’-GFP Over-Expression Disrupts MT Arrays. (A) Quantification of cells scored for MT phenotype in CC1-GFP and CC1B’-GFP over-expressing Cos7 cells, fixed and stained for tubulin. Error bars are S.D. for three independent experiments. (B) Representative images for unfocused MT arrays in CC1 and CC1B’ expressing cells. (Scale bars = 10 um)

Varying claims have been made about dynactin’s recruitment vs. activating role in membranous cargo transport. The original descriptions of dynactin argued that it is an activator of vesicular transport and it is not required for recruitment (Schroer and Sheetz, 1991). Some studies since have shown that disruption of the dynein-dynactin interaction prevents dynein recruitment to membranes (Splinter et al., 2012; Roghi and Allan, 1999; Muresan et al., 2001; Steffen et al., 1997; Waterman-Storer et al., 1997), while others have maintained that dynein can be recruited to membranes independently of dynactin (Yadav et al., 2012; Tan et al., 2011; Lacey and Haimo, 1994; Haghnia et al., 2007).

Since CC1 overexpression disrupts Golgi and vesicular distribution (Moughamian and Holzbaur, 2012; Quintyne et al., 1999; Flores-Rodriguez et al., 2011), we used it as a positive control for comparison to CC1B’ overexpression effects on Golgi and lysosomes. If dynactin is
a recruitment factor then CC1B’ should also be disruptive. Both CC1 and CC1B’ were potent inhibitors of lysosome clustering around the centrosome in the perinuclear region (Fig. 3-9 A and B), consistent with reports that dynactin recruits dynein to lysosomes through RILP (Watson and Stephens, 2006; Johansson et al., 2007). CC1B’ had a more moderate effect on Golgi morphology (Fig. 3-9 C and D), suggesting that some dynein might be recruited to the Golgi independently of dynein and this population is immune to dynactin displacement and potentially activated by CC1B’. Alternatively, our CD data indicate that CC1B’ unfolds at physiological temperatures, possibly reducing its ability to displace dynein. In this case we expect to see moderate effects on MT organization and lysosome localization, which we did not. Furthermore, CC1B’ co-sediments strongly with dynein on a sucrose gradient of lysates prepared from CC1B’ over-expressing cells (Fig. 3-3 E).

To distinguish between recruitment defects and inhibition, we observed CC1B’ effects on adenovirus transport to the nucleus, a cargo that does not rely on dynactin for recruitment. Our lab has shown that the dynein light intermediate chains (LICs) directly mediate the interaction with adenovirus and that p50 overexpression does not displace dynein from the virus. Despite this, p50 and CC1 overexpression strongly interfere with viral transport (Kim et al., 2007; Bremner et al., 2009). We hypothesized that free, full length p150 or CC1 inhibit transport by directly deactivating dynein, and that CC1B’ would allow normal or improved movement of virus to the nucleus. However, CC1B’ overexpression inhibited virus transport though the effects were modest in comparison to CC1 overexpression and the level of inhibition was not statistically significant (Fig. 3-10). Again, unfolded CC1B’ may be incapable of inducing processivity increases.
Figure 3-9. Effects of CC1 and CC1B’ Over-Expression on Membranous Organelles. (A) Quantification of CC1 and CC1B’ over-expression effects on lysosomes. Error bars are S.D. for three independent experiments. (B) Representative images of Lamp2 staining in CC1 and CC1B’ over-expressing Cos7 cells. Scale bars = 10 um. (C) Representative images of Golgi staining in CC1 and CC1B’ over-expressing Cos7 cells. Scale bars = 10 um. (D) Quantification of CC1 and CC1B’ over-expression effect on Golgi morphology. Error bars are S.D. for three independent experiments.
Figure 3-10. Effect of CC1 and CC1B’ Over-Expression on Adenovirus Transport to the Nucleus. (A) Quantification of the proportion of virus particles at the nucleus one hour post infection. Error bars are S.D. for three independent experiments. (**p<.005, two-tailed T-test) (B) Representative images of over-expressing Cos7 cells stained for tubulin and adenovirus.

**Discussion**

We initiated this study to determine if (1) dynactin’s MT binding activity is required to increase dynein’s run length and (2) if dynactin affects dynein’s force production. Because a stable purified mammalian dynein-dynactin complex cannot be formed without the addition of another protein (Mckenney et al., 2010; Splinter et al., 2012), and other studies have shown that dynactin containing truncated forms of p150 can increase dynein processivity (Vallee et al., 2012; Kardon et al., 2009; Kim et al., 2007), we tested the effects of dynein-interacting p150 fragments on dynein activity in single and multiple motor assays in vitro and on transport of cargo in vivo.

