The yeast dynein Dyn2-Pac11 complex is a dynein dimerization/processivity factor: structural and single-molecule characterization

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ABSTRACT Cytoplasmic dynein is the major microtubule minus end–directed motor. Although studies have probed the mechanism of the C-terminal motor domain, if and how dynein’s N-terminal tail and the accessory chains it binds regulate motor activity remain to be determined. Here, we investigate the structure and function of the Saccharomyces cerevisiae dynein light (Dyn2) and intermediate (Pac11) chains in dynein heavy chain (Dyn1) movement. We present the crystal structure of a Dyn2-Pac11 complex, showing Dyn2-mediated Pac11 dimerization. To determine the molecular effects of Dyn2 and Pac11 on Dyn1 function, we generated dyn2Δ and dyn2Δpac11Δ strains and analyzed Dyn1 single-molecule motor activity. We find that the Dyn2-Pac11 complex promotes Dyn1 homodimerization and potentiates processivity. The absence of Dyn2 and Pac11 yields motors with decreased velocity, dramatically reduced processivity, increased monomerization, aggregation, and immobility as determined by single-molecule measurements. Deleting dyn2 significantly reduces Pac11-Dyn1 complex formation, yielding Dyn1 motors with activity similar to Dyn1 from the dyn2Δpac11Δ strain. Of interest, motor phenotypes resulting from Dyn2-Pac11 complex depletion bear similarity to a point mutation in the mammalian dynein N-terminal tail (Loa), highlighting this region as a conserved, regulatory motor element.

INTRODUCTION

Cytoplasmic dynein is a microtubule (MT) minus end–directed motor complex involved in mitotic spindle formation and positioning, as well as in removing kinetochore-bound anaphase-wait checkpoint components (Pfarr et al., 1990; Steuer et al., 1990; Bader and Vaughan, 2010). In interphase, cytoplasmic dynein organizes the MT array and transports cargoes, including organelles and vesicles from peripheral regions to the cell center (Vale, 2003). When anchored at the cell cortex or at kinetochores, dynein captures MTs and pulls them toward its anchor site (Adames and Cooper, 2000; Tulu et al., 2006). In cilia, cytoplasmic dynein (cytoplasmic dynein 2) facilitates retrograde intraflagellar transport from the tip of the cilium back toward the cell body (Signor et al., 1999). Collectively, cytoplasmic dynein is the major MT minus end–directed processive motor in eukaryotic cells. However, the structural determinants that allow the native motor to dimerize and move processively remain to be determined.

The dynein heavy chain (HC) is composed of an N-terminal dimerization/cargo-binding domain and a C-terminal motor domain. The motor domain comprises a hexameric array of AAA+ ATPase domains (AAA+; ATPase associated with various cellular activities), a subset of which hydrolyze ATP to drive conformational changes of the force- and displacement-generating linker element and the
MT-binding stalk (Burgess et al., 2003; Carter et al., 2008, 2011), effectively coordinating phases of ATP hydrolysis and nucleotide exchange with MT binding, motor domain motions, and MT release (Kon et al., 2004, 2005; Reck-Peterson et al., 2006; Imamura et al., 2007). Although recent electron microscopy (Burgess et al., 2003; Roberts et al., 2009) and crystallographic studies (Kon et al., 2012; Schmidt et al., 2012) illuminate ATP-dependent structural changes underlying the dynein motility mechanism, little is known about the factors that bind the dynein N-terminal region and their potential to regulate dynein motor activity.

In contrast to the expansive MT plus-end-directed kinesin motor family, eukaryotes contain a single cytoplasmic dynein heavy chain (gene) (excluding many plant species that lack cytoplasmic dynein altogether). To perform specific biological functions, the cytoplasmic dynein heavy chain engages a subset of diverse proteins that facilitate differential cargo attachment and motor regulation. The dynein heavy chain N-terminal domain binds a number of accessory proteins directly, including the dynein intermediate chain (IC) and light-intermediate chain (LIC; Tynan et al., 2000). Human cytoplasmic dynein is essential to cellular function and performs diverse tasks by differentially recruiting a subset of accessory factors from a pool of 19 known proteins (Wickstead and Gull, 2007). In contrast, the Saccharomyces cerevisiae dynein heavy chain (Dyn1) is not essential and is exclusively used to position the mitotic spindle (Eshel et al., 1993; Li et al., 1993). Dyn1Δ cells are viable due to Cin8 activity, which redundantly positions the mitotic spindle, although dyn1Δ cells exhibit spindle-positioning defects, delayed mitosis, and an increased percentage of binucleate mother cells (Eshel et al., 1993; Saunders et al., 1995; Geiser et al., 1997). Restricted to this single function, yeast Dyn1 has a limited number of subunits and accessory proteins, which include the IC (Pac11), LIC (Dyn3), light chain (LC; Dyn2), Lis1 (Pac1), Nudel (Ndl1), and the dynactin complex (Lee et al., 2003; Li et al., 2005; Wickstead and Gull, 2007; Stuchell-Brereton et al., 2011).

Dynein ICs are composed of a predicted N-terminal coiled coil, followed by LC binding sites and a C-terminal WD-40 repeat/B-propeller domain (Figure 1A). The metazoan IC N-terminal region interacts with the p150Glued subunit of the dynactin complex (Nip100 in yeast; Moore et al., 2008), a key regulator of dynein motor activity (Karki and Holzbaur, 1995; King et al., 2003; Ross et al., 2006; Kardon et al., 2009). Metazoan ICs bind three distinct dynein LCs: TcTex, LC8, and LC7 (Mok et al., 2001; Makokha et al., 2002; Susalka et al., 2002). These three dynein LCs bind distinct motifs that bridge the IC’s N-terminal coiled coil and the C-terminal WD-40 repeat domain.

Absence of the mammalian dynein intermediate and light chains increases heavy-chain ATPase activity, reduces microtubule gliding activity, and causes overall structural instability (Gill et al., 1994; Kini and Collins, 2001; King et al., 2002). Recent work reconstituting the human cytoplasmic dynein complex from individually purified components found that the dynein heavy chain was unstable and aggregated in the absence of accessory chains and was monomeric when solubilized in high–ionic strength buffer (Trokter et al., 2012). In the same study, addition of the dynein intermediate and light intermediate chains promoted heavy-chain dimerization. The addition of all accessory chains (IC, LIC, and LCs) was required to attain the IC’s N-terminal coiled coil and the C-terminal WD-40 repeat domain. Here, we investigate the interaction between Dyn2 and Pac11 and probe the role of these accessory chains in dynein motor activity. We report the crystal structure of Dyn2 in complex with the second Pac11 Dyn2-binding site, determined to 1.9-Å resolution. We determine the affinity between Dyn2 and the second Pac11 Dyn2-binding site, characterize the determinants that confer Dyn2 dimerization and Pac11 binding, and show that the Dyn2-Pac11 complex enhances Dyn1 velocity, promotes Dyn1 homodimerization, and dramatically potentiates motor processivity.

**RESULTS**

**Yeast Pac11 contains two conserved Dyn2-binding motifs**

To compare and contrast the structural features of dynein intermediate chains from yeast with those from higher eukaryotes, we mapped the domain architecture of S. cerevisiae Pac11 and human

