The mammalian dynein–dynactin complex is a strong opponent to kinesin in a tug-of-war competition

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Kinesin and dynein motors transport intracellular cargos bidirectionally by pulling them in opposite directions along microtubules, through a process frequently described as a ‘tug of war’¹. While kinesin produces 6 pN of force, mammalian dynein was found to be a surprisingly weak motor (0.5–1.5 pN) in vitro, suggesting that many dyneins are required to counteract the pull of a single kinesin². Mammalian dynein’s association with dynactin and Bicaudal-D2 (BICD2) activates its processive motility³–⁶, but it was unknown how this affects dynein’s force output. Here, we show that formation of the dynein–dynactin–BICD2 (DBB) complex increases human dynein’s force production to 4.3 pN. An in vitro tug-of-war assay revealed that a single DBB successfully resists a single kinesin. Contrary to previous reports, the clustering of many dyneins is not required to win the tug of war. Our work reveals the key role of dynactin and a cargo adaptor protein in shifting the balance of forces between dynein and kinesin motors during intracellular transport.

Cytoplasmic dynein (‘dynein’ hereafter) is a minus-end-directed microtubule motor responsible for cargo trafficking, organelle positioning, and organization of the mitotic spindle in eukaryotic cells⁷. The core of the dynein complex comprises a homodimer of two massive heavy chains, each containing a AAA+ motor ring, a microtubule-binding domain separated from the ring by a ~15 nm coiled-coil stalk, and a flexible cargo-binding tail that also serves as the dimerization domain and the binding site for dynein light intermediate chains⁸,⁹. Previous studies on mammalian dynein revealed a striking mismatch between the motility of individual motors in vitro and their apparent in vivo functions. Despite high retrograde transport velocities observed in live cells¹⁰, single purified mammalian dyneins exhibited diffusive motility or short processive runs in vitro⁹,¹¹,¹² and were found to stall at forces of 0.5–1.5 pN (refs 9,13–17), significantly weaker than the 6 pN force production of plus-end-directed kinesin-1 motors¹⁸. It remained unclear how dynein generates the large forces required for its cellular roles. It has been proposed that multiple (4–7) dynein motors need to be engaged in transport per kinesin-1 to balance forces during tug of war², and that the spatial organization of dyneins on the surface of the cargo serves a fundamental regulatory role¹⁹. However, due to dynein’s large size, it may not be sterically feasible for multiple dynein motors to interact with a microtubule when transporting small cargos. Furthermore, measured dynein to kinesin ratios (~1:5:1) on mouse axonal membranous vesicles²⁰ are inconsistent with the ~5:1 coupling predicted by force-based models.

Recent studies with recombinant human dynein have begun to explore the mechanism of its motility. Both velocity and processivity of dynein are dramatically increased by the addition of dynactin, a multi-protein complex that associates with dynein in vivo, together with the amino terminus of the cargo activator Bicaudal-D2 (BICD2N), which increases the affinity of dynactin for dynein³–⁶ (Fig. 1a–d). In single-molecule motility experiments on sea urchin axonemes, we observed that human dynein is poorly recruited to axonemes (0.19 fluorescent spots μm⁻¹ min⁻¹ at 1 nM motor) and only 16% of dynein spots exhibited slow (79 ± 11 nm s⁻¹, mean ± s.e.m.), processive motility (Supplementary Video 1 and Supplementary Fig. 1a). The addition of dynactin and SNAPf-tagged BICD2N (hereafter BICD2N) at a 1:5:2 dynein/dynactin/BICD2N molar ratio increased the recruitment of dynein to 0.66 spots μm⁻¹ min⁻¹ at 1 nM motor, with a substantially higher percentage (52%) of processively moving spots (Supplementary Video 2 and Supplementary Fig. 1b), resulting in a tenfold increase in the number of walking molecules. These results are consistent with the enhancement of dynein recruitment and processivity in the presence of dynactin and BICD2N.

