Cryo–EM shows how dynactin recruits two dyneins for faster movement

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Dynein and its cofactor dynactin form a highly processive microtubule motor in the presence of an activating adaptor, such as BICD2. Different adaptors link dynein and dynactin to distinct cargoes. Here we use electron microscopy and single–molecule studies to show that adaptors can recruit a second dynein to dynactin. Whereas BICD2 is biased towards recruiting a single dynein, the adaptors BICDR1 and HOOK3 predominantly recruit two dyneins. We find that the shift towards a double dynein complex increases both the force and speed of the microtubule motor. Our 3.5 Å resolution cryo-electron microscopy reconstruction of a dynein tail–dynactin–BICDR1 complex reveals how dynactin can act as a scaffold to coordinate two dyneins side-by-side. Our work provides a structural basis for understanding how diverse adaptors recruit different numbers of dyneins and regulate the motile properties of the dynein–dynactin transport machine.

Cytoplasmic dynein-1 (dynein) is the main transporter of cargoes towards the minus ends of microtubules in animal cells. These cargoes move at a range of speeds and vary in size from large organelles to small individual proteins. Dynein is activated to form a highly processive motor by binding its cofactor dynactin and a cargo adaptor, such as BICD2 (bicaudal D homologue 2). Dynein contains two motor domains joined by a tail region, whereas dynactin is built around a short actin-like filament, capped at its pointed and barbed ends and decorated with a shoulder. A previous 8 Å resolution cryo-electron microscopy (cryo-EM) structure showed how a coiled coil in BICD2 recruits the tail of dynein to the filament of dynactin. Other adaptors that activate dynein and link it to different cargoes have been identified. These activating adaptors also contain long coiled coils; however, the sequence similarity between them is low and it is unclear whether they engage dynein and/or dynactin in the same way as BICD2 does. There is also evidence that certain adaptors—such as BICDR1 (BICD related-1, also known as BICDL1) and HOOK3—drive faster movement of dynein towards the minus ends of microtubules when compared with BICD2, although the mechanism underpinning this increased speed is not currently understood.

Dynactin can recruit two dyneins

We determined the cryo-EM structures of two previously unsolved dynein–dynactin–adaptor complexes. BICDR1, like BICD2, binds RAB6 vesicles, whereas HOOK3 links dynein and dynactin to early endosomes. We determined 7 Å resolution maps of both the dynein tail–dynactin–BICDR1 complex (hereafter termed TDR) and the dynein tail–dynactin–HOOK3 complex (hereafter termed TDH), which we compare to the previously determined structure of the dynein tail–dynactin–BICD2 complex (hereafter termed TDB)8 (Fig. 1a, Extended Data Fig. 1a–d, Extended Data Table 1).

The coiled coils of all three adaptors run along the length of the dynein filament (Fig. 1a). However, in contrast to previous predictions, each adaptor makes different interactions. BICD2 and BICDR1 diverge in their path and relative rotation (Fig. 1b). HOOK3 follows yet another route over the surface of dynactin (Fig. 1c). TDB also shows an extra coiled-coil density near the pointed end of dynactin (Fig. 1c) and extra globular density towards its barbed end (Extended Data Fig. 1e, f). The identity of the second coiled coil is unclear, but the globular density probably corresponds to the N-terminal Hook domain, which is required for HOOK3 to activate dynein and dynactin.

The most notable feature of TDB and TDH is the presence of two dynein tails (Fig. 1a). The tail of the first dynein (dynein-A) binds in an equivalent position to the dynein tail in TDB and to the full-length dynein in the dynactin–dynein–BICD2 complex (hereafter termed DDB). The second dynein (dynein-B) binds next to dynein-A near the barbed end of dynactin.

Adaptors determine dynein recruitment

We determined whether BICD2, BICDR1 and HOOK3 recruited different numbers of dyneins in moving dynein–dynactin complexes. We mixed dyneins that had been labelled with tetramethylrhodamine (TMR) or Alexa Fluor 647, and used single-molecule fluorescence microscopy to measure the frequency at which the two dyes colocalized on microtubules (Fig. 2a, b). In the presence of dynactin and BICD2, 13 ± 1% (s.e.m.) of processive complexes were labelled with both dyes; this was significantly higher than the colocalization observed for the dynein-only control (2.1 ± 0.3%). Using BICD1 or HOOK3 as an adaptor led to colocalization percentages of 31 ± 2% and 34 ± 1%, respectively (Fig. 2b). After correction for complexes that were double-labelled with the same colour, we estimate that 26% of BICD2 complexes contained two dyneins, compared to 61% for BICDR1 and 67% for HOOK3. We conclude that the majority of motile complexes that contain BICD2 have one dynein, whereas both BICDR1 and HOOK3 preferentially recruit two.

Although both this study and previous work are consistent with BICD2 predominantly recruiting one dynein, its ability to recruit a second was unanticipated. To verify this observation, we applied a mixture of BICD2, dynein tail and dynactin onto grids for negative-stain electron microscopy analysis (Fig. 2c). In agreement with our single-molecule data, 3D classification of adaptor complexes showed that 17 ± 1% of BICD2 complexes contained two dyneins;
Two dyneins increase force and speed

We next investigated how the recruitment of different numbers of motors affects the motile properties of the dynein–dynactin complex. We used an optical trap to measure the stall force of DDB, dynein–dynactin–BICDR1 (hereafter termed DDR) and dynein–dynactin–HOOK3 (hereafter termed DDH) (Fig. 2a). Similar to our previous measurements\textsuperscript{5,17}, the stall force of DDB is 3.7 ± 0.2 pN, which is significantly lower ($P < 0.0001$) than the stall force of the plus-end-directed motor kinesin-1 (5.7 ± 0.2 pN)\textsuperscript{23}. By comparison, the stall force of DDR is 6.5 ± 0.3 pN and that of DDH is 4.9 ± 0.2 pN (Fig. 2b), which suggests that recruiting higher numbers of dyneins to dynactin increases force production. This agrees with previous reports that concluded—on the basis of using dyneins with beads—that dyneins can team up efficiently for maximum mechanical output\textsuperscript{13,24}. The difference in stall force between the DDR and DDH dynein–dynactin complexes suggests that features other than motor number can also fine-tune force production.

The higher stall force of DDR also suggests that it competes more efficiently with kinesin than DDB does. This may explain why neuronal overexpression of BICDR1, but not of BICD2, counteracts kinesin-driven transport of RAB6 vesicles\textsuperscript{14} and may be relevant to the role of BICDR1 in opposing anterograde movement in early neuronal differentiation\textsuperscript{16}. The ability of some adaptors to recruit multiple dyneins could also contribute to the clustering and pairing of dynein motors required to transport large cargoes\textsuperscript{24,25}.