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FIGURE 1: Dynein ICs contain conserved dynein LC-binding motifs near the predicted N-terminal coiled-coil domain. (A) Domain diagrams of dynein intermediate chains from *S. cerevisiae* and *H. sapiens* consisting of a conserved, predicted N-terminal coiled-coil region followed by arrayed dynein LC-binding sites. The *S. cerevisiae* dynein IC contains two Dyn2-binding sites, whereas the human counterpart contains sequential binding sites for the light chains TcTex, LC8, and LC7. The Dyn2- and LC8-binding sites contain the invariant, signature QT motif. The ICs also contain C-terminal WD40 repeats predicted to form a β-propeller structure. The *H. sapiens* IC contains a p150Glued-binding site between residues 1 and 116. A sequence alignment across 12 species is shown below with identity contoured at 100% (green) and ≥60% (yellow) across all species shown. Two subgroups were delineated: subgroup 1 comprising *S. cerevisiae*, *Paracoccidioides brasiliensis*, *A. nidulans*, *Ajellomyces dermatitidis*, and *Arthroderma gypseum* with two known or predicted Dyn2/LC8-binding sites; and subgroup 2, comprising *Dictyostelium discoideum*, *D. melanogaster*, *Danio rerio*, *Xenopus tropicalis*, *Rattus norvegicus*, *Bos taurus*, and *H. sapiens* with known or predicted sequential TcTex- and LC8-binding sites. Within these subgroups, 60% identity is highlighted in light orange (subgroup 1) or purple (subgroup 2, TcTex site) and dark orange (subgroup 2, LC8 site). (B) Reported Dyn2 binding sites in *S. cerevisiae* Nup159 and Pac11 aligned with the LC8- and TcTex-binding sites from human and *D. melanogaster* dynein ICs. The Dyn2/LC8-binding sites display a highly conserved QT sequence motif that is QV in the *H. sapiens* IC TcTex-binding site and QA in the *D. melanogaster* site. A general consensus sequence is indicated below, where δ is a charged residue, X is any residue, and ϕ is V, L, or I. (C) Pac11 pep2 binds Dyn2 exothermically in a 2:2 (modeled as a 1:1) stoichiometry with *K*ₐ = 620 ± 270 nM as measured by isothermal titration calorimetry. Top, heat generated by sequential 19 × 2 μl injections of 1 mM Pac11 pep2, injected into the ITC cell containing 200 μl of 50 μM Dyn2. Bottom, data fit to a single-binding-site model from one of three independent experiments with averaged *K*ₐ (± SD).
Higher eukaryotic dynein ICs contain a predicted N-terminal coiled-coil domain followed by three sequential dynein LC–binding sites for TcTex, LC8, and LC7, respectively. Although mammalian IC–LC interactions have been biophysically and structurally characterized, a molecular and structural analysis of yeast dynein IC–LC interactions is incomplete. We created a sequence alignment of dynein ICs, spanning the first two LC-binding sites across diverse yeast species, as well as in higher eukaryotes (Figure 1A). Yeast contains a solitary dynein LC, Dyn2, which is homologous to LC8. Previous work identified two Dyn2-binding sites in the Pac11 IC (Stuchell-Brereton et al., 2011). Each binding site contains a signature LC-binding motif characterized by sequential QT residues. The first Dyn2-binding site substitutes for the corresponding TcTex-binding site in higher eukaryotes, whereas the second Dyn2-binding site is homologous to the higher eukaryotic IC LC8-binding site. The TcTex-binding site displays moderate homology to Dyn2/LC8-binding sites. TcTex and LC8/Dyn2 share a similar fold and target-binding mode, but TcTex is elongated relative to LC8, and the two families have little sequence homology (Williams et al., 2005). The two tandem Pac11 Dyn2-binding sites are conserved across other yeast species (Figure 1A). Dyn2-binding sites characterized in S. cerevisiae proteins include a pentameric Dyn2-binding array present in the nucleoporin Nup159, where Dyn2 is believed to promote Nup159 oligomerization (Stelter et al., 2007) and scaffold extension of the cytoplasmic fibrill to effectively gate nuclear transport. We aligned the Pac11 and Nup159 Dyn2-binding sites, which highlights the invariant QT motif. Although residues flanking the QT motif exhibit variance, a general consensus sequence is evident: δ-X-G/S-ϕ-Q-T-D/E, where δ is a charged residue, X is any residue, and ϕ is V, L, or I (Figure 1B).

Dyn2 binds Pac11 site 2 with submicromolar affinity

To determine the affinity between Dyn2 and the individual Pac11 Dyn2-binding sites, we performed isothermal titration calorimetry (ITC). Two peptides, each corresponding to the Pac11 Dyn2-binding sites, were synthesized and designated Pac11 pep1 and pep2, respectively. Pac11 pep1 proved insoluble, likely due to its high hydrophobic content, and precluded further investigation. Pac11 pep2 was solubilized and injected into the ITC cell containing Dyn2 at pH 6.8 (Figure 1C). Pac11 pep2 bound Dyn2 exothermically at 26 °C. To fit the data, we used a single-site binding model as used in other LC-binding studies (Radnai et al., 2010; Romes et al., 2012). The Dyn2 homodimer has two sites that bind peptides independently, enabling the 2:2 (Dyn2:peptide) interaction to be effectively modeled as a 1:1 interaction. The data were well described by a single-site binding model, yielding an average binding affinity $K_d = 620 \text{nM} \pm 270 \text{nM}$ (Figure 1C; $N = 1.05 \pm 0.02$ sites, $\Delta H = -5175 \pm 139 \text{cal/mol}$, $\Delta S = 11.3 \pm 1.5 \text{cal/mol per deg})$. Previous Dyn2/LC8-binding experiments found that light chains bind peptides either endothermically or exothermically, depending on the peptide sequence (Hödi et al., 2006; Wagner et al., 2006; Benison et al., 2008; Radnai et al., 2010; Nyarko et al., 2011; Rapali et al., 2011; Romes et al., 2012). Previous characterization of Dyn2-Nup159 interactions showed both thermal binding modes, with $K_d = 13–18 \text{μM}$ (Romes et al., 2012), indicating that the Pac11 pep2 interaction is an order of magnitude tighter than Dyn2-binding sites characterized to date. Mammalian LC8 binds similar-length peptides exothermically with $K_d = 1 \text{μM}$ for the apoptosis regulator Bmf and 7 μM for neuronal nitric oxide synthase (Radnai et al., 2010). Thus the Dyn2-Pac11 pep2 interaction is on par with known high-affinity LC8 interactions. Although the Pac11 N-terminal region contains a predicted coiled-coil, previous analytical ultracentrifugation studies indicated that this region is unlikely to confer Pac11 homodimerization in the absence of Dyn2 (Stuchell-Brereton et al., 2011) (a coiled coil may form, however, once Dyn2 mediates initial Pac11 dimerization). Because the Pac11-Dyn2 complex is formed through multivalent interactions, the measured Dyn2 affinity for Pac11 pep2 is likely weaker than its affinity for native Pac11.

Structure of the Dyn2-Pac11 complex

To determine the molecular architecture of the Pac11-Dyn2 complex, we crystallized Dyn2 in complex with Pac11 pep2. Dyn2:Pac11 pep2 crystals were obtained from a solution containing a 1:1.2 M ratio of Dyn2 and Pac11 pep2. A complete diffraction data set was collected to 1.90-Å resolution in space group C2221. The structure was solved by molecular replacement using a single Dyn2 chain derived from the Dyn2-Nup159 structure (PDB 4DS1; Romes et al., 2012). Clear electron density for all Pac11 pep2 chains was evident in the molecular replacement electron density map ($F_o - F_c$), confirming the presence of bound peptide. The structure was built and refined to R and $R_{free}$ factors values of 16.0 and 21.2, respectively, and is presented in Figure 2A. Multiple Dyn2-Pac11 complexes were present in the asymmetric unit, and each yielded a similar structure (Figure 2B; root-mean-square deviation, 0.18 Å across 168 Cα atoms) indicative of a relatively rigid, locked complex. Crystallographic data, refinement, and model statistics are presented in Table 1. A final, refined $2F_o - F_c$ electron density map for Pac11 pep2 is shown in Figure 2C.

The Dyn2 homodimer induces parallel homodimerization of Pac11 pep2 chains

In the Dyn2-Pac11 pep2 complex, two Pac11 chains form β-strands that symmetrically extend the central β-sheets of the Dyn2 homodimer (Figure 2A). Each β-sheet is composed of six antiparallel β-strands: four from one Dyn2 subunit, a fifth from the dimeric Dyn2 mate (features of which are designated with a prime symbol), and the sixth from one of the Pac11 pep2 strands, ordered β1-β4-β5-β2-β3'-Pac11 pep2. The two core β-sheets, related by a twofold symmetry axis, form a β-sandwich, flanked by Dyn2 helices α1 and α2. The α2 helix lines one side of the Pac11 pep2-binding site. The two Pac11 peptides in the complex are positioned parallel to one another on opposite sides of the Dyn2 homodimer. The distance between equivalent residues in each of the two Pac11 peptides (as measured between Cα carbon atoms) varies from 14 to 22 Å. Thus, within Pac11’s LC-binding region, the Dyn2 homodimer drives parallel Pac11 dimerization. This structural arrangement is consistent with an N-terminal parallel coiled coil in Pac11.