Circular dichroism and multi-angle light scattering indicate that the fragments are well-behaved coiled-coil dimers, though the minimal dynein interacting fragment, CC1B’ is less
stable than the other fragments (Fig. 3-4), which was recently confirmed by another group (Watanabe et al., 2011; Siglin et al., 2013). Pull down experiments here and elsewhere (Vaughan and Vallee, 1995; Mckenney et al., 2011; Karki and Holzbaur, 1995) indicate that the primary mode of interaction for CC1B’ is through the dynein IC N-terminal region. CC1B’ containing p150 fragments cosediment with dynein on sucrose gradients and do not appear to discernably alter dynein structure (Fig. 3-3).

**p150 Fragment Effects on Single Molecule Dynein Behavior**

We did not detect any alterations to force production of processive dynein motors by any p150 fragments. Other single molecule measurements reveal mixed effects on dynein’s activity. CC1B’ and p135-CC1 increase dynein processivity to the same extent reported for whole dynactin complex (Mckenney et al., 2011; Kardon et al., 2009; King and Schroer, 2000). Curiously, p135-CC1 also causes dynein to become diffusive in 50% of cases. CC1, on the other hand, is completely inhibitory and decreases bead binding fraction by 65%. Of the beads that do bind, 70% show diffusive movement, compared to 10-25% for controls. Additionally, non-diffusive CC1-dynein complexes have velocities that are half that of controls. We hypothesize that the CC1A’ may sterically hinder dynein since it forms a coiled-coil predicted to be 20 nm. This region may be less inhibitory in the context of p135-CC1 because it contains part of the basic domain, which may allow transient interactions with MTs preventing the coiled-coil from interfering with the motor domain. Interestingly CC1B’ seems to enhance coordination in dynein stepping by reducing back steps and side steps, while increasing the number of large forward steps. This supports a recent study showing that dynein heads are more coordinated when step sizes are larger (Siglin et al., 2013; Qiu et al., 2012).
Some evidence exists that dynactin may have inhibitory effects on dynein. GFP tagged dynein-dynactin purified from mouse brain moved bi-directionally, with 30% of movements in the plus end direction (Tokito et al., 1996; Ross et al., 2006). The extent of dynein-dynactin complex formation in this study is unclear and there is no direct comparison to dynein purified under the same conditions, but the basic domain of p150 is capable of mediating one-dimensional diffusion of the dynactin complex along MTs (Siglin et al., 2013; Culver-Hanlon et al., 2006), so the plus-end directed motions may have been diffusive. Ross et al. argue plus-end movements were not diffusive, since their velocity was ATP dependent, however ATP-depend dynein diffusion has been observed (Mckenney et al., 2011; Wang and Sheetz, 1999). Diffusion along MTs may allow dynein to move around roadblocks by moving to different protofilaments or to search the MT for the plus end where it awaits cargo. Indeed several kinesins display both diffusive and processive movement (Ori-McKenney et al., 2010; Cooper and Wordeman, 2009; Harms et al., 2012). The dual properties of p135-CC1 may represent a way for dynein to switch between modes, though we have not observed both behaviors for a single molecule. While the mechanism of CC1 inhibition remains unclear, it is an important observation given the ubiquitous use of CC1 to displace dynactin from dynein and indicates additional mechanochemical inhibition.

**p150 Fragment Effects on Ensemble Dynein Behavior**

Interestingly p135-CC1 effects on several multi-motor dynein behaviors is consistent with increased processivity, namely slowed MT gliding and increased dynein sedimentation in the presence of ATP. Neither of these effects were observed with CC1B’ however, perhaps due to its thermal instability. Though, gliding and MT sedimentation assays were performed at only a few degrees above single molecule assays, the loss of dimeric CC1B’ in the population may
have been great enough to prevent a detectable effect. Alternatively, the diffusivity conferred by half of the p135-CC1-dynein complexes may have enhanced interactions with MTs in these assays. CC1 also showed behavior consistent with inhibition in the single molecule assays. Though CC1 did not change dynein co-sedimentation with MTs, it did alter the affinity for MTs as measured from Michealis-Menten kinetics in ATPase assays. Possibly the concentration of MTs and dynein used in the sedimentation assays were not in the correct range to detect alterations in dynein’s sedimentation. The increased speed of MTs in gliding assays, may be consistent with the presence of more weakly bound diffusive motors that decrease the drag on the MT, though this is admittedly difficult to reconcile with decreased single molecule speeds. Interestingly, NudE, another dynein regulatory factor which decreases dynein’s interaction with MTs (Lau et al., 1984; Mckenney et al., 2010) also increases dynein’s MT gliding speed (Richard McKenney, unpublished data).