We next explored whether the recruitment of more dyneins to dynactin had an effect on speed. Previous work on BICDR1 in cells\textsuperscript{14} and HOOK3 in vitro\textsuperscript{5,10,11,19} has shown that complexes containing these adaptors move faster than those containing BICD2. Our data raise the possibility that these faster speeds are due to an increased number of complexes with two dyneins. However, previous reports have suggested that, although artificially tethering dyneins increases run length, it has little or no effect on velocity\textsuperscript{26,27}.

To determine whether motor number affects the movement speed of dynein–dynactin complexes, we first directly compared all three adaptors in our in vitro motility assay. As expected, the run lengths of DDR and DDH were longer than that of DDB (Extended Data Fig. 2a). Notably, the average velocities of DDR (1.35 ± 0.2 μm s\textsuperscript{-1}) and DDH (1.23 ± 0.04 μm s\textsuperscript{-1}) were significantly faster than that of DDB (0.86 ± 0.04 μm s\textsuperscript{-1}, $P < 0.0001$) (Fig. 2c).

To investigate whether this difference in speed required the presence of two active dyneins, we mixed Alexa Fluor 647-labelled dynein with dynactin as strongly as the dynein tail does\textsuperscript{8,9} but moves at wild-type velocities (Extended Data Fig. 2b, d). We compared the speeds of dynactin–BICDR1 (hereafter termed DDR) and dynein–dynactin–HOOK3 (hereafter termed DDH) (Electron Microscopy Data Bank (EMDB) code: EMD-2862) is shown in Figure 1. The EMDB code: EMD-2862 is included for comparison. Surface representation molecular models of BICDR1 and HOOK3 on dynactin show the divergent paths of the coiled coils. Figure 1, Dynactin can recruit two dyneins. a, Sub-7 Å cryo-EM maps of TDR and TDH complexes, coloured according to their components. TDB complex (Electron Microscopy Data Bank (EMDB) code: EMD-2862) is included for comparison. b, Surface representation molecular models of BICDR1 and HOOK3 on dynactin show the divergent paths of the coiled coils. c, Comparison of HOOK3 and BICD2 on dynactin.

69 ± 4% contained only one dynein, and the rest were ambiguous. The ability of BICD2 to bind two dyneins also agrees with a cryo-electron tomography reconstruction of microtubule-bound DDB\textsuperscript{20}. Negative-stain electron microscopy of BICDR1 and HOOK3 complexes showed that 94 ± 2% and 88 ± 1% of these complexes, respectively, contained two dyneins (Fig. 2c). This suggests an even higher degree of second dynein recruitment than is indicated by our single-molecule data. Our data suggest that the number of dyneins bound to dynactin can be controlled by the identity of the adaptor.
moving complexes that contained only full-length dynein (‘dynein-only’) with those that contained one tail and one active dynein (‘tail–dynein’). As expected, dynein-only complexes moved at a similar speed (1.25 ± 0.04 μm s⁻¹, Fig. 3e) to DDR (1.22 ± 0.05 μm s⁻¹, Extended Data Fig. 2d). However, tail–dynein complexes moved significantly more slowly (0.84 ± 0.03 μm s⁻¹, P < 0.0001, Fig. 3e, Extended Data Fig. 2e) than either DDR or dynein-only complexes. This suggests that the presence of a second dynein increases the velocity of dynein–dynactin complexes.

We propose that the increase in speed on the recruitment of two dyneins is linked to the way in which dynein recruits them side-by-side (Fig. 1a). This may restrict the inherent sideways and backwards movements of the motor domains29 and cause the complex to take a more direct and faster route along the microtubule. Other dynein regulators, such as LIS1 (otherwise known as PAFAH1B1), have been reported to increase the speed of dynein–dynactin complexes29,30 and could act by increasing motor copy number. For LIS1, however, quantitative fluorescence measurements suggest that this is not the case29. The velocity of BICD2 complexes containing both fluorophores and therefore two dyneins (1.08 ± 0.03 μm s⁻¹, Extended Data Fig. 2f) was significantly faster than the average DDB velocity (P < 0.0001), but not as fast as DDR. This suggests that, in addition to recruiting two dyneins, certain adaptors also affect speed through small differences in how they recruit the motors to dynactin.

The dynein–dynactin–BICDRI structure
To investigate how dynactin recruits two dyneins, we collected data sufficient to determine the TDR structure to an overall resolution of 3.5 Å (Extended Data Fig. 3, Extended Data Table 1). To improve the tail density, we performed multiple rounds of particle signal subtraction, focused 3D classification and refinement on regions that moved as rigid blocks, which improved the definition of the blocks at each iteration (Extended Data Fig. 4). This produced a set of 3.4 Å maps that covered the entire length of the dynein tail (Extended Data Table 1).

Previous low-resolution structures showed that the dynein tail comprises two heavy chains (HCs), which consist of a series of helical bundles held together by an N-terminal dimerization domain (NDD)8,9. Each HC binds one intermediate chain (IC) and one light intermediate chain (LIC)7,9,31. The IC N-terminal regions are held together by the dynein light chains, ROBL1 (otherwise known as DYNLRB1), LC8 (otherwise known as DYNLL1) and TCTEX1 (also known as DYNLT1)32,33. We used our high-resolution maps to build an atomic model of the dynein tail. We de novo traced helical bundles 1–6 of the HC and the WD40 domain of the IC (Fig. 4a, Extended Data Figs 5a, b, 6a, Extended Data Table 1). We also placed helices for part of helical bundle 7 and rebuilt homology models for the LIC31 and ROBL132 (Fig. 4a, Extended Data Figs 5c, 6b, c). Our structure reveals that the IC WD40 domain makes extensive contacts with HC bundles 4 and 5, and that its central cavity is filled by a loop-helix from bundle 4 (Extended Data Fig. 6a). By contrast, the LIC globular domain interacts only with two helices from bundle 6. The tight binding of the LIC to the HC34 is the result of its N- and C termini, which span out from the globular domain and form an integral part of HC bundles 5 and 7, respectively (Extended Data Figs 5c, 6b).

We assembled and refined a model of the whole TDR complex (Fig. 4b, Extended Data Table 1, Supplementary Video 1) into our 3.5 Å map. We used a previous dynactin structure8 and a model of the BICDRI coiled-coil region. For each dynein, we fit in two copies each of HC, IC and LIC, one ROBL1 dimer and a new 1.9 Å crystal structure of the human NDD (Extended Data Fig. 6d, e, Extended Data Table 2).