Dyn2 engages Pac11 pep2 using extensive hydrogen bonding, van der Waals contacts, and canonical QT-motif recognition determinants

Pac11 pep2 residues Y76–Q82 form a β-strand running antiparallel to Dyn2-β3 (Figure 2A). Pac11 pep2 binds the Dyn2 homodimer through standard antiparallel β-sheet hydrogen bonding, as well as via distinct side-chain interactions that engage both Dyn2 subunits in the homodimer (Figure 3, A and B). The largest number of hydrogen bonds and van der Waals contacts are contributed by the canonical LC8 target motif determinants, Pac11 pep2 residues Q82 and T83 (Figure 3C). Overall, the number of hydrogen bonds and van der Waals interactions likely contribute to both the stability and specificity of the Dyn2-Pac11 pep2 interaction. Dyn2 recognizes similar target determinants in Nup159 (Supplemental Figure S1) and binds Nup159 in an analogous, antiparallel β-strand configuration (Romes et al., 2012). To compare and contrast LC target binding
glutamate or glutamine) at position −2 in higher eukaryotic LC-binding sites. In yeast Dyn2, a bulky arginine side chain projects from the flanking α2 helix to interact with the target glycine at position −2. In contrast, in Drosophila, a less bulky lysine residue (K36) occupies the position equivalent to the yeast arginine, leaving space for a glutamine at target position −2 (Figure 4, A–D).

An F76K/Y78E double mutation in the Dyn2 peptide-binding site abrogates Pac11 pep2 binding

To confirm the role of Dyn2 target-binding determinants observed in our crystal structure, we mutated two residues at the Pac11 pep2-binding site and examined the effect on Pac11 binding. Residues

![Diagram](image_url)
An H58K mutation in the Dyn2 dimerization interface abrogates dimerization and target peptide binding

Residues from each of the Dyn2 molecules in the homodimer comprise the Pac11 peptide-binding site. To test whether dimerization is required for target binding, we analyzed residues at the dimerization interface that could be mutated to prevent dimerization. An LC8 mutation, H55K, was reported to transform LC8 from a homodimer to a monomer (Nyarko et al., 2005). The equivalent residue in Dyn2, H58, resides at the dimer interface, stacked 5.4 Å from its dimeric mate (H58; Figure 5D). We mutated Dyn2 H58 to lysine and analyzed the oligomeric state of the mutant using SEC-MAALS (Figure 5B). In contrast to homodimeric WT Dyn2 (Supplemental Figure S2), Dyn2 H58K eluted primarily as a monomer (Figure 5E). To determine whether monomeric Dyn2 can bind Pac11 pep2, we performed ITC using the Dyn2 H58K mutant. No binding between Dyn2 H58K and Pac11 pep2 was observed using a range of Dyn2 and Pac11 pep2 concentrations. These results indicate that Dyn2 homodimerization is required for target binding (Figure 5E and Supplemental Figure S3, B, D, and E). Collectively, Dyn2 is a homodimer, Dyn2 homodimerization is required for Pac11 binding, and Pac11 pep2 binds in the composite groove formed by Dyn2 homodimerization (as observed in our Dyn2-Pac11 pep2 crystal structure). Mutations in Dyn2’s peptide-binding site or dimerization interface disrupt Pac11 pep2 binding.

Dyn2/Pac11 facilitates dynein single-molecule processivity

The results of our structural and biochemical investigations of the Dyn2-Pac11 interaction imply a role for Dyn2 in Pac11 dimerization. To investigate whether Dyn2-mediated Pac11 dimerization plays a role in the oligomerization of the dynein heavy chains and the dimerization-dependent motile single-molecule properties (velocity and processivity), we created strains in which either the dyn2 gene or both the dyn2 and pac11 genes were deleted, respectively. Previous single-molecule experiments with full-length dynein labeled at its C-terminus with the HaloTag and covalently bound to tetramethylrhodamine (TMR) demonstrated that single Dyn1 motors containing both subunits are highly processive (take multiple steps along MT filaments without detaching) with an average run length of 1.7 μm (Reck-Peterson et al., 2006).

We used a custom-built total internal reflection fluorescence (TIRF) microscope and performed kymograph analyses to determine the average velocities and run lengths of single dimeric Dyn1 molecules in the presence and absence of Dyn2 (dyn2Δ) and Dyn2/Pac11 (dyn2Δpac11Δ), respectively. Dyn1 was C-terminally tagged with a HaloTag and site-specifically labeled with TMR. We performed experiments under decreased ionic strength (in dynein motility buffer [DMB]; see Materials and Methods) to enhance the detection of changes in run length of the Dyn1 motor purified from WT, dyn2Δ, and dyn2Δpac11Δ strains with sufficient precision (preventing significant underscoring of short runs that occur within the detection limit of the microscope). Under these conditions, the velocities of Dyn1 motors purified from dyn2Δ and dyn2Δpac11Δ strains were slightly decreased (113 and 118 nm/s respectively, p < 0.0001) compared with the 133-nm/s velocity of WT dynein (Figure 6, A and B). Surprisingly, when we analyzed motor processivity, we noted that Dyn1 molecules purified from dyn2Δ and dyn2Δpac11Δ strains move significantly shorter distances (1.25- and 1.24-μm average run lengths, respectively) compared with the average 2.2-μm run length of WT dynein (Figure 6, A and C). These results demonstrate that Dyn2/Pac11 affect dynein processivity significantly and that loss of Dyn2 has similar effects to loss of both Dyn2 and its binding partner, Pac11. To investigate why the single and
double deletions have very similar effects on dynein processivity and velocity, we next performed Western blot analysis to reveal differences in the amount of Pac11 that copurifies with the dynein HC in the presence and absence of Dyn2 (Figure 7A). This analysis revealed that the absence of Dyn2 dramatically reduces the relative amount of Pac11 that copurifies with the dynein HC (Figure 7A), suggesting that Dyn2 promotes the Pac11-Dyn1 interaction and that the loss of Dyn2 reduces stable Pac11-Dyn1 complex formation. Western blot analysis reflects an initial motor concentration of 80 nM. TIRF assays were performed after diluting motors to ~1 nM, which would favor Pac11-Dyn1 dissociation. To further test this idea, we performed multicolor single-molecule fluorescence analysis to probe for Pac11-Dyn1 complex formation under the conditions used in our single-molecule motility studies. In agreement with our hypothesis, Dyn1 purified from the WT strain shows significant colocalization between TMR-tagged Dyn1 and Cy5-labeled anti-Myc antibodies (which targets Myc-tagged Pac11), whereas Dyn1 purified from the dyn2Δ (or dyn2Δpac11Δ) strain did not reveal any associated Pac11 (Figure 7B; see also Supplemental Figure S4 and Supplemental Materials and Methods for details underlying this analysis). Thus loss of Dyn2 increases the dissociation of Pac11 from Dyn1, which explains why motors purified from dyn2Δ and dyn2Δpac11Δ strains behave similarly.

Dyn2/Pac11 help to maintain dynein heavy chains in a dimeric motile state
It was recently demonstrated that the Loa mutation (F580Y) in the mammalian dynein heavy chain's IC-binding site results in decreased motor run length (Ori-McKenney et al., 2010). Of interest, brain cytosol from Loa−/− mice contains a greater fraction of free IC than in
Dyn2-Pac11 complex helps to maintain Dyn1 in a homodimeric, motile state. To determine whether there is a population of monomeric motors and examine monomeric motor activity at the single-molecule level, we analyzed the fluorescence intensities of the purified motors in our single-molecule TIRF assay (Figure 8, A–C, and Supplemental Table S2). Because dynein dimers are expected to have at most two TMR molecules bound (the labeling efficiency is ∼65%; see legend to Figure 8B), whereas a monomer is expected to have no more than one TMR bound, we can distinguish between monomeric, immobile and dimeric, motile states of dynein by analyzing the intensities associated with motile versus immobile motors (see arrows 1–3 and corresponding intensity distributions, Figure 6A). In agreement with our hypothesis, ∼65% of motile motors exhibit a fluorescence intensity that corresponds to a maximum of two dyes (Figure 8B), whereas the majority of immobile motors show single-dye fluorescence (Figure 8C). Of note, the fraction of motile motors decreased from 60 to 30% as a result of Dyn2 and Pac11 deletion (Figure 8A). The decrease in the fraction of motile motors from the dyn2Δ and dyn2Δpac11Δ strains correlates with an increase of the fraction of immobile, MT-bound fluorescent spots containing predominantly one dye (Figure 8C) and an increasing number of bright fluorescent spots bound to the coverslip surface containing more than two dyes (Supplemental Figure S5). These correlations suggest that the loss of the Dyn2-Pac11 complex promotes Dyn1 monomerization and aggregation, which agrees with previous studies on mammalian dynein that showed that IC removal resulted in decreased heavy-chain stability (Gill et al., 1994; King et al., 2002; Trokter et al., 2012).