The autoinhibition of dynein may be mediated by a large-scale rearrangement of the tail domain, as is the case for some members of myosin²¹ and kinesin²² families, or by the back-to-back stacking of the motor domains⁶. The tail-inactivation hypothesis, wherein the

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Figure 1 Activation of human dynein motility by dynactin, BICD2N and artificial cargos. (a) Schematic depiction of the DDB complex on a microtubule. The putative orientation of the p150 subunit of dynactin is shown with a semi-transparent outline and omitted from illustrations elsewhere in the manuscript. (b) Sample kymographs representing the motility of individual dyneins alone and DDB complexes on axonemes. (c) Denaturing SDS–PAGE gel of purified dynein fractions. Bands corresponding to all dynein subunits can be observed. (d) Denaturing SDS–PAGE gel of purified BICD2N. Unprocessed original scans of gels corresponding to all dynein subunits can be observed. (e) Illustration of dynein with a quantum dot (QD) attached to the tail (top) and sample kymograph (bottom). The experiment was repeated four times. (f) Illustration of dynein with a 200 nm bead attached to the tail (top) and sample kymograph (bottom). The experiment was repeated three times. (g) Illustration of dynein with an optically trapped 860 nm bead attached to the tail (top) and sample trajectory of dynein pulling the bead against a constant 0.4 pN hindering force in force-feedback mode (bottom). The experiment was repeated four times. (h) Effect of cargo size and type on dynein velocity in comparison with the DDB complex. MT, microtubule. n = 51, 83, 21, 19, 15 runs from 3 independent experiments in order from top to bottom. Vertical bars represent median values and quartiles. Median values, from top to bottom, are 373, 188, 213, 29 and 56 nm s⁻¹. Mean ± s.e.m., from top to bottom, are 513 ± 58, 257 ± 26, 200 ± 23, 49 ± 11, and 79 ± 11 nm s⁻¹.

tail folds over onto the motor domains and inhibits their activity, has previously been rejected because attaching quantum dots (QDs) to human dynein's tail did not enhance its motility. However, QDs are similar in size (~20 nm in diameter) to dynein itself, and may be too small to release inhibition. To test this possibility, we attached a range of artificial cargos to dynein's tail and tested its motility in vitro. In agreement with the findings of ref. 9, binding a QD to dynein's tail did not increase dynein's velocity as the QD-labelled dynein walked at 49 ± 11 nm s⁻¹ and the majority of QDs did not exhibit any processive motility along axonemes (n = 19 walking QDs out of 319 observed, Fig. 1e). However, attachment of larger cargos to dynein's tail resulted in fast processive runs. Single dynein motors carried 200-nm-diameter beads at 200 ± 23 nm s⁻¹ in unloaded conditions (Fig. 1f and Supplementary Video 3) and 860-nm-diameter beads at 257 ± 26 nm s⁻¹ under 0.4 pN constant hindering force (Fig. 1g), which was applied to allow for reliable detection of dynein unbinding from the microtubule.

This ~4-fold increase in dynein's velocity indicates that release of dynein from the autoinhibited state can be partially stimulated by attachment of a large cargo to the tail domain, consistent with reports of bead motility driven by nonspecifically adsorbed mammalian dyneins. Importantly, the DDB complex moves ~2-fold faster than dyneins carrying a large bead (Fig. 1h). Therefore, dynactin and BICD2 binding probably leads to additional conformational changes of the heavy chains, such as alignment of the motor domains and reorientation of the carboxy terminus.