Structural basis of two–dynein recruitment
Our TDR structure shows the two dyneins binding to grooves on the surface of dynactin that are formed by its β-actin subunit and the three actin-related protein 1 (ARP1, also known as ACTR1A) subunits ARP1F, ARP1D and ARP1B (Fig. 4b). The two dynein-A chains,
A1 and A2, and the first dynein-B chain, B1, all bind in a similar way, by making contacts to both sides of their respective grooves. Although the precise interactions overlap, they are all slightly different from one another (Fig. 5a). The second dynein-B chain, B2, binds between ARP1B and the barbed-end capping protein CAPZ\(\beta\) (CAPZ\(\beta\) is also known as CAPZB) and is rotated by 90° along its long axis, relative to the other dynein chains.

The two dyneins also make extensive interactions with one another. These consist of the IC WD40 domain of A2 binding the HC of B1; direct HC-to-HC interactions; and contacts between the A2 LIC and the HC and IC of B1 (Fig. 5b). These contacts are highly conserved across higher eukaryotes (Extended Data Fig. 7a). They contribute to a cascade of interactions (Supplementary Video 2) between the four dynein chains that include contacts between the IC WD40 domain of each chain and the neighbouring HC (Extended Data Fig. 7b). This network of connections stabilizes the binding of the second dynein and ensures all four HCs are held in a rigid orientation with respect to one another. This is likely to keep the dynein motor domains properly aligned and may be important for the increase in speed when two dyneins are recruited to the dynactin scaffold.

Our structure reveals the key role BICDR1 has in recruiting two dyneins to the dynactin subunits (green). Contact residues on dynactin are shown as yellow spheres. For each HC, helix α6 is highlighted (dynein-A, dark blue; dynein-B, dark pink). By contrast, A2 makes extensive interactions with B1. Interaction sites are shown as yellow and red spheres. c, Interactions of dynactin subunits (green). Contact residues on dynactin are shown as yellow spheres. For each HC, helix α6 is highlighted (dynein-A, dark blue; dynein-B, dark pink). By contrast, A2 makes extensive interactions with B1. Interaction sites are shown as yellow and red spheres. c, Interactions of dynein-A (top) and B1 (bottom) with BICDR1. Interaction sites marked in dark blue and red. Extra density from A2 LIC mediates the connection between B1 and BICDR1. d, Negative-stain electron microscopy reconstructions of DDB containing two dyneins (top) or dynein-A only (bottom), sliced to highlight BICD2. Arrows depict alternative positions of BICD2 at the barbed end of dynactin.

**Adaptor position controls dynein number**

All three dynein–dynactin–adaptor complexes recruit dynein-A in a similar way, despite the differences in the positions of the adaptors themselves (Extended Data Fig. 8a). In TDR and TDH, dynein-B can contact the adaptor because the BICDR1 and HOOK3 N termini follow downward paths, stabilized by interactions with CAPZ\(\beta\). By contrast, in TDB no contact site for dynein-B is available because the adaptor is shifted upward towards the shoulder domain to contact ARP1A (Fig. 1b, Extended Data Fig. 8b).

We investigated which structural changes allow BICD2 to recruit a second dynein (Fig. 2). We combined our negative-stain electron microscopy TDB datasets (Fig. 2c) to determine structures of sufficient quality to resolve the position of the coiled coil. We found that TDB with two dynein tails has BICD2 in a lower position, similar to BICDR1 and HOOK3 and different from its position in single-dynein-bound TDB (Fig. 5d). Our data suggest that a switch in the position in the N terminus of the adaptor is sufficient to account for dynein-B recruitment.

In conclusion, we show that dynactin can act as a natural scaffold to line up two dyneins in close proximity. This arrangement results in a dynein–dynactin complex that moves faster and can produce larger forces when compared with complexes containing a single dynein. Our observations provide a mechanism by which cargo can control the output of the dynein–dynactin machine via the identity of its activating adaptor.
RESEARCH ARTICLE

Online Content Methods, along with any additional Extended Data display items and Source Data, are available in the online version of the paper; references unique to these sections appear only in the online paper.

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METHODS

Cloning. The following adaptors were codon-optimized for expression in S9 cells (Epoch Life Science): mouse BICDR1 (BICD1L), human HOOK3 residues 1-522 and mouse BICD2 residues 1-400. Adapters were subcloned into pOMniBac- and pACEBac-derived vectors for expression in S9 cells8. Tags were added for purification (a His6–ZZ tag with a TEV protease cleavage site (TEV), or a PreScission protease site (Psc) followed by a 2× strep affinity tag) or protein labelling (GFP or SNAPf–NEBD). The following constructs were generated: pOMniBac-His–ZZ–TEV–SNAPf–BICD1–400, pOMniBac-His–ZZ–TEV–BICD1R, pOMniBac-His–ZZ–TEV–SNAPf–BICD1R, pOMniBac-His–Z1–TEV–BICD1–SNAPf, pOMniBac-His–Z1–TEV–BICD1–SNAPf–Psc–2× strep and pACEBac1–HOOK3–522–SNAPf–Psc–2× strep.

We generated a new dynein tail construct containing residues 1–1,455 of the human dyninc HC. The fragment of the S9-codon optimized DYNHC1 gene was cloned into a pACEBac1 vector containing an N-terminal His6–ZZ–TEV tag and fused to pDyN2 (containing genes for human IC2C, LIC2, TCTEX1, LC8 and ROBL1) as described8.

Protein purification. Dynactin was purified from pig brains using the large-scale SP sepharose protocol9. Wild-type human dyninc and a mutant open dynein8 were expressed and purified using baculovirus as described8. The two dynein tail constructs (HC1455 and SNAPf–HC11,073–GST) were purified as described8.

The following adaptors were codon-optimized for expression in Escherichia coli strain as described37.

For cryo-EM of TDR.

The structure was solved in PHENIX39, built in COOT40 and refined using the Relion v.2.044 unless otherwise stated. Using CTFFIND3_13030743. Subsequent processing used Relion v.2.044 unless otherwise stated. Using automated data collection (EPU, FEI). Seven images were collected per hole.

Falcon II detector (300 kV, 32 frames, 2-s exposure, 1.34 Å per pixel, 52 e− per A2) using automated data collection (EPU, FEI). Seven images were collected per hole (26,906 images in total, over 11 separate sessions). Drift correction was performed using MotionCor242 and contrast transfer function (CTF) parameters estimated using CTFFind3_13030743. Subsequent processing used Relion v.2.044 unless otherwise stated.