DISCUSSION

Many single-molecule dynein experiments examined the dynein motor domain fused to an artificial dimerization domain that ensures motor domain homodimerization, a critical prerequisite for processivity (Reck-Peterson et al., 2006; Numata et al., 2011). Although this facilitates robust analysis of motor domain function, it excludes analysis of any role the heavy chain's N-terminal domain and its associated binding partners may play in dynein motor domain activity. The dynein heavy-chain N-terminal domain and its associated chains are highly conserved from yeast to humans, suggesting a key functional role in the motor complex. Here, we marshaled biochemical, structural, and single-molecule analyses to probe the role of the light and intermediate chains in the dynein motility mechanism.
canonical Dyn2-target interaction, with the Dyn2 homodimer bound to two parallel Pac11 chains. Previous work analyzing the Pac11-Dyn2 interaction using analytical ultracentrifugation showed Dyn2-dependent Pac11 dimerization, yielding a complex mass on par with a Pac11-Dyn2 complex that, with our crystal structure, supports parallel Pac11 chains bridged at two sites by Dyn2 homodimers (Stuchell-Brereton et al., 2011).

We find that Dyn2 binds Pac11’s second Dyn2-binding site with an affinity of 620 nM. This interaction is one of the higher-affinity LC8/Dyn2 family-target interactions determined and our measurement likely underestimates the affinity between Dyn2 and full-length Pac11 due to the avidity effects of Dyn2 homodimers interacting with two Pac11 chains, each with dual, arrayed Dyn2-binding sites. Our structural analysis of the Dyn2-Pac11 pep2 complex shows a canonical Dyn2-target interaction, with the Dyn2 homodimer bound to two parallel Pac11 chains. Previous work analyzing the Pac11-Dyn2 interaction using analytical ultracentrifugation showed Dyn2-dependent Pac11 dimerization, yielding a complex mass on par with a Pac11-Dyn2 complex that, with our crystal structure, supports parallel Pac11 chains bridged at two sites by Dyn2 homodimers (Stuchell-Brereton et al., 2011). This predicted Pac11-Dyn2 interaction...
FIGURE 6: Dynein from dyn2Δpac11Δ cells shows decreased run lengths compared with motors purified from WT cells. (A) Kymographs were generated by stacking line scans along the long MT axis using custom-made analysis software (Supplemental Material and Methods). Diagonal lines represent moving molecules, with velocity equal to the inverse of the slope. The run length of individual molecules can be easily determined (double-headed arrow). An increasing number of stationary Dyn1 molecules can be seen (vertical lines) for Dyn1 purified from the deletion strains. Analysis of the fluorescence intensities (post background subtraction) reveals that moving and immobile Dyn1 molecules contain predominantly one or two TMR molecules and very rarely more than two dyes (example in bottom inset and statistics in Figure 8 and Supplemental Table S2). Pooling the two distributions with the lowest mean intensities (1 and 2) and taking the average of both means yields 1.9 a.u. (ΔI; note that when bleaching occurs during the image acquisition time, the lowest mean intensities in our kymograph-based intensity analysis show one-step photobleaching, confirming single-dye identity; Supplemental Figure S6A). Dashed lines are placed at integral multiples of ΔI, revealing that the two moving motors carry two and three dyes, respectively (3 and 4). The latter observation suggests the rare occurrence of a moving Dyn1 aggregate (see also Supplemental Figure S5). (B) Histograms of velocities (v > 0 nm/s) of single Dyn1 molecules at 1 mM ATP (WT, top; dyn2Δ, center; dyn2Δpac11Δ, bottom). Values are means ± SEM (n = 500–1000). (C) Histograms of run lengths of single Dyn1 molecules moving along MTs (WT, left; dyn2Δ, middle; dyn2Δpac11Δ, right). Values are means ± SEM (n = 500–1000).
arrangement resembles the arrangement observed in crystal structures of the Drosophila Ic2-TcTex2-LC82 complex (Williams et al., 2007; Hall et al., 2010). In these animal complexes, the TcTex light chain replaces the first Dyn2 binding site in yeast. Based on our affinity measurements and since Dyn2 is a homodimer, we predict that the Pac112-(Dyn22)2 complex is highly stable and that Pac11 and Dyn2 work as a complex in dynein motor function. Our data show that the Pac11-Dyn2 complex promotes Dyn1 homodimerization and that loss of Dyn2 or Dyn2 and Pac11 enhances Dyn1 homodimerization.

**FIGURE 7:** Absence of Dyn2 reduces Pac11-Dyn1 complex formation. (A) Copurification of Dyn2 and Pac11 with full-length Dyn1 through IgG affinity purification of cell lysate from WT, dyn2Δ, and dyn2Δpac11Δ strains. Western blotting was performed with antibodies against Dyn2 (Stelter et al., 2007) and against the Myc tag in Pac11. Subsequent SDS–PAGE and Western blot analysis confirms the absence of Dyn2 in the dyn2Δ and dyn2Δpac11Δ deletion strains and the absence of Pac11 in dyn2Δpac11Δ cells and reveals a significant reduction of Pac11 copurified with Dyn1 from dyn2Δ cells (for more details, see Materials and Methods). (B) TIRF-based, single-molecule fluorescence analysis confirms that the majority of TMR-labeled Dyn1 molecules purified from the WT strain are bound to Pac11-Myc, as revealed by the ~60% colocalization (yellow) of TMR-labeled Dyn1 (red) and Cy5-labeled anti-Myc antibodies (green) (top). In contrast, Dyn1 motors purified from the dyn2Δ (center) and dyn2Δpac11Δ strains (bottom) do not show significant TMR-Cy5 colocalization (see Supplemental Materials and Methods for details underlying this analysis). Images represent 150 × 150-pixel subregions of the original 512 × 512–pixel EMCCD images (see Supplemental Figure S4 for larger field).

**FIGURE 8:** Single-molecule fluorescence analysis shows that Dyn2/Pac11 facilitate Dyn1 motility by promoting HC homodimerization. (A) Deletion of Dyn2 or both Dyn2 and Pac11 results in the same increase of the fraction of immobile Dyn1 molecules. (B) Analysis of the fluorescence intensities reveals that the majority of moving motors have two TMR-tagged Dyn1 heavy chains. The insignificant fraction of motile motors with more than two dyes (indicating higher oligomers) together with the negligible photobleaching (Supplemental Figure S6) allows the estimation of ~65% HC fluorescence labeling efficiency. (C) Analysis of the imaged fluorescence intensities reveals that the increased fraction of immobile Dyn1 in the absence of Dyn2 or both Dyn2 and Pac11 (A) is largely caused by HC monomerization, as indicated by the large fraction of immobile Dyn1 molecules with single-dye intensities. Note that not all Dyn1 motors purified from the WT and deletion strains are active and that the inactive, immobile motors purified from the WT strain have a higher likelihood to be dimers, which have a higher likelihood to be labeled with two dyes. Values are means ± SEM (see Supplemental Table S2 for corresponding data).
dissociation, leading to nonprocessive motors (Figure 9). How the Pac11-Dyn2 complex structurally interacts with the Dyn1 homodimer remains to be determined.