To test whether formation of the DDB complex enhances dynein's work output, we measured the force generation of dynein, dynein–dynactin and the DDB complex using an optical trap. We sparsely attached recombinant human dynein motors containing an N-terminal GFP to micrometre-sized polystyrene beads coated with anti-GFP antibody (Fig. 2a). With the trap held in a fixed position, the minus-end-directed motility of beads driven by individual dynein
Figure 2 Effect of dynactin and BICD2N on dynein force production. (a) Top: the optically trapped bead, represented with a force arrow, is attached to GFP on dynein’s tail via an anti-GFP antibody. Middle: trace showing a typical stall of a bead driven by GFP–dynein in a fixed-trap assay. The red arrowhead represents the detachment of the motor from a microtubule after the stall. Bottom: the histogram of observed stalls reveals the mean stall force (mean ± s.e.m., n = 50 stalls from 12 beads in 4 independent experiments). (b) Stall force of GFP–dynein with the addition of 5× molar excess of dynactin (n = 41 stalls from 10 beads in 4 independent experiments). (c) Stall force measurement of GFP–dynein with the addition of 5× molar excess of dynactin and 2× molar excess of BICD2N. The underlying distribution of the observed stalls is fitted to two Gaussians (blue curve; n = 195 stalls from 47 beads in 19 independent experiments) using a Gaussian mixture model. A three-Gaussian fit, shown with a dashed red line, is not statistically warranted as determined by the Bayes information criterion (see Supplementary Fig. 2). (d) Stall force measurement of dynein with the addition of 5× molar excess of dynactin and 2× molar excess of BICD2N with a C-terminal GFP (BICD2N–GFP). The BICD2N–GFP is the attachment point (n = 45 stalls from 14 beads in 4 independent experiments). (e) Stall force measurement of kinesin-1 with a C-terminal GFP fusion as the attachment point (n = 37 stalls from 15 beads in 4 independent experiments).

Motors stalled at 2.04 ± 0.02 pN (mean ± s.e.m.) resistive forces, slightly higher than previously reported stall forces of human dynein. Addition of dynactin to human dynein at a fivefold molar excess caused only a modest (P = 0.0025, Welch’s t-test) increase in stall force to 2.48 ± 0.06 pN (Fig. 2b), consistent with previous findings that dynactin alone has little effect on dynein motility.

We next assembled the DDB complex at a 1:5:2 molar ratio of dynein/dynactin/BICD2N. Single-molecule motility assays showed robust processive motility at this ratio (Fig. 1b). With all three components present, stall forces exhibited a bimodal distribution with a lower peak at 2.1 ± 0.3 pN, comprising 34% of all motors, and a higher peak at 4.4 ± 0.5 pN, comprising 70% of the 195 observed motors (Fig. 2c and Supplementary Fig. 2). A third peak is not statistically warranted as determined by the Bayes information criterion (see Supplementary Fig. 2). It is likely that the lower peak at 2.1 pN corresponds to incomplete complex formation because in single-molecule motility assays, only 52% of GFP-tagged dynein motors move processively following addition of dynactin and BICD2N. The stall forces near 4.3 pN appear only when all three components are present, suggesting that they correspond to the force output of DDB. To confirm this, we measured the stall force of DDB by attaching the bead directly to the N-terminal 400 residues of BICD2 via a C-terminal GFP tag (BICD2N–GFP), ensuring that any observed motility is driven by DDB rather than dynein alone. The beads stalled at 4.3 ± 0.2 pN (Fig. 2d), similar to the 4.3 pN peak observed in Fig. 2c. A peak in stall events near 2.1 pN was not observed. Thus, we concluded that DDB complexes produce forces above 4 pN. To make a direct comparison with yeast cytoplasmic dynein, whose motility mechanism has been studied in detail, we independently measured the stall force of full-length yeast dynein to be 3.6 ± 0.2 pN (Supplementary Fig. 3). Therefore, force production of mammalian dynein is similar to yeast cytoplasmic dynein28 and less than human kinesin-129 (5.8 ± 0.1 pN; Fig. 2e).