Micrographs were first manually examined to remove images with a large amount of contamination, very low number of particles (<15), substantial uncorrected drift, a large astigmatism, extreme defocus values (<1 μm or >60 μm) and/or abnormally Fourier patterns. We selected 23,945 micrographs that had good signal to noise at least 8 Å. For the first dataset, a small set of particles was manually picked from 8× binned micrographs and subjected to reference-free 2D classification. A selection of 2-D class averages that represented a range of different size and shape particles present in micrographs (not just TDR complexes) was selected, centred using the Relion shift_com function, low-pass filtered to 50 Å and used as references for autopicking all binned micrographs using Relion v.1.445. For optimal particle picking of other datasets, we used 2-D classes obtained from multiple sessions. We also used a value of 1.1 for the ‘maximum standard deviation of the background noise’ setting to ensure we picked all good TDR particles with high contrast. This value resulted in substantial levels of contaminants but these were removed by extensive manual screening, as indicated below. Initially, particles with a high ‘autopick figure of merit’ values were screened to remove false positives. Then, the particles were cleaned by several cycles of 2D classification. At each cycle, the particles that were clearly not TDR complexes were discarded. The particles were then refined using all 11 datasets yielded a 3.5 Å resolution map.

The 3D refinement using the unbinned particles from the first dataset yielded a 6.5 Å resolution map, and the 3D refinement using all 11 datasets yielded a 3.5 Å resolution map.

3D classification (see above) revealed movement of the dynein tails, which resulted in a lower resolution for these parts of the map. As a result, we conducted focused 3D classification and 3D refinement of the dynein tail as described8, except that we used multiple rounds of mask optimization. First, we generated overlapping binary masks covering the N- and C-terminal densities of all four dynein chains and used the particle subtraction function in Relion to subtract the density outside these regions from the raw TDR particles. Next, 3D refinement was used to align these ‘subtracted particles’ on the basis of the remaining density. Then we used 3D classification with no alignments to investigate which parts of the structure moved as a rigid block. We then made new masks around the rigid block and repeated all the steps of particle subtraction, 3D refinement and 3D classification. This process was repeated several times to obtain the optimal mask.

In the case of the N-terminal region of the tail, the optimal mask was used for a final round of particle subtraction and 3D refinement, which resulted in a 3.4 Å map. To further improve the densities for the IC WD40 domain, we performed local sub-volume averaging with Chimera1,107. Similarly, for the C-terminal region of the dynein tail we performed a round of particle subtraction and 3D refinement using the optimal mask. We then used 3D classification with no alignment to identify the most homogenous particles for different local regions. For HC residues 500–927, we performed 3D classification using the whole of the optimal mask. For ROBL1 or LIC, we performed 3D classification using the local mass around their respective regions. In all cases, selected particles were refined using the whole optimal mask. All density maps were corrected for the modulation transfer function of the detector, and then sharpened by applying a negative B factor that was estimated either using automated procedures within Relion or by using M梦见113,114.

Model building and refinement. Building was performed in COOT and refinement in PHENIX. The dynein HC residues 201–710 from dynein-B1 and the IC from dynein-A2 were de novo built and refined into the ‘N-terminal tail’ map guided by maps generated by local sub-volume averaging, with improved density for flexible loops. HC residues 500–927 from dynein-A2 were built and refined into the ‘C-terminal tail’ map. A ‘LIC-mask’ map was used to model secondary structure elements for HC residues 927–1,057 and to rebuild a Phyre248 homology model for the ROBL1–IC-extended-N termini complex and identify local sub-volume averaging with Chimera1,110. For the N-terminal region of the dynein tail we performed a round of particle subtraction and 3D refinement using the optimal mask. We then used 3D classification with no alignment to identify the most homogenous particles for different local regions. For HC residues 500–927, we performed 3D classification using the whole of the optimal mask. For ROBL1 or LIC, we performed 3D classification using the local mass around their respective regions. In all cases, selected particles were refined using the whole optimal mask. All density maps were corrected for the modulation transfer function of the detector, and then sharpened by applying a negative B factor that was estimated either using automated procedures within Relion or by using M梦见113,114.

Model building and refinement. Building was performed in COOT and refinement in PHENIX. The dynein HC residues 201–710 from dynein-B1 and the IC from dynein-A2 were de novo built and refined into the ‘N-terminal tail’ map guided by maps generated by local sub-volume averaging, with improved density for flexible loops. HC residues 500–927 from dynein-A2 were built and refined into the ‘C-terminal tail’ map. A ‘LIC-mask’ map was used to model secondary structure elements for HC residues 927–1,057 and to rebuild a Phyre2 homology model for human LIC2 (both dynein-A2). A ‘Robl-mask’ map was used to rebuild a homology model for the ROBL1–IC-extended-N termini complex and identify local sub-volume averaging with Chimera1,110. Similarly, for the C-terminal region of the dynein tail we performed a round of particle subtraction and 3D refinement using the optimal mask. We then used 3D classification with no alignment to identify the most homogenous particles for different local regions. For HC residues 500–927, we performed 3D classification using the whole of the optimal mask. For ROBL1 or LIC, we performed 3D classification using the local mass around their respective regions. In all cases, selected particles were refined using the whole optimal mask. All density maps were corrected for the modulation transfer function of the detector, and then sharpened by applying a negative B factor that was estimated either using automated procedures within Relion or by using M梦见113,114.

The structure of TDR was assembled and real-space refined into an overall TDR map that was not sharpened, and was filtered to 8 Å resolution.

We used four copies of the HC–IC–LIC consensus model, two copies of the NDD, a model of dynactin8 and a stretch of coiled coil for BICDR1. The combined model was fit for autopicking all binned micrographs using Relion v.1.445. For optimal particle picking of other datasets, we used 2-D classes obtained from multiple sessions. We also used a value of 1.1 for the ‘maximum standard deviation of the background noise’ setting to ensure we picked all good TDR particles with high contrast. This value resulted in substantial levels of contaminants but these were removed by extensive manual screening, as indicated below. Initially, particles with a high ‘autopick figure of merit’ values were screened to remove false positives. Then, the particles were cleaned by several cycles of 2D classification. At each cycle, the particles that were clearly not TDR complexes were discarded. The particles were then refined using all 11 datasets yielded a 3.5 Å resolution map.
to the 3.5 Å overall map. Sections of dynactin, including the CAP250x5 dimer and the N terminus of subunit p50, were rebuilt. Corrections were made to secondary structure elements in the pointed end and shoulder domains. An approximate model for the BICDR1 coiled coil (residues 105–392) was generated by placing helices into density, and assigning registry on the basis of fitting residues Phe159 and 1° offset search range set to 3 pixels and step to 0.5 pixels, which yielded 3D classification. It was followed by 15 iterations with an angular sampling interval of 5 pixels and step to 1 pixel. The gradual reduction in sampling yielded the best TDR: 1,861, 1,782 and 5,782 and 5,388). For the best particles was picked from 4 micrographs of each sample were recorded using FEI EPU on a FEI Falcon II microscope. Two replicate samples were made on separate days. Between 200 and 400 micrographs of each sample were recorded using FEI EPU on a FEI Titan Krios with a FEI Falcon III detector in linear mode: 300 kV, 59 frames during a 1.5 s exposure; 1.42 Å per pixel; 45 e− per Å2; and 5 images per hole. Correlation of inter-frame movement for each pixel and dose-weighting was performed using MotionCor2. CTF parameters were estimated using GCETP5. A reference set of 2D classes was generated using Relion v.2.0 from a small set of particles picked by the EMAN2 Snap boxing tool. Gautomatch (http://www.mrc-lmb.cam.ac.uk/kzhang/) was used to pick particles from all micrographs (4× binned) using this reference set. Relion v.2.0 was used for 2D classification to clean the autopicked particles. An 8 Å resolution level was low-pass filtered to 60 Å, was used as an initial model for a first round of 3D refinement. The dataset was cleaned by 3D classification using the output from 3D refinement as a reference. The cleaned particles were re-extracted from unbinned micrographs and used for a final round of 3D refinement, yielding a 6.7 Å map.