The Pac11-Dyn2 complex enhances Dyn1 homodimer stability, slightly increasing velocity and enhancing processivity significantly, likely by facilitating the alternating forward motions of dynein’s two motor domains. Intriguingly, the IC-LC binding site overlaps the site of the mouse Loa mutation (F580Y) that causes neurodegenerative disease. Single-molecule experiments analyzing Loa-mutant dynein movement in vitro found increased lateral stepping and an increased ATP affinity, suggesting disrupted motor domain coordination that results in premature ATP binding in the rear head and subsequent release from the MT (Ori-McKenney et al., 2010). Loa phenotypes show overlap with the behavior we observed for Dyn1 operating without Dyn2 or the Pac11-Dyn2 complex. Independent studies investigating whether the Loa mutation affects IC-HC complex stability found contrasting results, some showing decreased IC association (Ori-McKenney et al., 2010) and others showing unaltered (Sivagurunathan et al., 2012) or even increased IC association (Deng et al., 2010). Whether absence of the IC-LC complex and the Loa mutation modulate HC activity through the same, overlapping, or independent mechanisms remains an open question. Nevertheless, in vivo analyses of phenotypes resulting from the Loa mutation provide insights that parallel our findings, such as decreased velocities and run lengths of vesicles transported by Loa dynein (Ori-McKenney et al., 2010; Sivagurunathan et al., 2012). Both the loss of the IC-LC complex and the Loa mutation suggest that key conserved elements in the HC N-terminal domain modulate motor domain activity. Additional evidence for the HC N-terminal domain exerting regulatory action on the motor domain comes from a recent study in Aspergillus nidulans, in which a F208V mutation in the dynein HC, located N-terminal to the IC-binding site, causes early endosomes to move less frequently and with a diminished velocity without affecting the HC-IC interaction (Qiu et al., 2013). When the valine mutation was replaced with a bulkier hydrophobic side chain, isoleucine, WT vesicle motility was restored. The A. nidulans F208V mutation and the mouse Loa F580Y mutation both change the size of a hydrophobic residue. We suspect that these mutations alter the packing of the dynein N-terminal domain, each debilitating the regulatory action that this domain exerts on the motor domain. Additional support comes from reconstitution studies of human cytoplasmic dynein in which the addition of the IC and LIC promoted HC dimerization, and all accessory chains were required (including the LCs) for the motor complex to appear in its native conformation, as determined by negative–stain electron microscopy (Trokter et al., 2012). Our results highlight commonality between mammalian and yeast cytoplasmic dynein. It is likely that the structure of the HC’s N-terminal domain and its IC-LC–potentiated dimerization are critical for its regulatory action. Removal of the IC-LC complex or mutations within the HC N-terminal domain itself may therefore yield similar, yet distinct effects on HC motor activity. Future single-molecule studies will probe the role of the IC-LC complex in motor domain coordination using differentially labeled motor domains, as well as optical trapping to examine accessory-chain–dependent effects on force generation. Probing a direct interaction between the HC N-terminal tail and motor domain during active walking in the presence and absence of Dyn2/Pac11 awaits future single-molecule studies combined with Förster resonance energy transfer.

MATERIALS AND METHODS

Cloning, expression, and purification of full-length Dyn2

Full-length Dyn2 was cloned from S. cerevisiae S288c as described (Romes et al., 2012). Briefly, Dyn2 was cloned into pGEX-6P2 (GE Healthcare, Piscataway, NJ), yielding an N-terminal, cleavable glutathione S-transferase (GST) tag. GST-Dyn2 was expressed in BL21 DE3 (pLysS) and induced using 0.1 mM isopropyl-1-thio-β-D-galactopyranoside at 18°C for 16 h. Cells were harvested, resuspended in lysis buffer, and lysed by sonication. Dyn2 was purified using a glutathione sepharose column, eluted, and incubated with PreScission protease (GE Healthcare) to cleave the GST tag. Final purification was performed using an SP Sepharose Fast Flow column (GE Healthcare). The Dyn2 peak was collected and exchanged into buffer A (50 mM NaCl, 25 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid [HEPES], pH 6.8, and 0.1% β-mercaptoethanol). The final, purified Dyn2 protein was concentrated to 5 mg/ml, snap frozen in liquid nitrogen, and stored at −80°C. The final Dyn2 protein contained an N-terminal GPLGS cloning artifact.

Cloning, expression, and purification of Dyn2 mutants

Dyn2 H58K and F76K/Y78E mutants were generated using the QuikChange method (Strategene, Santa Clara, CA) with the complementary oligonucleotides for the H58K mutant, 5′-CGGAGTCTAAATACGCGCAATACCTTGGAAGGTGTGTGCAGAAAGACCTTTGGG-3′ (where the underlined portion codes for the mutated codon) and 5′-CCTCGAGTATTTCCAGGCAATACACTTCCAGGTATTGCGTATTTGACATCC-3′; and the complementary oligonucleotides for F76K/Y78E, 5′-GGTCAACGAAAGGGCCTAAAGTTGGAATTCTATATCGGTCCACTGGG-3′ and 5′-CCGCGGACGGAATCAATTCATGATTTGCA-3′. Both the
single and double mutants were sequence confirmed. The expression and purification of Dyn2 H58K and Dyn2 F76K/Y78E followed the same protocols and buffers as described for WT Dyn2.

**Pac11 peptide synthesis**

Pac11 peptide 1 (pep1; YMVSVSVDQ, residues 45–55) and peptide 2 (pep2; ITYDKGIQTDO, residues 75–85) were synthesized at the University of North Carolina Microprotein Sequencing and Peptide Synthesis Facility. Peptide 1 was designed with an amino-terminal tyrosine in order to quantify the peptide concentration. Lyophilized peptides were added to buffer A. Peptide 1 proved insoluble in buffer A, and attempts to solubilize it in buffers containing increased salt, different pH ranges, or various detergents did not result in solubilization.

**Crystallization**

Final concentrations of 0.5 mM Dyn2 and 0.6 mM Pac11 pep2 were incubated in buffer A for 30 min at ambient temperature. Crystals were obtained by hanging-drop vapor diffusion using 2 μl of the Dyn2-Pac11 pep2 mixture and 1 μl of the 1-mL well solution (0.4 M sodium phosphate monobasic, 0.1 M 1,6-hexanediol, and 25% polyethylene glycol 3350). Crystals grew at 20°C into thin, individual plates over a 2-wk period. Crystals were transferred into a cryoprotectant containing well solution supplemented with 20% ethylene glycol and flash frozen in liquid nitrogen.

**Data collection, structure determination, and refinement**

Diffraction data were collected on a single Dyn2-Pac11 pep2 crystal at the Advanced Photon Source SER-CAT beamline 22-ID at 100 K with 1° oscillations over 180° from a single crystal. Data were indexed, integrated, and scaled in the space group C222; using HKL2000 (Otwinski and Minor, 1997). The structure was determined using the AutoMR molecular replacement program (PHENIX crystallographic suite; Adams et al., 2010) and a modified 4DS1 coordinate file (Romes et al., 2012) in which a monomeric, apo Dyn2 search model was used. The model was built using AutoBuild (PHENIX; Adams et al., 2010) and refined iteratively through manual builds in Coot (Emsley et al., 2010) followed by refinement runs using phenix.refine (PHENIX; Adams et al., 2010). Refinement statistics were monitored using a free R, calculated using 7.8% of the data, randomly excluded from refinement (Brünger, 1992).

**Isothermal titration microcalorimetry**

Pac11 pep2 was exchanged into buffer A using G-25 Sephadex Quick Spin Columns (Roche, Indianapolis, IN). Pac11 pep1 proved insoluble under all buffer conditions analyzed. WT Dyn2, Dyn2 H58K, and Dyn2 F76K/Y78E were individually exchanged into buffer A. Binding was measured by ITC at 26°C on an AutoITC200 (Microcal/GE Healthcare). Sequential injections, 19 × 2 μl, of 1.0 mM Pac11 pep2 were automatically injected into 200 μl of 50 μM WT Dyn2. The data were analyzed with the Origin (for ITC) 7.0 software package (Microcal/GE Healthcare), and the resulting isotherm was fitted to a single-site, independent-binding model. The experiment was performed in triplicate and the mean Kd and SD calculated. Mutant Dyn2–Pac11 pep2 binding experiments were performed with Pac11 pep2, Dyn2 H58K, and Dyn2 F76K/Y78E in buffer A using similar injection routines as described for WT Dyn2. Three different initial concentrations of injectant (Pac11 pep2) and cell (Dyn2 mutant construct) components were analyzed: 0.5 mM Pac11 pep2, 50 μM mutant Dyn2; 1.0 mM Pac11 pep2, 50 μM mutant Dyn2; and 3.0 mM Pac11 pep2, 0.3 mM mutant Dyn2. The 1.0 and 3.0 mM Pac11 pep2 stocks were resuspended directly in buffer A, and the 0.5 mM Pac11 pep2 stock was obtained after exchanging into buffer A to limit the background noise due to ionic interference. There was a significant heat of dilution recorded in the Pac11 pep2 control experiment, which used 3.0 mM Pac11 pep2 stock injected into 200 μl of buffer A. This component was subtracted from the raw heat, where 3.0 mM Pac11 pep2 was injected into the ITC cell containing either 0.3 mM Dyn2 H58K or 0.3 mM Dyn2 F76K/Y78E. No binding was observed across all three concentrations of Pac11 pep2 and mutant Dyn2 constructs analyzed.