We sought to rule out the possibility that multiple dyneins aggregate in fluorescence or trapping assays. First, we counted the number of photobleaching steps of microtubule-bound dynein dimers tagged with a single GFP on each monomer. Ninety-five per cent of the GFP spots bleached in either one or two steps in the presence and absence of dynactin and BICD2N (Fig. 3a–c), suggesting that they correspond to a single dynein dimer. In the trapping experiments, to prevent the possibility of dynactin- and BICD2N-induced aggregation of dynein on the surface of beads, we pre-bound dynein sparsely to beads, removed the free motors, and then added dynactin and BICD2N (Fig. 3d). Under these conditions, dynein still produced high stall forces, 4.0 ± 0.2 pN, similar to the case in which all three components were mixed before bead binding (Fig. 3e). Therefore, the measured increase in stall force of DDB is not an artefact of aggregation, but rather caused by the binding of dynactin and BICD2N to individual dynein motors. Finally, to ensure that trapped beads are driven by a
Figure 3  Processive motility of DDB complexes is driven by single dynein dimers. (a) GFP–dynein molecules tightly bound to microtubules in the absence of ATP. (b) Intensity traces of GFP–dynein alone in the presence and absence of dynactin and BICD2N show one- and two-step photobleaching. (c) Histograms showing the number of photobleaching steps of dynein in the absence and presence of dynactin and BICD2N. \( n = 127 \) for dynein alone and \( n = 192 \) for DDB. The experiment was repeated three times with dynein from two independent preparations. (d) Schematic depiction of the modified sample preparation for optical trapping. Dynein is mixed with beads and excess dynein is removed by centrifugation. BICD2N and dynactin are added after the removal of free motors to rule out dynactin- and BICD2N-dependent aggregation of dynein motors on beads. (e) Representative stall trace of a DDB complex and distribution of stall forces of the DDB complexes (mean ± s.e.m.). The experiment was repeated three times. (f) Fraction of dynein-coated beads moving as a function of dynein concentration. Values are represented as the mean ± the square root of \( F(1 - F)/n \), with \( n \) being the number of beads tested. The solid red line represents a fit to the Poisson probability \( 1 - e^{-Cn} \) that the bead is carried by one or more motors, where \( C \) is dynein concentration and \( i \) is the fit parameter (reduced \( \chi^2 = 0.26 \)). The dashed blue line represents a fit to the probability \( 1 - e^{-Cn} - (JC)e^{-JC} \) that the bead is carried by two or more motors (reduced \( \chi^2 = 4.62 \)). For each data point, from left to right, \( n = 23, 25, 10, 482, 28, 35, 20 \) and 24 beads from 3 independent experiments, except for the 20 pM data point, which was obtained from 19 independent experiments. The mean values, from left to right, are 0.087, 0.16, 0.2, 0.22, 0.22, 0.33, 0.61 and 0.88.

All trapping experiments in this work were performed at 20 pM dynein at which >97% of all beads were driven by single motors.

To investigate the effect of dynein activation by its adaptor proteins on the motor’s ability to transport cargo in competition with motors single motor, we quantified the fraction of motile beads as a function of dynein concentration (Fig. 3f). The data fitted well to the model that each bead is carried by one or more motors and did not fit well to the model that a minimum of two motors is required to carry a bead.
of opposing polarity, we established an in vitro tug-of-war assay, allowing us to pit one dynein against one human kinesin-1. A dynein construct with an N-terminal SNAPf tag was first labelled sub-stoichiometrically with a 74-nucleotide-long single-stranded DNA, and then with an excess of Alexa647 at its tail. This labelling strategy ensured that most dynein dimers were labelled with either one DNA molecule and one Alexa647 fluorophore, or two Alexa647 fluorophores. A similar strategy was used to label kinesin with a complementary DNA and a tetramethylrhodamine (TMR) at its tail using HaloTag (see Methods and Fig. 4a–c). The labelled motors were linked to each other through DNA hybridization, and assayed for motility on microtubules polymerized from pig brain tubulin.