Negative stain electron microscopy analysis. The dynein–tail–actin complex triple complexes were assembled by mixing 1.7 μl of 1 mg ml−1 dynein tail (HC-45), 1 μl of 1.35 mg ml−1 dynactin and 2 μl of 1.3 mg ml−1 cargoadaptor (BICD2(210–400), GFP, HOOK3(352–522)–SNAPf and BICDR1–GFP). The incubates were stained for 15 min before diluting eighth fold for preparation of negative stain grids as described. Two replicate samples were made on separate days. Between 200 and 400 micrographs of each sample were recorded using FEI EPU on a FEI Titan Krios with a FEI Falcon II detector, fitted to a FEI F20 electron microscope operated at 200 kV: 1.5 s exposure; 2.08 Å per pixel; 20 e− per Å2; 0.8–1.2 μm underfocus. A small set of particles was picked from 4× binned micrographs using the EMAN2 Snap boxing tool. Subsequent processing was done by Relion v.2.0 unless otherwise stated. Particles were extracted and subjected to reference-free 2D classification. From two to five 2D-class averages of triple complex were centred using a shift command, low-pass filtered to 50 Å resolution and used as references for automated particle picking within Relion v.1.4 of all 4× binned micrographs. Autopicked particles were extracted, split in half and subjected to 2D classification as above. Ten 2D-class averages representing different particle orientations were selected and used for another round of autopicking. This third round of autopicking was used to obtain the optimal particle selection (fewest missed particles) and used for a final round of 3D refinement, yielding a 6.7 Å map.

To determine the path of BICD2, all particles of the complexes from both TDB (BICD2(210–400)–GFP) datasets were combined (42,823 particles). Binned particles from these complexes were subjected to 3D refinement as described above. These coordinate files were used to re-centre—as described above—and extract particles from unbinned micrographs, with CTF parameters that were determined using CTFFIND3_130307. Extracted particles were subjected to 3D refinement, followed by 3D classification. In both steps, the CTF correction was set to ignore CTFs until the first peak. Particles from 3D classes with dynein-A only, or with dynein-A and -B, were selected separately. Each dataset was subjected to another round of 3D refinement and subsequent 3D classification (using 5,782 angular sampling interval, offset search range of 5 pixels and step of 1 pixel) to obtain the best particles for each complex (13,278 and 14,070 particles for TDB with dynein-A only and with two dyneins, respectively). These were used in a final round of 3D refinement. Molecular models of dynactin, cargo adaptors and dynactin tails were fitted into the density maps and used to colour different segments in Chimera.

Single molecule assays. SNAPf–dynactin–cargo–adaptor complexes were labelled with TMR-Star or Alexa Fluor 647 (NEB) and purified separately, and the percentage labelling was quantified as previously described. Single-molecule assays were conducted as previously described, with slight modifications in order to optimize the number of moving complexes. Under these optimized conditions, DBB complexes moved faster than observed in previous publications. All adaptor complexes were measured under identical conditions. The percentage of processive events was 56% for DDB, 76% for DDH and 75% for DDR.

Microtubules were made by mixing 26 μl of 5.2 mg ml−1 unlabelled pig tubulin, 5 μl of 2 mg ml−1 HiLyte Fluor 488 tubulin and 10 μl of 2 mg ml−1 biotin tubulin (Cyto skeleton) in BB800 buffer (80 mM PIPES pH 6.8, 1 mM MgCl2, 1 mM EGTA, 1 mM DTT). The mixture was incubated on ice for 5 min before adding 41 μl of polymerization buffer (2× BB800 buffer plus 20% (v/v) tubulin solution). Microtubules were polymerized at 37°C for 30–60 min. The sample was diluted with 200 μl of microtubule buffer (BB800 plus 40 μM Paclitaxel). Excess tubulin was removed by pelleting (20,238 r.c.f., 8 min; at room temperature). The microtubule pellet was washed with 200 μl of microtubule buffer and re-pelleted as above. The microtubule pellet was re-suspended in 200 μl of microtubule buffer and stored at room temperature, and covered from light for at least half a day (to a maximum of three days) before use. Microtubules were visualized (see below) and if the density was too low, or the free tubulin concentration was too high, they were re-pelleted and re-suspended in a smaller volume of microtubule buffer.

Motility chambers were made by applying two strips of double sided-tape (Tesa) approximately 8–10 mm apart on a glass slide and placing a cleaned coverslip on top. The glass surface was functionalized with 0.4 mg ml−1 biotinylated poly-L-lysine)–g-poly(ethylene-glycol) (SuSoS AG). The chamber was immediately washed with 40 μl of assay buffer (30 mM Hepes–KOH pH 7.2, 2 mM MgSO4, 1 mM EGTA, 1 mM DTT). Subsequently, 10 μl of 1 mg ml−1 streptavidin (NEB) was flowed through and immediately washed with 40 μl of assay buffer. The chamber was then incubated with freshly diluted microtubules (typically 3× of microtubules and 10 μl of assay buffer). Microtubules were immediately washed out with assay buffer supplemented with 1.25 mg ml−1 (α-casein) (Sigma–Aldrich).