**Size exclusion chromatography and multangle light scattering**

WT Dyn2, Dyn2 H58K, and Dyn2 F76K/Y78E were exchanged into buffer A using 3-kDa Amicon Ultra Spin Concentrators (Millipore, Billerica, MA). Dyn2 WT was concentrated to 6.0 mg/ml, Dyn2 H58K to 10.0 mg/ml, and Dyn2 F76K/Y78E to 6.0 mg/ml. All Dyn2 constructs remained soluble during the concentration step. A 50-μl amount of each concentrated sample (Dyn2 WT, Dyn2 H58K, and Dyn2 F76K/Y78E) was individually injected onto a Superdex 200 10/300 GL size exclusion column (GE Healthcare) in buffer A supplemented with 0.2 g/l sodium azide and then passed consecutively through a Wyatt DAWN HELEOS II light scattering instrument and a Wyatt Optilab REX refractometer (Wyatt Technology, Santa Barbara, CA). The light scattering and refractive index data were used to calculate the weight-averaged molar mass and the mass fraction in each peak using Wyatt Astra V software.

**Generation of yeast deletion strains**

All S. cerevisiae deletion strains were derived from the haploid strain VY263 (Kardon et al., 2009), which has the dyn11 heavy chain gene (DYN1) tagged at its 3′ end with DNA encoding a HaloTag and the NIP100 gene deleted (genotype, see Supplemental Table S1). DYN1 is also fused at its 3′ end to DNA encoding an N-terminal ZZ tag for binding to immunoglobulin G (IgG) beads, a TEV cleavage site, a linker that promotes the efficiency of TEV protease, and green fluorescent protein. Gene deletion was performed via homologous recombination. Yeast transformations were performed by standard LiAc/polyethylene glycol protocol (Goldstein and McCusker, 1999). Stitched DNA fragments were amplified by standard PCR protocol. For deletion of DYN2, the pAG25 plasmid encoded marker nourseothricin N-acetyltransferase was used as template. For PAC11 deletion, the pAG32 plasmid encoded marker hygromycin B phosphotransferase was used as template. Deletions were confirmed by DNA sequencing.

**Dynine expression, purification, and TMR labeling**

Dynine was purified using an established protocol (Reck-Peterson et al., 2006). A 2-l yeast culture was grown to OD600 ~1.0 and harvested by centrifugation at 1500 × g for 8 min at 4°C. The pellet was washed once with double-distilled H2O (ddH2O) and then added dropwise into liquid nitrogen. To lyse cells, frozen droplets were ground in a coffee grinder (KitchenAid, Benton Harbor, MI) and solubilized in lysis buffer (30 mM HEPES, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM ethylene glycol tetraacetic acid [EGTA], 10% [vol/vol] glycerol, and 0.2% [vol/vol] Triton X-100, pH 7.4, supplemented with protease inhibitors, 1 mM dithiothreitol [DTT], and 100 μM MgATP). Lysate was clarified by centrifugation at 387,000 × g for 30 min at 4°C. Supernatant was incubated with 250 μl of IgG beads (GE Healthcare) and nated at 4°C for 1 h. The bead slurry was poured into a column (Bio-Rad, Hercules, CA), and washed twice with 5 ml of lysis buffer. To label Dyn1, 0.5 μl of 1 mM TMR-labeled HaloTag ligand (Promega, Madison, WI) was
added, mixed well with the IgG beads, incubated at room temperature for 10 min, and washed twice with 5 ml TEV buffer (10 mM Tris-HCl, 150 mM KCl, 10% [vol/vol] glycerol, and 0.1% [vol/vol] Triton X-100, pH 7.5, 500 μM Pefabloc, 1 mM DTT, and 100 μM MgATP). IgG beads were transferred to a 2-ml Eppendorf tube, and 2.5 μl of ProTEV protease (Invitrogen, Carlsbad, CA) was added to cleave the dynein off the beads. The mixture was nuted at 16°C for 1 h and centrifuged at 20,800 × g, and the dynein-containing supernatant was aliquotted, flash frozen in liquid nitrogen, and stored at −80°C. Further purification to select for active Dyn1 by microtubule affinity and ATP-induced release is often performed. However, we did not do so here in order to preserve the different populations of Dyn1 complexes caused by the deletion of Dyn2/Pac11 (i.e., active/inactive monomeric, dimeric and aggregated Dyn1, respectively; Figures 6 and 8).

**Single-molecule dynein motility assay**

To prepare coverslips for single-molecule fluorescence assays, coverslips were submerged into 25% HNO₃ for 10 min, washed twice with ddH₂O, transferred to 2 M NaOH, and incubated for 10 min. Coverslips were then washed six times with ddH₂O and air dried on a heat block. Flow chambers were generated by placing a coverslip onto a glass slide with parallel tracks of double-sided sticky tape. Taxol-stabilized MTs (Cytoskeleton, Denver, CO) were labeled with NHS-monofunctional Cy5 (GE Healthcare) following a published protocol (Peloquin et al., 2005). DMB was used in all experiments unless otherwise specified (30 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 10 μM Taxol, pH 7.2). To immobilize MTs on the coverslip, 10 μl of 0.2 mg/ml monomeric rigor kinesin-1 (193N mutant; Nakata and Hirokawa, 1995) was flowed into the chamber and incubated for 2 min, followed by 20 μl of blocking solution (2 mg/ml bovine serum albumin [BSA] and 1 mg/ml β-casein) for 20 min. Twenty microliters of 0.1 mg/ml Cy5-labeled MTs (labeled tubulin:unlabeled tubulin ratio, 1:20) was flowed in the chamber and incubated for 2 min. Dynnein was diluted into DMF supplemented with 2 mg/ml BSA, 2 mM Trolox, 1 mM MgATP, oxygen scavenger system 0.4% [wt/vol] glucose, 1 mg/ml glucose oxidase, and 0.04 mg/ml catalase; Shi et al., 2010) and ATP regeneration system (1% pyruvate kinase and 10 mM phosphoenolpyruvate). Of this solution, 2 × 20 μl was flowed into the −100-μl chamber, and the system was sealed with vacuum grease.

The sample was mounted on a TIRF microscope (Eclipse Ti; Nikon, Melville, NY) with a 100x/numerical aperture 1.45 oil immersion objective. The sample was excited by a 635-nm laser (Radius; Coherent, Santa Clara, CA) to visualize Cy5-labeled MTs and by a 532-nm laser (Sapphire; Coherent) to visualize TMR-labeled motors. The TMR fluorescence signal was collected for 5 min with an acquisition time of 500 ms per frame under constant illumination on an electron-multiplying charge-coupled device (EMCCD) camera (Xon Ultra; Andor, Belfast, United Kingdom) using NIS-elements imaging software (Nikon). Velocity, processivity, and the number of TMR-labeled motors from a diffraction-limited spot were analyzed using a custom Matlab, version 7.10.0 (MathWorks, Natick, MA) program described in the Supplemental Materials and Methods. The velocity for each motor was averaged over its moving distance, and long pause times were excluded from the calculation. For processivity calculation, a lower run-length cutoff of 0.5 μm was applied (the pixel size is 160 nm, and at least three pixels are necessary to distinguish a moving trace). Data were processed using Origin (OriginLab, Northampton, MA). Note that the run-length measurements are not significantly affected by the photobleaching of TMR: the characteristic time before photobleaching of TMR under our experimental conditions is 271 s (fbleach = 0.0037 s⁻¹; Supplemental Figure S6), whereas the WT motor needs on average only a time of 16.5 s for its characteristic run length of 2.2 μm (v = 133 nm/s; Figure 6, B and C).

**Labeling of anti-Myc antibody with Cy5**

NHS-monofunctional Cy5 (GE Healthcare; 0.4 μl of 10 mg/ml) was added to 20 μl of 1 mg/ml anti-Myc antibody (ab9106, rabbit polyclonal; Abcam, Cambridge, MA) and incubated at room temperature for 30 min in the dark. The free dye molecules were removed by centrifugation: the solution was transferred to a 0.5-ml centrifugal filter unit with a 100-kDa membrane (Millipore Amicon Ultra) and washed four times with 0.5 ml of 1× phosphate-buffered saline each time by centrifugation at 20,000 × g for 5 min at 4°C. The labeling ratio was determined using a NanoPhotometer (Implen, Westlake Village, CA), and the labeled antibody was stored at 4°C in the dark.