Simultaneous imaging of TMR–kinesin and Alexa647–dynein on microtubules revealed co-localization and correlated movement of dynein–kinesin complexes. By relying on sub-stoichiometric DNA labelling, we ensured that any observed co-localizers were comprised of one dynein and one kinesin motor. In the absence of dynactin and BICD2N, the velocities of co-localizers were nearly the same as those of kinesins alone (Fig. 4d,e and Supplementary Video 4), revealing that dynein on its own is unable to resist kinesin’s pull.
However, the behaviour of the co-localizers was markedly different following the addition of dynactin and BICD2N. The median velocity of DDB–kinesin co-localizers (26 nm s\(^{-1}\)) was reduced over 20-fold compared with that of dynein–kinesin (464 nm s\(^{-1}\)) towards the microtubule plus end. Furthermore, 22% of DDB–kinesin co-localizers walked towards the microtubule minus end (Fig. 4g and Supplementary Fig. 4 and Supplementary Video 5), which was not observed in the absence of dynactin and BICD2N. The velocity distribution of the co-localizers is distinct from those of both DDB alone and kinesin alone, indicating that both motors are contributing to the overall motility rather than one being passively carried by the winning motor. Interestingly, we did not observe frequent reversals of a DDB–kinesin co-localizer’s motility. This agrees with the absence of reversals in the artificial linking of multiple yeast dyneins to multiple human kinesins\(^{31}\). Reversals of cargo motility observed \textit{in vivo}\(^{1}\) may result from the regulatory factors on the cargo that modulate motor activity, such as JIP1\(^{13}\), or transient association of key regulatory proteins such as dynactin and BICD2 in cells\(^{1}\).

Next, we tested whether the dramatic slowing down and reversal of DDB motility can be explained by a purely mechanical response to kinesin’s pulling force towards the plus end. Operating an optical trap in a force-feedback mode, we pulled single DDB complexes towards the plus end with a force of 6 pN, corresponding to the reported stall force of kinesin-1 (Fig. 2e)\(^{18}\). Under this condition, the median velocity of DDB was 10 nm s\(^{-1}\) towards the plus end, with 29% of the motors walking towards the minus end (Fig. 4g,h). These values are remarkably similar to the velocities of DDB–kinesin co-localizers, which is consistent with the predictions of the tug-of-war model.

Our results demonstrated that mammalian dynein complexes are strong motors capable of transporting cargos towards the microtubule minus end against large resistive forces. Contrary to the earlier suggestion that 4–7 dyneins are needed to counteract the force production of a single kinesin-1\(^{34}\), the activation of human dynein by dynactin and BICD2N allows it to dramatically slow down and sometimes defeat kinesin-1 in a one-to-one mechanical competition. A large force output of DDB is consistent with high minus-end-directed forces exerted on lipid droplets in Drosophila embryos\(^{32}\) and single phagosomes inside mouse macrophage cells\(^{33}\). Furthermore, formation of strong DDB complexes bypasses the previously postulated requirement for a highly ordered spatial organization of dyneins on the surface of cellular cargos. It remains to be seen how diverse cargo adaptor proteins other than BICD2 affect the force production of the dynein–dynactin complex and regulate dynein’s activity throughout the cell.

Previous \textit{in vivo} optical trapping recordings have detected periodic peaks spaced at 1–2 pN intervals in the force distributions of dynein-driven cargos. It has been proposed that the peak periodicity represents the force production of single\(^{35}\) or a pair\(^{34}\) of dynein motors, and that the larger collective forces originate from multiple (up to 12) dyneins simultaneously engaging with the microtubule to transport the cargo. This model relies on the assumption that the measured peak forces in the retrograde direction represent the maximal force production of a single motor multiplied by the motor copy number\(^{36}\). Our results are not fully consistent with this simple mechanical model, because the stall force of single DDB complexes (4.3 pN) is significantly higher than the peak periodicity of these force distributions. We propose that the wide range of cargo stall forces measured during multiple-motor transport \textit{in vivo} can be affected by the modulation of kinesin and dynein activity by cargo adaptor proteins\(^{37}\), premature release of the motors from the microtubule before they come to a complete stall, and nonlinear mechanical coupling between multiple motors engaged with the cargo transport\(^{38}\). These possibilities remain to be rigorously tested by characterizing the force production of cargos containing predetermined numbers of motors and their regulators \textit{in vitro}, such as the artificial cargos employed in this study.

\textbf{METHODS}

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of this paper.

\textbf{Note:} Supplementary Information is available in the online version of this paper.