Dynein–actin–cargo–adaptor complexes were prepared by mixing 1 μl of 1 mg ml−1 fluorescently labelled dynein, 1 μl of 1.35 mg ml−1 dynactin and 2 μl of 1.3 mg ml−1 cargo adaptor (SNAPf–BICD2(210–400), HOOK3(352–522)–SNAPf and SNAPf–BICDR1). For experiments with SNAPf–tail, an additional 1 μl of 1 mg ml−1 TMR-labelled tail was added. The complex was incubated on ice for 10–15 min, and then diluted with assay buffer to a final volume of 10 μl. One microlitre of complex solution was added to 19 μl of assay buffer containing 1.25 mg ml−1 α-casein, an oxygen scavenging system (0.2 mg ml−1 catalase (Calbiochem) and 1.5 mg ml−1 glucose (Sigma–Aldrich)), 0.4% (w/v) glucose, 1% BME, 25 mM KCl and 5 mM MgATP. Taxol was omitted from this buffer to reduce the non-specific background and non-moving events. The motility mix was flowed into the chamber, and washed with assay buffer supplemented with 1.25 mg ml−1 α-casein for a second time. The sample was analysed immediately at 23 ± 1°C with a total internal reflection fluorescence microscope, as previously described. Colocalization and velocity were determined from the same datasets (4, 8, 9 and 7 chambers overall, for dynein-only, DDB, DDH and DDR, respectively). Data were collected on two different days. Tail and full-length dynein speed data were collected from three chambers. Data were analysed using ImageJ. Tilt stacks were Z-projected to identify the position of microtubules and segments drawn along them and kymographs generated using the relucise function. Processive movements were defined as previously described. Velocity was calculating using a pixel size of 105 nm and a frame rate of 236 ms per frame.

Colocalization data were collected using a DV2 beam splitter (Photometrics), which projected each channel (TMR and Alexa Fluor 647) onto a different half
of the image. Movie tif-stacks were split and kymographs generated for each channel. Kymographs were overlaid using the ‘Colour:Merge’ function to generate a composite image. The kymographs were manually scored for processive events that showed colocalization, followed by those events that only appeared in individual channels. Colocalization in the dynein-alone control chambers was determined for all microtubule binding events longer than 2 frames.

The fraction of complexes containing two dyneins, \( d \), was calculated from the fraction of total events with signal for TMR-only (\( R_{\text{obs}} \)), Alexa Fluor 647-only (\( G_{\text{obs}} \)) and colocalized signal (\( Y_{\text{obs}} \)). Colocalized events represent dynein–dynactin–adaptor complexes with two dyneins, and single-coloured events represent a mixture of both one- and two-dynein complexes. We can express this using the following three equations:

\[
R_{\text{obs}} = (s \times r) + (d \times r^2)
\]

\[
G_{\text{obs}} = (s \times r) + (d \times g^2)
\]

\[
Y_{\text{obs}} = d \times 2(r \times g)
\]

Where \( s \) is the fraction of complexes that contain one dynein, \( r \) is the fraction of TMR-labelled dynein and \( g \) is the fraction of Alexa Fluor 647-labelled dynein flowed into the chamber. These equations hold at high labelling efficiency—our dynein was >94% labelled for a dynein monomer, or >99.64% per dimer—where dynein can be labelled by either TMR or Alexa Fluor 647 (r + g = 1). We can therefore solve these equations for \( d \) without knowing \( r \) or \( g \).

**Stall force measurements.** Eight-hundred-nanometre carboxy latex beads (Life Technologies) were functionalized with anti-GFP antibodies. Dynein, dynactin and cargo adaptor (BICD1–400–GFP, HOOK1–132–GFP and BICDR1–GFP) were mixed at a 1:5:20 molar ratio and incubated for 10 min on ice in dynein motility buffer (DMB: 30 mM HEPES pH 7.0, 5 mM MgSO\(_4\), 1 mM EGTA). BSA. The diluted mixture was incubated with the anti-GFP beads for 10 min on ice. CyS-labelled axonemes were introduced to the sample flow chamber, which was washed with 40 \( \mu \)l of DMB plus 1 mM TCEP (100 \( \mu \)g/ml-1 casein). The protein–bead mixture was introduced to the chamber in imaging buffer (DMB with 1 mM TCEP, 500 \( \mu \)g/ml-1 casein, 2.5 mM protocatechuate acid, 35 \( \mu \)g/ml-1 protocatechuate 3,4-dioxygenase (PCD), 2 mM MgATP). Kinesin was diluted in BRB80 supplemented with 1.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 1 mg/ml-1 BSA. The diluted mixture was incubated with the anti-GFP beads for 10 min on ice. CyS-labelled axonemes were introduced to the sample flow chamber, which was washed with 40 \( \mu \)l of DMB plus 1 mM TCEP (100 \( \mu \)g/ml-1 casein). The protein–bead mixture was introduced to the chamber in imaging buffer (DMB with 1 mM TCEP, 500 \( \mu \)g/ml-1 casein, 2.5 mM protocatechuate acid, 35 \( \mu \)g/ml-1 protocatechuate 3,4-dioxygenase (PCD), 2 mM MgATP). Kinesin was diluted in BRB80 supplemented with 1.5 mM tris(2-carboxyethyl)phosphine (TCEP) and 1 mg/ml-1 BSA. The diluted mixture was incubated with the anti-GFP beads for 10 min on ice. Kinesin-coated beads were introduced to the sample chamber in motility imaging solution (BRB80 supplemented with 2 mM DTT and 1.5 mg/ml-1 casein, 2.5 mM protocatechuate acid, 35 \( \mu \)g/ml-1 PCD, 2 mM MgATP). For each experiment, the protein concentration was adjusted until less than 30% of the tested beads exhibited any binding and motility when brought in contact with axonemes. This ensured that 95% of the beads were driven by a single processive motor complex.

Trapping experiments were performed on a custom-built fully automated optical trap microscope setup. To generate stall force histograms, position data were randomized before analysis; other experiments were not randomized, and the investigators were not blinded to allocation during experiments and outcome assessment.

**Code availability.** Code used is available from A.P.C.

**Data availability.** Cryo-EM maps have been deposited in the EMDB under accession codes EMD-4168 (whole TDR complex), EMD-4169 (N-terminal tail), EMD-4170 (C-terminal tail/ICD complex), EMD-4171 (LIC region), EMD-4172 (ROB1L region) and EMD-4177 (TDR complex). Coordinates are available from the RCSB Protein Data Bank under accession codes 6FIT (whole TDR complex), 6F1U (N-terminal tail), 6F1Y (C-terminal tail), 6F1Z (LIC region), 6F3Z (ROBL1 region), 6F38 (TDD complex) and 6F3A (TDB complex). Raw data are available from A.P.C.