**Single-molecule Dyn1-Pac11 colocalization assay**

The coverslip was cleaned and the flow chamber was constructed as done for the motility assays. The following steps were performed sequentially: 1) labeled antibody was diluted 10,000× in 30 mM HEPES, 2 mM MgCl₂, and 1 mM EGTA with 0.5 mg/ml BSA (pH 7.2), and 20 μl of diluted solution was flowed into the chamber and incubated for 2 min; 2) 20 μl of blocking buffer (BB; 30 mM HEPES, 2 mM MgCl₂, 1 mM EGTA, with 5 mg/ml BSA and 1 mg/ml β-casein, pH 7.2) was flowed into the chamber and incubated for 5 min; 3) 20 μl of diluted motors (the same dilution used for the motility assays) in BB was added and incubated for 5 min; 4) 20 μl of diluted antibody solution was flowed into the chamber and incubated for 5 min; 5) the chamber was then washed twice with 20 μl of BB containing an oxygen scavenger system and 2 mM Trolox; and 6) the chamber was sealed with vacuum grease. The acquisition parameters were the same as for the motility assays, except that 10 frames were collected instead of 600. The images shown in Figure 7B and Supplemental Figure S4 are the averages of the 10 image frames acquired for each experiment.

This approach resulted in sparse nonspecific binding to the glass surface by antibodies, Dyn1, Pac11-Dyn1, and Pac11, in addition to specific binding of Pac11 and Pac11-Dyn1 to the surface-bound antibodies. The second round of antibody incubation ensured recognition of Pac11 on nonspecifically bound complexes as well (e.g., on Dyn1-Pac11 complexes bound to the glass via Dyn1), although some Dyn1-Pac11 complexes could have bound the surface in a manner that prevented antibody access. Although an initial increase of the incubation time of 2–5 min for the second round of antibody incubation led to an increased binding of anti-Myc antibody to Dyn1-associated Pac11 (resulting in an increased Cy5-TMR colocalization for Dyn1 purified from the WT strain), we refrained from increasing the incubation time further due to an increased background signal resulting from the nonspecific binding of the Cy5-labeled antibody to the coverslip surface. Thus, the degree of Pac11-Dyn1 colocalization obtained from this method represents a lower boundary, and the actual degree of Pac11-Dyn1 colocalization could be significantly greater.

For all three strains, the binding of Dyn1 to the coverslip was similar. However whereas ~60% of TMR-labeled Dyn1 colocalized with the Cy5-labeled anti-Myc antibody in the WT (implying complex formation of 60% or more of Dyn1 and Pac11 proteins), no colocalization was observed in either of the deletion strains. This simultaneously demonstrates that the antibody does not cross-react with Dyn1 and that in the absence of Dyn2, Pac11 fails to bind to Dyn1.
Western blot analysis

The protein concentrations of TEV-released Dyn1 purified from WT, dyn2Δ, and dyn2Δpac11Δ strains were determined by SDS-PAGE analysis using Krypton stain (Pierce, Rockford, IL) and BSA protein concentration standards. Western blot analysis was conducted on the TEV-released motor fraction from each strain after loading amounts of Dyn1 that had been normalized across strains. Pac11 and Dyn2 were detected using rabbit anti-Myc antibody (1:2000; Abcam) and rabbit anti-Dyn2 antibody (1:200; Stelter et al., 2007), respectively, and an Alexa Fluor 680-labeled anti-rabbit secondary antibody (1:5000; Life Technologies, Carlsbad, CA).

Coordinate deposition

Atomic coordinates and structure factors for the Dyn2-Pac11 pep2 complex have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (www.rcsb.org/) under accession code 4HT6.

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REFERENCES

Adames NR, Cooper JA (2000). Microtubule interactions with the cell cortex causing nuclear movements in Saccharomyces cerevisiae. J Cell Biol 149, 863–874.
Adams PD et al. (2010). PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr 66, 213–221.
Bader JR, Vaughan KT (2010). Dynine at the kinetochore: timing, interactions and functions. Semin Cell Dev Biol 21, 269–275.
Benison G, Karplus PA, Barbar E (2008). The interplay of ligand binding and quaternary structure in the diverse interactions of dynine light chain LCB8. J Mol Biol 384, 954–966.
Brünger AT (1992). Free R value: a novel statistical quantity for assessing the accuracy of crystal structures. Nature 355, 472–475.
Burgess SA, Walker ML, Sakakibara H, Knight PJ, Oiwa K (2003). Dynein structure and power stroke. Nature 421, 715–718.
Carter AP, Cho C, Jin L, Vale RD (2011). Crystal structure of the dynein motor domain. Cell 144, 1159–1165.
Carter AP, Garbarino JE, Wilson-Kubalek EM, Shipley WE, Cho C, Milligan RA, Vale RD, Gibbons IR (2008). Structure and functional role of dynine’s microtubule-binding domain. Science 322, 1691–1695.
Deng W, Garrett C, Dombert B, Soura V, Banks G, Fisher EM, van der Brug MP, Hafiezparast M (2010). Neurodegenerative mutation in cytoplasmic dynein alters its organization and dynine-dynactin and dynine-kinesin interactions. J Biol Chem 285, 39922–39934.
Emsley P, Lohkamp B, Scott WG, Cowtan K (2010). Features and development of Coot. Acta Crystallogr D Biol Crystallogr 66, 486–501.
Eshel D, Urestarazu LA, Vissers S, Jauniaux JC, van Vliet-Reedijk JC, Planta RJ, Gibbons IR (1993). Cytoplasmic dynein is required for normal nuclear segregation in yeast. Proc Natl Acad Sci USA 90, 11172–11176.
Farkasovsky M, Küntzel H (2001). Cortical Num1p interacts with the dynein intermediate chain Pac11p and cytoplasmic microtubules in budding yeast. J Cell Biol 152, 251–262.
Geiser JR, Schott EJ, Kingsbury TJ, Cole NB, Totis LJ, Bhattacharyya G, He L, Hoyt MA (1997). Saccharomyces cerevisiae genes required in the absence of the Cin8-encoded spindle motor act in functionally diverse mitotic pathways. Mol Biol Cell 8, 1035–1050.
Gennrich A, Carter AP, Reck-Peterson SL, Vale RD (2007). Force-induced bidirectional stepping of cytoplasmic dynein. Cell 131, 952–965.
Gett Bariller CW, Schroer TA (1994). Characterization of DLA-A and DLC-B, two families of cytoplasmic dynein light chain subunits. Mol Biol Cell 5, 645–654.
Goldstein AL, McCusker JH (1999). Three new dominant drug resistance cassettes for gene disruption in Saccharomyces cerevisiae. Yeast 15, 1541–1553.
Hafiezparast M et al. (2003). Mutations in dynein link motor neuron degeneration to defects in retrograde transport. Science 300, 808–812.
Hall J, Song Y, Karplus PA, Barbar E (2010). The crystal structure of dynine intermediate chain-light chain blockroad complex gives new insights into dynine assembly. J Biol Chem 285, 22566–22575.
Ho Y et al. (2002). Systematic identification of protein complexes in Saccharomyces cerevisiae by mass spectrometry. Nature 415, 180–183.
Hödi Z, Nemeth AL, Radnai L, Hétenyi C, Schlett K, Bodor A, Perzel A, Nyitrai L (2006). Alternatively spliced exon B of myosin Va is essential for binding the tail-associated light chain shared by dynine. Biochemistry 45, 12582–12595.
Hrabé de Angelis MH et al. (2000). Genome-wide, large-scale production of mutant mice by ENU mutagenesis. Nat Genet 25, 444–447.
Imamura K, Kon T, Ohkura R, Sutoh K (2007). The coordination of cyclic microtubule association/disociation and tail swinging of cytoplasmic dynein. Proc Natl Acad Sci USA 104, 16134–16139.
Kardon JR, Reck-Peterson SL, Vale RD (2009). Regulation of the processivity and intracellular localization of Saccharomyces cerevisiae dynine by dynactin. Proc Natl Acad Sci USA 106, 5669–5674.
Karki S, Holzbaur EL (1995). Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. J Biol Chem 270, 28806–28811.
King SJ, Bonilla M, Rodgers ME, Schroer TA (2002). Subunit organization in cytoplasmic dynein subcomplexes. Protein Sci 11, 1229–1250.
King SJ, Brown CL, Maier KC, Quintyne NJ, Schroer TA (2003). Analysis of the dynein-dynactin interaction in vitro and in vivo. Mol Biol Cell 14, 5089–5097.
Kini AR, Collins CA (2001). Modulation of cytoplasmic dynein ATPase activity by the accessory subunits. Cell Motil Cytoskeleton 48, 52–60.
Kon T, Mogami T, Ohkura R, Nishura M, Sutoh K (2005). ATP hydrolysis cycle-dependent tail motions in cytoplasmic dynein. Nat Struct Mol Biol 12, 513–519.
Kon T, Nishura M, Ohkura R, Toyoshima YH, Sutoh K (2004). Distinct functions of nucleotide-binding/hydrolysis sites in the four AAA modules of cytoplasmic dynein. Biochemistry 43, 11266–11274.
Kon T, Oyama T, Shimo-Kon R, Imamura K, Shima T, Sutoh K, Kurisu G (2012). The 2.8 Å crystal structure of the dynein motor domain. Nature 484, 345–350.
Lee WL, Kaiser MA, Cooper JA (2005). The offloading model for dynein function: differential function of motor subunits. J Cell Biol 168, 201–207.
Lee WL, Oberle JR, Cooper JA (2003). The role of the lissenccephaly protein Pac1 during nuclear migration in budding yeast. J Cell Biol 160, 355–364.
Li J, Lee WL, Cooper JA (2005). NudEL targets dynine to microtubule ends through LIS1. Nat Cell Biol 7, 686–690.
Li YY, Yeh E, Bloom K (1993). Disruption of mitotic spindle orientation in a yeast dynein mutant. Proc Natl Acad Sci USA 90, 10996–10100.
Liang J, Jaffrey SR, Guo W, Snyder SH, Clardy J (1999). Structure of the PIN/LC8 dimer with a bound peptide. Nat Struct Biol 6, 735–740.
Makokha M, Hare M, Li M, Hays T, Barbar E (2002). Interactions of cytoplasmic dynein light chains Tctex-1 and LC8 with the intermediate chain LC8. J Biol Chem 277, 14067–14074.
Mok YK, Lo KW, Zhang M (2001). Structure of Tctex-1 and its interaction with cytoplasmic dynein intermediate chain. J Biol Chem 276, 14067–14074.
Moore JK, Li J, Cooper JA (2008). Dynactin function in mitotic spindle positioning. Traffic 9, 510–527.
Nakata T, Hirokawa N (1995). Point mutation of adenosine triphosphate-binding motif generated rigor kinesin that selectively blocks anterograde lysosome membrane transport. J Cell Biol 131, 1039–1053.
Nakata T, Shima T, Ohkura R, Kon T, Sutoh K (2011). C-sequene of the Dictyostelium cytoplasmic dynein participates in processivity modulation. FEBS Lett 585, 1185–1190.
Nyarko A, Cochran L, Norwood S, Pursifull N, Voth A, Barbar E (2005). Ionization of His 55 at the dimer interface of dynein light-chain LC8 is coupled to dimer dissociation. Biochemistry 44, 14248–14255.