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\textbf{AUTHOR CONTRIBUTIONS} V.B., A.P.C. and A.Y. designed the study. M.A.S. and H.F. prepared the constructs and purified the protein. V.B. and A.E.R. performed the optical trapping experiments. V.B. labelled dynein and kinesin with DNA, performed the fluorescence motility experiments, and analysed the data. V.B., A.P.C. and A.Y. wrote the manuscript.

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METHODS

Cloning and plasmid construction. Genes for DHC (DYNCH1H1, accession number NM_001376.4), DIC (DYNCH12, IC2C, AF344477), DLIC (DYNCH12, LIC2, NM_006142.2), Tctex (DYNLT1, Tctex1, NM_006591.2), LC8 (DYNLL1, LC8-1, NM_006142.2) and Robl (DYNNLRB1, Robl1, NM_014183.3) were codon-optimized (Epoch Life Sciences) and cloned into the vector pET28a (Novagen, Madison, WI). The genes were inserted into the vector with an N-terminal His-ZZ-LTLT tag followed by the N-terminal 400 amino acids of a codon-optimized mouse DICD2 via microtubule bind and release (MTBR).

Protein expression and purification. Human dynein and BICD2N expression and purification using the baculovirus insect cell system was performed as described previously. Briefly, constructs for either the full dynein complex or BICD2N were integrated into the EMBac or EMBacY baculovirus genome and p2 baculovirus was used to infect insect cells. For protein expression, a 500 ml 500 ml S9 cell suspension was infected with 5 ml p2 baculovirus and incubated at 27 °C while shaking at 124 r.p.m. for 70-75 h. A 2.5 g cell pellet was lysed using a tissue homogenizer (Wheaton), cleared by centrifugation and incubated with 2 ml IgG Sepharose 6 Fast Flow (GE Healthcare). The protein was removed from the beads with TEV protease and purified by size-exclusion chromatography using either a G4000SW column (TOSOH Bioscience) or a Superose 6 column (GE Healthcare) on an Etan LC system (GE Healthcare). The appropriate fractions were concentrated, snap frozen in liquid nitrogen and stored at −80 °C. Dynactin was purified from pig brain using the large-scale SP-Sepharose purification protocol. Protein size and purity were confirmed by SDS–PAGE using Novex 4–12% Bis–Tris precast gels using either MOPS or MES buffer (Life Technologies) and stained with Instant Blue (Supplementary Fig. 5). Protein concentration was measured using the Quick Start Bradford kit (Bio-Rad).

Full-length yeast cytoplasmic dynein tagged with GFP at the N-terminal tail and with DHA (Promega) at the C terminus (GFP–Dynactin–DHA) was expressed and purified as described previously. Briefly, frozen yeast pellets were ground in a commercial steel coffee grinder and the resulting powder was thawed in lysis buffer (150 mM HEPES, 250 mM K acetate, 10 mM Mg acetate, 5 mM EGTA, 5 mM phenylmethanesulfonyl fluoride, 500 mM MgATP, 50% glycero, pH 7.4). Following centrifugation at 270,000 g for 45 min, the supernatant was incubated with IgG Sepharose beads (GE Healthcare Life Sciences, 17-0969-01) at 4 °C for 1 h. The beads were washed twice and transferred into TEV protease buffer (150 mM KCl, 10 mM Tris-HCl, 1% glycero, 1 mM TCEP, 1 mM phenylmethanesulfonyl fluoride, 100 mM ATP, pH 8.0). Dynactin was cleaved from the beads by incubation with TEV protease at 16 °C for 1 h.

Axonemes were extracted from live sea urchins. Tubulin used for the assembly of microtubules from fresh pig brains through two polymerization–depolymerization cycles in a high-molarity buffer. To assemble microtubules for motility experiments, tubulin was polymerized in the presence of 20 µM taxol in BRB80 at 37 °C for 30 min and remaining free tubulin dimers were removed by centrifugation.