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Extended Data Figure 1 | Single particle cryo-EM analysis of TDR and TDH. 

a. Cryo-EM reconstruction of the TDR complex analysed by ResMap and showing resolution distribution from 4 to 12 Å.

b. The gold-standard Fourier shell correlation (FSC) curve of the 6.5 Å TDR map.

c. Cryo-EM reconstruction of the TDH complex, showing resolution distribution from 4 to 12 Å.

d. The gold-standard FSC curve of the 6.7 Å TDH map.

e. Cryo-EM density for TDR low-pass filtered to 6.7 Å resolution (coloured according to cartoon) and to 20 Å (transparent outline). Density at the N terminus of BICDR1 is boxed.

f. Cryo-EM density for TDH low-pass filtered to 6.7 Å (coloured according to cartoon) and to 20 Å (transparent outline) showing the putative Hook domain, an extension of the HOOK3 coiled coil ending in extra density near dynein-B (dashed box).
**Extended Data Figure 2 | Single-molecule assay speed distributions.**
a. A one-cumulative frequency distribution plot showing run-lengths of DDB, DDR and DDH, with fit to a one-phase exponential decay. The decay constant (run length) and $R^2$ value (least squares regression) of the fit are shown. We measured 785, 677 and 684 events for DDB, DDH and DDR, respectively, from microtubules of at least 20 μm in length from three chambers. 
b–f, Distribution of mean velocities of processive (unidirectional, minus-end-directed) events for DDB ($n = 3,343$) and DDR ($n = 3,162$) (b); DDB and DDH ($n = 3,744$) (c); active mutant dynein in complex with dynactin and BICD2 (mtDDB, $n = 905$) or BICDRI (mtDDR, $n = 1,183$) (d); the colocalized mtDDR complexes containing both TMR–dynein tail and Alexa Fluor 647-full-length dynein (tail–dynein, $n = 939$) or Alexa Fluor 647-only complexes containing only full-length dynein (dynein-only, $n = 1,004$) (e); and all DDB complexes and complexes with both fluorophores, and hence two dyneins (colocalizers, $n = 660$) (f). Mean ± s.e.m. values were estimated by fitting the histograms to a Gaussian distribution (dashed lines).
Extended Data Figure 3 | Single-particle cryo-EM analysis of TDR complex at 3.5 Å resolution. a, Micrograph of the TDR complex (representative of 26,906 micrographs). b, Typical 2D-class averages of TDR in different orientations. c, The overall density map of TDR was analysed by ResMap, showing a resolution distribution from 3 to 8 Å. d, The gold-standard FSC curve of the overall TDR map. e, Mesh representation of 3.4 Å resolution density map of α helices from dynein-B1 obtained by focused 3D classification and refinement. f, Sample density obtained by local sub-volume averaging, showing β strands from IC WD40.
Extended Data Figure 4 | Cryo-EM data procedures of TDR. Focused 3D classification and refinement procedures used in this study to improve density maps for dynein tails.
Extended Data Figure 5 | Secondary structure diagram of dynein HC. a, Secondary structure elements of dynein HC are matched against the primary sequence showing the NDD (purple) and the dynein helical bundles (blue; cyan; green; yellow; pale yellow; orange; red; pink). b, Secondary structure elements of IC. Extended N-terminal regions are coloured purple and other elements are coloured according to the blade of the WD40 domain to which they belong, except sheet 35, which associates with 330–32. c, Secondary structure elements of LIC, showing the globular domain helices and sheets (blue) and the two helices that pack against the HC (red). Jpred53 secondary structure predictions of features not seen in the electron microscopy map are shown in grey.
Extended Data Figure 6 | Interactions between dynein subunits. a, The dynein HC (yellow) interacts with the IC WD40 domain (blue) using bundles 4 and 5, with a helical segment (red cartoon) sitting in the WD40 central cavity. Dynein-A2 is shown. Interacting residues are shown as sticks (bottom panel), with HC residues in red and IC residues in green. b, Density map and model showing how the LIC (density and cartoon, blue) N- and C-terminal regions extend from the globular domain and pack against the HC (density, coloured by bundle number). Dynein-A2 is shown. c, ROBL1 (cartoon, light and dark pink) makes contacts with the IC N-terminal helices (cartoon, light and dark blue), which mediate the interaction between ROBL1 and the IC WD40 (surface). d, Representative density from the 1.9 Å resolution NDD crystal structure. e, Cartoon model of the NDD showing one chain in rainbow spectrum.
Extended Data Figure 7 | Dynein–dynein contacts and interactions at the BICDR1 N terminus. a, Conservation diagram showing sequence similarity between A2 and B1 interacting residues. Residues coloured white with red background are completely conserved, whereas residues coloured red show sequence similarity at that position. Residues at each interaction site are numbered below the alignment (A2 residues in yellow circles, B1 residues in red circles). These numbers label the accompanying cartoon to show the dynein chains that constitute each interaction. Alignment generated by ESPript54 (http://espript.ibcp.fr). b, Intermediate chain interactions showing connections between the IC of A1 and the HC of A2; the IC of A2 and the HC of B1; and the IC of B1 and the HC of B2. Interacting sites on each IC are shown as yellow spheres; sites on each HC are shown as red spheres. c, B1 (pink) contacts extra density (labelled, blue) adjacent to the BICDR1 coiled coil. The cartoon below shows the location of the area depicted (correspondingly coloured). d, Weak density connects the extra density with the LIC A2 helix 13 (blue). A cartoon representation of the area depicted is shown below.
Extended Data Figure 8 | Comparison between different adaptors recruiting dynein. a, The TDR structure (left) is compared to models of TDH (middle) and TDB (right). Although the paths of BICDR1 (yellow), HOOK3 (magenta) and BICD2 (orange) vary along the surface of dynactin (green surface), dynein-A HCs (light blue) bind at the same sites in each complex. b, Zoomed-in views of the barbed end of dynactin show that BICD2 adopts an upwards position to contact ARP1A (grey), whereas BICDR1 and HOOK3 adopt lower positions to bind dynein-B using the region coloured in red. The BICD2–ARP1A interaction site is highlighted in purple.
Extended Data Table 1 | Cryo-EM data collection parameters of TDR and TDH structures and model refinement statistics of the 3.5 Å resolution TDR structure

| Data collection and processing | TDR_1 | TDR_2 | TDH |
|-------------------------------|-------|-------|-----|
| Voltage (kV)                  | 300   | 300   | 300 |
| Electron exposure (e-/Å²)     | 52    | 52    | 45  |
| Pixel size (Å)                | 1.34  | 1.34  | 1.42|
| Number of sessions            | 1     | 11    | 5   |
| Micrographs                   | 2,459 | 26,906| 5,464|
| Symmetry imposed              | C1    | C1    | C1  |
| Final particle images (no.)   | 12,420| 205,611| 23,407|
| Map resolution (Å)            | 6.5   | 3.5   | 6.7 |
| FSC threshold                 | 0.143 | 0.143 | 0.143|