Nyarko A, Hall J, Hall A, Hare M, Kremerskothen J, Barbar E (2011). Conformational dynamics promote binding diversity of dynein light chain LC8. Biophys Chem 159, 41–47.

On-Mckenney KM, Xu J, Gross SP, Vallee RB (2010). A cytoplasmic dynein tail mutation impairs motor processivity. Nat Cell Biol 12, 1228–1234.

Otwinowski Z, Minor W (1997). Processing of x-ray diffraction data collected in oscillation mode. Methods Enzymol 276, 307–326.

Peloquin J, Komarova Y, Borisny G (2005). Conjugation of fluorophores to tubulin. Nat Methods 2, 299–303.

Pfarr CM, Coue M, Grissom PM, Hays TS, Porter ME, McIntosh JR (1990). Cytoplasmic dynein is localized to kinetochores during mitosis. Nature 345, 263–265.

Qi Qiu R, Zhang J, Xiang X (2013). Identification of a novel site in the tail of dynein heavy chain important for dynein function in vivo. J Biol Chem 288, 2271–2280.

Radnai L et al. (2010). Affinity, avidity, and kinetics of target sequence binding to LC8 dynein light chain isoforms. J Biol Chem 285, 38649–38657.

Rapali P, Szemes A, Radnai L, Bakos A, Pal G, Nyitray L (2011). DYNLL/LC8: a light chain subunit of the dynein motor complex and beyond. FEBS J 278, 2980–2996.

Reck-Peterson SL, Yildiz A, Carter AP, Gennerich A, Zhang N, Vale RD (2006). Single-molecule analysis of dynein processivity and stepping behavior. Cell 126, 335–348.

Roberts AJ et al. (2009). AAA+ Ring and linker swing mechanism in the dynein motor. Cell 136, 485–495.

Romes EM, Tripathy A, Slep KC (2012). Structure of a yeast Dyn2-Nup159 complex and molecular basis for dynein light chain-nuclear pore interaction. J Biol Chem 287, 15862–15873.

Ross JL, Wallace K, Shuman H, Goldman YE, Holzbaur EL (2006). Processive bidirectional motion of dynein-dynactin complexes in vitro. Nat Cell Biol 8, 562–570.

Saunders WS, Koshland D, Eshelf D, Gibbons IR, Hoyt MA (1995). Saccha-romyces cerevisiae kinesin- and dynein-related proteins required for anaphase chromosome segregation. J Cell Biol 128, 617–624.

Schmidt H, Gleave ES, Carter AP (2012). Insights into dynein motor domain function from a 3.3-A crystal structure. Nat Struct Mol Biol 19, 492–497, 5491.

Shi X, Lim J, Ha T (2010). Acidification of the oxygen scavenging system in single-molecule fluorescence studies: in situ sensing with a ratiometric dual-emission probe. Anal Chem 82, 6132–6138.

Signor D, Wedaman KP, Orozco JT, Dwyer ND, Bargmann CI, Rose LS, Scholey JM (1999). Role of a class DHC1b dynein in retrograde transport of IFT motors and IFT raft particles along cilia, but not dendrites, in chemo-sensory neurons of living Caenorhabditis elegans. J Cell Biol 147, 519–530.

Sivagurunathan S, Schnittker RR, Nandini S, Plamann MD, King SJ (2012). A mouse neurodegenerative dynein heavy chain mutation alters dynein motility and localization in Neurospora crassa. Cytoskeleton (Hoboken) 69, 613–624.

Stelter P, Kunze R, Flemming D, Hopfner D, Diepholz M, Philippien P, Bottcher B, Hurt E (2007). Molecular basis for the functional interaction of dynein light chain with the nuclear-pore complex. Nat Cell Biol 9, 788–796.

Steuer ER, Wordeman L, Schroer TA, Sheetz MP (1990). Localization of cytoplasmic dynein to mitotic spindles and kinetochores. Nature 345, 266–268.

Stuchelli-Brereton MD, Siglin A, Li J, Moore JK, Ahmed S, Williams JC, Cooper JA (2011). Functional interaction between dynein light chain and intermediate chain is required for mitotic spindle positioning. Mol Biol Cell 22, 2690–2701.

Susalka SJ, Nikulina K, Salata MW, Vaughan PS, King SM, Vaughan KT, Pfister KK (2002). The roadblock light chain binds a novel region of the cytoplasmic dynein intermediate chain. J Biol Chem 277, 32939–32946.

Tang X, Germain BS, Lee WL (2012). A novel patch assembly domain in Num1 mediates dynein anchoring at the cortex during spindle positioning. J Cell Biol 196, 743–756.

Trotker M, Mucke N, Surrey T (2012). Reconstitution of the human cytoplasmic dynein complex. Proc Natl Acad Sci USA 109, 20895–20900.

Tulu US, Fagerstrom C, Ferenz NP, Wadsworth P (2006). Molecular requirements for kinetochore-associated microtubule formation in mammalian cells. Curr Biol 16, 536–541.

Tynan SH, Gee MA, Vallee RB (2000). Distinct but overlapping sites within the cytoplasmic dynein heavy chain for dimerization and for intermediate chain and light intermediate chain binding. J Biol Chem 275, 32769–32774.

Vale RD (2003). The molecular motor toolbox for intracellular transport. Cell 112, 467–480.

Wagner W, Fodor E, Ginsburg A, Hammer JA 3rd (2007). Structural and thermodynamic characterization of a cytoplasmic dynein light chain-intermediate chain complex. Proc Natl Acad Sci USA 104, 10028–10033.

Williams JC, Xie H, Hendrickson WA (2005). Crystal structure of dynein light chain TcTex-1. J Biol Chem 280, 21981–21986.