Functionalization of complementary DNA oligonucleotides. Two complementary amine-modified 74 bp oligonucleotides (IDT) with the sequences /5AmMC12/5 and the DHC gene. The reaction was allowed to proceed for 30 min at room temperature, after which the DNA was desalted and exchanged into dynein motility buffer (DMB: 30 mM HEPES, 5 mM MgSO4, 1 mM EDTA, pH 7.0 with KOH). Antibodies were reduced with 2 mM DTT for 30 min and residual DTT was removed by three runs through 7,000 MWCO spin de-salting columns. Activated QD585s were then incubated with a fourfold molar excess of the reduced antibodies for 1 h. The reaction was quenched with the addition of 20 mM Tris pH 8.0 and spin-concentrated to obtain the desired final concentration.

Optical trap assay. Dynactin concentration was determined for each batch of protein. The protein was diluted until less than 30% of beads exhibited any activity when brought in contact with an axoneme, ensuring that >95% of observed events could be attributed to the actions of single motors. When BICD2N and/or dynactin were added to the assay, they were mixed with dynein at 1:52 molar ratio (dynein/dynactin/BICD2N) and incubated at 4 °C for 5 min before adding the composite to beads. Dynactin with any auxiliary proteins was then allowed to bind to 800-nm diameter latex beads for 10 min at 4 °C before proceeding with sample preparation. The sample chamber was loaded by first flowing Cy5-labeled axonemes in dynein motility buffer (DMB: 30 mM HEPES, 5 mM MgSO4, 1 mM EDTA, pH 7.0 with KOH), followed by a solution of dynein- or DDB-coated beads in motility/imaging buffer (DMB supplemented with 35 µg/ml PCD, 2.5 mM PCA, 10 mM DTT, 1 mM casein and 2 mM ATP). The samples were imaged using a custom-built optical trap microscope as described previously. Cy5-labeled axonemes were brought to the centre of the view of a piezo-driven servo XY stage (M-687, Physik Instrumente). Beads were trapped with a focused 1.064 nm beam using a 100x 1.49

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N.A. apochromat oil-immersion objective (Nikon). The trap was steered with a pair of perpendicular acousto-optical deflectors (AA Opto-Electronic) and lowered to the surface of the axoneme by moving the trapping objective with a piezo flexure objective scanner (P-721 PIFOC, Physik Instrumente). The position of the bead relative to the centre of the trap was monitored by imaging the back-focal plane of a 1.4 N.A. oil-immersion condenser (Nikon) onto a position-sensitive detector (First Sensor). Signals were acquired at either 20 kHz or 5 kHz, and position feedback was performed at up to 200 Hz. Detector response was calibrated by rapidly raster-scanning the laser across a trapped bead and trap stiffness was obtained from the Lorentzian fit to the power spectrum of a trapped bead. Typical stiffness values used in these assays ranged from 0.008 pN nm\(^{-1}\) to 0.06 pN nm\(^{-1}\). Trap stiffness is adjusted to allow motors to travel 100 nm on average before stalling.

Motility and photobleaching assays. Single-molecule motility assays were carried out on a custom-built objective-type TIRF set-up, built around the body of a commercial Nikon Ti-E microscope. Fluorophores were visualized with a 100 × 1.49 N.A. apochromat oil-immersion objective (Nikon) and imaged onto an electron-multiplying CCD (charge-coupled device) camera (Andor). Assay preparation was identical to the optical trap sample preparation described in a previous section, except without the addition of latex beads. In the dynein–kinesin crosslinking motility experiments, dynein–DNA and kinesin–DNA were allowed to react with each other for 10 min at 4°C before being diluted to the final desired concentration. Photobleaching experiments were performed without the addition of ATP to ensure that motors remain stationary on axonemes. More than 95% of spots bleached completely over the course of a 200-frame video.

For dynein-driven bead motility experiments, 200 nm latex beads coated with GFP antibodies were sparsely decorated with dynein (with or without dynactin and BICD2N) and imaged with either bright-field illumination or scattered-light fluorescence. To ensure single-molecule conditions, the concentration of dynein was reduced until >90% of beads did not visibly interact with axonemes following contact. The trap was used to measure the velocity of individual dynein motors carrying 860-nm-diameter beads, because these beads are too large to encounter microtubules with sufficient frequency by diffusion alone.