| Refinement | N-terminal tail | C-terminal tail | TDR | TDR(ordered) |
|------------|-----------------|-----------------|-----|--------------|
| Map        | EMD-4169        | EMD-4170        | EMD-4168 | EMD-4168 |
| Map resolution (Å) | 3.4 | 3.4 | 8 | 3.5 |
| FSC threshold | 0.143 | 0.143 | 0.143 | 0.143 |
| Map sharpening B factor (Å²) | -50 | -30 | 0 | -70 |
| Map CC (around atoms) | 0.76 | 0.70 | 0.79 | 0.65 |
| Non-hydrogen atoms | 28,871 | 5,164 | 92,789 | 90,725 |
| Protein residues | 3,555 | 628 | 13,982 | 13,567 |
| Ligands (ADP/ATP) | 0/0 | 0/0 | 9/1 | 9/1 |
| R.m.s. deviations | | | | |
| Bond lengths (Å) | 0.02 | 0.01 | 0.01 | 0.01 |
| Bond angles (°) | 1.59 | 1.93 | 1.60 | 1.60 |
| Validation | | | | |
| MolProbity score | 2.12 | 2.21 | 2.05 | 2.05 |
| Clashscore | 11.18 | 13.19 | 9.08 | 9.08 |
| Poor rotamers (%) | 0.29 | 0.36 | 0.44 | 0.44 |
| Ramachandran plot | | | | |
| Favorcd (%) | 89.83 | 88.75 | 89.13 | 89.17 |
| Disallowed (%) | 0.11 | 0.16 | 0.14 | 0.14 |
| Cβ deviations (%) | 0.00 | 0.00 | 0.00 | 0.00 |

The TDR_1 dataset is included in TDR_2 dataset. The N-terminal tail model consists of HC of A2 (residues 201–829), HC of B1 (residues 201–629), HC of B2 (residues 201–575), IC of A2 WD40 domain, BICDR1 (residues 132–210), ARP1B, ARP1D, ARP1F, CAPZα and CAPZβ. The C-terminal tail model consists of HC of A2 (residues 517–927) and HC of B1 (residues 453–702). The TDR (ordered) model consists of all parts of TDR for which density was seen.

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Extended Data Table 2 | Crystal structure data collection parameters and model refinement statistics of the 1.9 Å resolution structure of the human dynein NDD

| Data collection | NDD (PDB 5OWO) |
|-----------------|----------------|
| Space group     | P 2 2 1 2 1     |
| Cell dimensions |                |
| \(a, b, c\) (Å) | 50.5, 101.8, 176.19 |
| \(\alpha, \beta, \gamma\) (°) | 90, 90, 90       |
| Resolution (Å)  | 50.45-1.86 (1.94-1.86) * |
| \(R_{merge}\)   | 0.291(3.939)    |
| \(I/\sigma I\)  | 3.9(0.4)        |
| Completeness (%)| 94.3(98.5)      |
| Redundancy      | 3.1 (2.9)       |

| Refinement      |                |
|-----------------|----------------|
| Resolution (Å)  | 1.80           |
| No. reflections | 48901          |
| \(R_{work} / R_{free}\) | 27.09/29.24   |
| B-factor, from Wilson plot (Å) | 25.7 |
| R.m.s. deviations |          |
| Bond lengths (Å) | 0.02          |
| Bond angles (°)  | 2.15           |

*Values in parentheses are for the highest-resolution shell.
Life Sciences Reporting Summary

Nature Research wishes to improve the reproducibility of the work that we publish. This form is intended for publication with all accepted life science papers and provides structure for consistency and transparency in reporting. Every life science submission will use this form; some list items might not apply to an individual manuscript, but all fields must be completed for clarity.

For further information on the points included in this form, see Reporting Life Sciences Research. For further information on Nature Research policies, including our data availability policy, see Authors & Referees and the Editorial Policy Checklist.

1. Sample size
   Describe how sample size was determined.
   No sample-size calculation or pre-specification of sample size was used in our experimental design. In general, no fewer than fifteen individual molecules were used to make any conclusions. The sample size chosen for the velocity and colocalization data (>900) is larger than examples from previous publications (Schlager et al. 2014, EMBO J, n=331; McKenney et al. 2014, Science, n=>129). The sample size chosen for stall force experiments (>53) is in line with previous stall force studies (Belyy et al. Nature Cell Biol., n=50).

2. Data exclusions
   Describe any data exclusions.
   Processive dynein/dynactin complex movements in TIRF motility assays were defined as established in Schlager et al., 2014. Any beads that did not show clear motility or a clear stall (defined in Methods) are not included in data analysis.

3. Replication
   Describe whether the experimental findings were reliably reproduced.
   TIRF data were reliably reproduced from 6 replicates (3 replicates collected on two separate days). Optical trap data were reliably reproduced from at least 5 independent experiments. Estimation of the number of dyneins bound by negative stain EM were reliably reproduced from two repeats collected on two separate days.

4. Randomization
   Describe how samples/organisms/participants were allocated into experimental groups.
   For calculation of the gold standard FSC, cryo-EM particles were randomly split into two halves using the RELION software.

5. Blinding
   Describe whether the investigators were blinded to group allocation during data collection and/or analysis.
   Investigators analyzing colocalization and speed data were not told which protein sample each dataset belonged to.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.
6. Statistical parameters
For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

| n/a | Confirmed |
|-----|-----------|

- [x] The exact sample size ($n$) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
- [x] A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- [x] A statement indicating how many times each experiment was replicated
- [x] The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
- [x] A description of any assumptions or corrections, such as an adjustment for multiple comparisons
- [x] The test results (e.g. $P$ values) given as exact values whenever possible and with confidence intervals noted
- [x] A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
- [x] Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

Software

Policy information about availability of computer code

7. Software
Describe the software used to analyze the data in this study.

ImageJ (v. 1.51j) and Graphpad PRISM 7 were used to analyze TIRF data. Matlab was used to process and analyze optical trap data. Figures were plotted using Matlab and GraphPad PRISM. The following EM softwares were used: Relion 1.4, Relion 2.0, EMAN2, Motioncorr2, GCTF. Gautomatch, CTFFIND3. Structures were built and refined using Coot, PHENIX, Chimera and Pymol.

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). Nature Methods guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability
Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.

- No unique materials were used in this study.

9. Antibodies
Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).

Carboxyl latex beads (Life Technologies) were coated with custom-made rabbit polyclonal anti-GFP antibodies (Covance Inc). Antibodies were purified through GFP affinity chromatography and validated by ELISA.

10. Eukaryotic cell lines
a. State the source of each eukaryotic cell line used.

Sf9 cells were used only for protein production, originally purchased from ThermoFisher Scientific (cat no. 11496015).

b. Describe the method of cell line authentication used.

No cell line authentication was used.

c. Report whether the cell lines were tested for mycoplasma contamination.

Cells were not tested for mycoplasma contamination.

d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.

No commonly misidentified cell lines were used.
### Animals and human research participants

Policy information about *studies involving animals*; when reporting animal research, follow the [ARRIVE guidelines](#).

**11. Description of research animals**

Provide details on animals and/or animal-derived materials used in the study.

| No animals were used in this study. |

Policy information about *studies involving human research participants*

**12. Description of human research participants**

Describe the covariate-relevant population characteristics of the human research participants.

| No human research participants were involved in this study. |