**p150 Fragment Effects Dynein Activity In Vivo**

Despite its thermal instability, we found that CC1B’ and CC1 equally inhibit formation of focused MT arrays and lysosome transport, suggesting that dynein localization to lysosomes or MTOC maintenance requires the entire dynactin complex. In support of this the C-terminus of p150 interacts with RILP, whose overexpression leads to highly compact lysosomal cluster at the MTOC (Kardon et al., 2009; Johansson et al., 2007; Kim et al., 2007). Furthermore p150 CC2 and p24 over-expression disrupt MT organization though they do not affect the stability of dynactin (Quintyne et al., 1999), implying they might have dynein-independent centrosomal functions and that CC1B’ disrupts transport of dynactin to the centrosome.

Dynein is recruited to the Golgi directly by golgin 160 (Svoboda and Block, 1994; Yadav et al., 2012) and via dynactin through spectrin (Mckenney et al., 2010; Muresan et al., 2001;
King and Schroer, 2000), Rab6 and BicD2 (King and Schroer, 2000; Hoogenraad et al., 2003; Matanis et al., 2002), and possibly huntingtin and huntingtin associated protein 1 (Whitby et al., 1992; Caviston et al., 2007). The extreme Golgi disruption caused by over-expressed CC1 may represent dynein displacement and inhibition of golgin 160-recruited dynein while the CC1B’ phenotype is more subtle because it does not inhibit dynein in addition to displacing it. An interesting experiment would be to artificially recruit CC1 or CC1B’ to mitrochondria and see if they equally drive minus-end transport.

Though adenovirus recruits dynein via LIC1, viral transport is inhibited by over-expression of CC1 and N-terminally truncated p150, which has an N-terminal boundary similar to CC1. This can be explained by CC1’s in vitro inhibition. The moderate CC1B’ phenotype may reflect that while dynactin does not recruit dynein to adenovirus, other portions of the dynactin complex are required. Specifically, the p150 N-terminal region, though not required for processive motion might be necessary to localize dynein to MT plus-ends where it can encounter incoming viruses.

Conclusions and Future Studies

The finding that CC1B’ can change dynein’s stepping pattern and increase run length is particularly surprising and suggests very long range allosteric regulation of the dynein motor. However, the in vivo studies are compromised by CC1B’ thermal instability and complicated by dynactin’s recruitment functions and it remains to be determined if CC1B’ can activate dynein in a biological setting. The existence of p135 implies that there are dynactin functions that do not involve its MT binding, however, these could be purely recruitment roles. Further in vivo studies with p135-CC1 may resolve these questions. For example, based on our findings, targeting CC1
or p135-CC1 to mitochondria should recruit dynein, however p135-CC1 should result in more efficient transport to the MTOC than CC1.

Additionally it will be important to observe in vitro dynein activities with a p150 fragment that contains the N-terminal binding region (residues 1-555). When expressed in bacteria, this protein is prone to aggregation, but we have successfully expressed and purified it from insect cells and are currently testing its effects on dynein. We will also phosphorylate this protein at a site know to decrease its interaction with MTs and examine how this alters dynein’s behavior.

The physical mechanisms of activation and inhibition for all fragments tested here are unknown. We are presently working to visualize dynein in complex with these fragments by EM after they are labeled with nickel-conjugated gold nanoparticles, which bind to histidine tags on the fragments’ N or C terminus. This will hopefully allow us to locate the position of the fragments relative to dynein and to detect changes in dynein structure that may account for its altered behaviors. The long range allostery that we have described support the possibility that other dynein interactors and/or specific subunit isoforms can have profound effects on dynein activity, which are exciting prospects and will be major areas of study for the dynein field.
Materials and Methods

Cloning and Protein Expression

All p150 constructs for recombinant protein expression were cloned from full-length rat (EDL91133.1) into pGEX 6P-1 (Amersham Biosciences) using the EcoRI and XhoI restriction sites with C-terminal Flag and 6X His tags. Additionally, p150 CC1 and CC1B’ were cloned into pEGFP-N1 (Clontech) using EcoRI and XmaI sites. BL21-CodonPlus RIPL competent cells (Agilent Technologies, #230280) were transformed with pGEX 6P-1 containing the gene of interest. Overnight cultures were used to inoculate 0.5-1 L of LB or Terrific Broth (Sigma, T5574). All cultures were grown at 37°C to an OD (λ600) of 0.5-0.7 and construct expression was induced with 0.5mM IPTG for 4-6 hours at 20°C after 10 minutes cold shock. Following induction cultures were pelleted and frozen at -80°C until purification. For purification, pellets were resuspended in cold PBS with 1mM DTT and 1:500 protease inhibitor cocktail (Sigma, P8340), sonicated on ice for 10 minutes, and centrifuged at 4°C for 30 min at 150,000g with a final concentration of 1% Triton-X. The supernatant was incubated with glutathione beads (GE, 17-0756-01) for 1 hour at 4°C, collected on a column and washed. Beads were washed into cleavage buffer (50mM Tris-HCl pH 7.0, 150mM NaCl, 1mM EDTA) supplemented with 1mM DTT and incubated overnight at 4°C with Precission Protease (GE, 27-0843-01). The supernatant was collected and supplemented with a final concentration of 5% glycerol before aliquoting, flash freezing and storage at -80°C. Dynein GST-motor domain was generously provided by Peter Hook.

Dynein Purification

Dynein was purified from calf brain as described previously(Qiu et al., 2012; Paschal et al., 1991; DeWitt et al., 2011; Mallik et al., 2004). Briefly, calf brains were collected from the
abattoir immediately after animals were scarified, transported on ice, packed into 50mL falcon tubes, flash frozen and stored at -80°C. On the day of the prep, 120-150g brains were thawed at 37°C, homogenized in 120-150 mL P/H buffer (50mM HEPES, 50mM PIPES, 2mM MgCl2,1mM EDTA, pH 7.4), supplemented with 1mM DTT and 1:200 protease inhibitors (Sigma P8340), and insoluble material was removed by sequential centrifugation at 17,000 x g for 30 min and 140,000 x g for 1 hr. MTs were polymerized by addition of 20uM taxol (Sigma, T7402) and incubation at 37°C for 20 min, and sedimented through at 7.5% sucrose cushion at 17,000 x g for 1hr. MT pellet was washed in P/H buffer and 20uM taxol, incubated at 37°C for 10 min, sedimented at 42,000 x g at 25°C. Pellet was resuspended in P/H buffer with 20uM taxol and 3mM GTP (Sigma G8877), incubated and sedimented as before. Dynein was released by resuspending the pellet in P/H buffer with 20uM taxol and 10mM fresh ATP (Sigma A9187), incubated at 37°C for 20 min and spun 75,000 x g for 30 min at 25°C. Supernatant was applied to 11mL 5-20% Tris-KCl (20mM Tris, 50mM KCl, 1mM MgSO4,1mM EDTA, pH7.4) sucrose gradients and centrifuges at 16 hrs at 130,000 x g at 4°C. Fractions were collected, analyzed by Coomassie staining, aliquoted, flash frozen and stored at -80°C.

**Immunoprecipitation**

All IPs were performed in PEM-35 (35 mM PIPES, 5 mM MgSO4, 1 mM EGTA, .5 mM EDTA, pH 7.0) supplemented with .05 ug/ul BSA + 1 mM DTT + .1% Tween for 1-2 hrs at 4°C with protein A beads (Invitrogen). IP and/or blotting antibodies are: 74.1 (gift from Kevin Pfister), anti-Flag (Sigma, F1804 M2) and anti-DDDDK (Abcam ab1162).

**Sucrose Gradients**

For purified proteins, gradients were made by overlaying 4 layers of 30, 22, 14, and 6% sucrose in PEM-35 (35 mM PIPES, 5 mM MgSO4, 1 mM EGTA, .5 mM EDTA, pH 7.0), which were
allowed to linearize for 3 hrs. Purified proteins were mixed, incubated on ice for 1 hr and applied to the gradient. For cells, lysates were made in PEM-35 (35mM PIPES, 5mM MgSO4, 1mM EGTA, .5mM EDTA, pH 7.0) supplemented with 1mM DTT and protease inhibitor cocktail (Sigma P8340) and cells were disrupted by passage through a fine gauge needle. Soluble portion of lysates were applied to gradients. Gradients were centrifuged 3 hrs at 20,000 x g and fractions were analysed by Western blot against dynein IC and flag (listed above), or GFP (Sigma, G1544), p150 (BD Transduction, 612709), and dynein IC.

**Circular Dichroism**

All proteins were dialyzed overnight into 50 mM sodium phosphate buffer pH 7.0 and CD measurements were taken on a Jasco-J815 spectropolimeter in the lab of Scott Banta (Columbia University). Fixed temperature measurements were collected in 0.1mm cuvettes at 185-260 nm wavelengths, 0.1nm data pitch, continuous scanning mode, at standard sensitivity, with scanning speed of 50nm/sec, response pf 8 sec., and bandwidth of 1nm. For each sample 3 data sets were accumulated per run. For melting curves data were accumulated at 222 nm from 5-85°C (for CC1) and 5-60°C (for CC1B’) in a 1mm cuvette with data pitch of 0.5°C, a 10 sec. delay, a temperature slope of 40°C/hr, standard sensitivity, 8 sec. response, and bandwidth of 1 nm. The cooling curve was collected in the same manner reversing the temperature 15 sec. after reaching the maximum. Molar ellipticities were calculated as described (Böhm et al., 2000; Kelly et al., 2005). Secondary structure content predictions were made using the online server DICHROWEB (http://dichroweb.cryst.bbk.ac.uk/html/home.shtml).

**Multi-Angle Light Scattering**

MALS experiments were performed in the lab of John Hunt (Columbia University). Proteins were dialyzed overnight into 50 mM sodium phosphate buffer and data were collected after
analytical gel filtration (Shodex KW-803-4E) using miniDAWN Light Scattering instrument (Wyatt Technology) at 4°C. Data analysis was performed with Astra (Wyatt Technology).

**Microtubule gliding assay**

MT gliding was performed in chambers constructed from acid washed cover slips adhered to glass slides with double-sided tape. Rhodamine labeled MTs (Cytoskeleton, Inc, TL620M) were polymerized in BRB80 (80 mM K-Pipes pH 6.9, 1 mM MgCl2, 1 mM EGTA) supplemented with 20 uM taxol (Sigma, T7402) and 1 mM DTT. Purified bovine dynein (88 nM) was incubated in the chamber for 5 min, MTs were flowed in for 5 min in buffer containing 1 mM ATP (Sigma, A-9187), 10-fold molar excess p150 fragments, and an oxygen scavenging system (gloxy). 25X gloxy contains 25mg/ml glucose oxidase (Sigma, G7141), 5mg/ml catalase (Sigma, C60), 10% glucose (Sigma, G5767) and 25% BME (Sigma, M6250). After a 5 min incubation, excess MTs were washed out with buffer containing 1mM ATP, 10X p150 fragments, and gloxy. Chamber ends were sealed with nail polish and imaged after 10 min at 26-27°C on an Olympus IX81 inverted microscope with 100X objective and CCD Orca-R2 Hamamatsu camera. Images were acquired every second for one minute and movies were analyzed with the manual tracking plugin for ImageJ.

**Microtubule Sedimentation Assays**

Purified bovine dynein (8nM) mixed with 10X p150 fragment and 2.5uM taxol-stabilized MTs (Cytoskeleton Inc, TL238) with or without 10mM ATP (Sigma, A9187) in BRB80 (80 mM K-Pipes pH 6.9, 1 mM MgCl2, 1 mM EGTA) supplemented with 20 uM taxol, 1 mM DTT, and .05 ug/ul BSA was incubated for 30 min at room temperature, and centrifuged for 45 min at 35,000 x g. Supernatants and pellets were analyzed by Sypro staining (Molecular Probes, S-6653) scanned at 700nm on a Lic-or imaging system.
ATPase Assays

ATPase assays were performed using malachite green phosphate detection as described (Mckenney et al., 2010; Baykov et al., 1988; Huang et al., 2012). 1 ug of purified bovine dynein was mixed with 20X p150 fragment and taxol stabilized MTs (Cytoskeleton Inc, TL238) in PEM 30 (30mM K-PIPES, 2mM MgSO4, 2mM EGTA, pH 7.0) in 50ul reactions. 1mM ATP was added just before samples were incubated at 37°C for exactly 15 min. 400 ul malachite green and 50 ul 34% sodium citrate were added to each tube and measurements were taken at 650 nm on a spectrophotometer (Beckman).

p150 Fragment Over-Expression Studies

Cos7 cells were transfected with GFP constructs using Effectene Transfection System (Qiagen) for 24 hrs, fixed in methanol, stained for tubulin, and Golgi or lysosomes. For adenovirus studies cells were infected for one hour as described (Paschal et al., 1991; Bremner et al., 2009), before fixing and staining. Antibodies used for staining were: anti tyrosinated tubulin (Millipore, MB1864), anti-GFP (Sigma, G1544), anti-GM130 (BD Transduction, 558712), anti- LAMP2 (Santa Cruz H4B4), anti-adenovirus (Abcam).
References


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