The dynein–kinesin co-localization motility experiments were performed on microtubules. We observed that using axonemes versus microtubules had a modest effect on dynein recruitment to the tracks and initiation of motility, but not on its velocity or processivity following recruitment. Microtubules were used for the co-localization experiments mainly to obtain longer tracks for processive motility. For surface immobilization of microtubules, 1% of biotinylated tubulin was incorporated into the microtubule polymerization reaction. Biotinylated microtubules were attached to the coverslips pre-coated with streptavidin and BSA-biotin. Following microtubule attachment, coverslips were passivated for 5 min by incubation in DMB supplemented with 1 mg ml\(^{-1}\) casein.

Data analysis. To extract run velocities from fluorescence videos, kymographs were created along each individual axoneme using ImageJ. Motile dynein motors were then identified manually from the kymographs. Only molecules that travelled >530 nm (5 pixels along x) and remained bound for more than 2.5 s (5 pixels along y) were included in the analysis. Diffusive molecules (those that exhibited >530 nm excursions in both directions along the axoneme) were excluded. For molecules that transitioned from static behaviour (>30 s with <100 nm displacement) to motile behaviour, only the motile segments were analysed.

To generate dynein stall force histograms, position data from trap recordings were downsampled to 250 Hz or 500 Hz for ease of visualization and stall events were manually selected. To qualify as a stall, the position trace had to reach a plateau and remain stationary (with mean deviations of less than ±10 nm) for at least 100 ms before terminating in a ‘rip’. A ‘rip’, indicating that the motor fully released from the microtubule, had to constitute a rapid (<2 ms) jump towards the trap centre of at least 50 nm, larger than the maximum step a dynein molecule can be expected to take. Image analysis was performed in ImageJ.

Statistics and reproducibility. At least three independent repetitions were performed to obtain every published result, and the exact number of repetitions is reported for each experiment. Each statistical analysis method is explicitly stated in the main text and/or figure legend.

Data availability. All data that support the conclusions are available from the authors on request.

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Supplementary Figure 1. Temporally color-coded projections of free dyneins and DDB complexes on microtubules. (a) (Top) Interactions of single free dyneins with microtubules are visualized by color-coding a 2D projection of Supplementary Video 1. Scale bar: 5 µm. (Bottom) Fluorescence kymograph along one of the three visible axonemes in Supplementary Video 1. Motile dyneins walked at 79 ± 11 nm s⁻¹ (s.e.m., n = 15). At 1 nM motor, the density of fluorescent spots on the axoneme was 0.19 per micron length of axoneme per minute, n = 93. (b) (Top) Processive motility of DDB complexes on microtubules is visualized by color-coding a 2D projection of Supplementary Video 2. (Bottom) Fluorescence kymograph along the axoneme in Supplementary Video 3. At 1 nM motor, the density of fluorescent spots on the axoneme was 0.66 per micron length of axoneme per minute, n = 98.
**Supplementary Figure 2** Individual steps in trapping traces and Gaussian mixture model fitting of stall distributions. The kinesin (a) and dynein (b) stall traces are zoomed-in looks on the traces shown in Fig. 2e and Fig. 2c, respectively. Red arrowheads represent the detachment of the motor from the microtubule after the stall. (c,d) Comparison of 2-component and 3-component Gaussian mixture model fits to the DDB stall force distribution shown in Fig. 2e. The fitting is carried out on the underlying distribution and is thus independent of the binning of the histogram. Blue curves are the scaled results of 2-component (c) and 3-component (d) Gaussian fits. Numbers represent center values ± standard errors of each peak. The Bayes Information Criterion (BIC) is lower for the 2-Gaussian fit, indicating that adding a third Gaussian peak is not statistically warranted.
Supplementary Figure 3  Stall force measurements of full-length yeast cytoplasmic dynein. (a) Full-length yeast cytoplasmic dynein with an N-terminal GFP (GFP-Dyn471kD-DHA) is attached to an optically trapped bead via anti-GFP antibody. (b) Trace showing a typical stall of a bead driven by full-length yeast dynein in a fixed trap assay. Red arrowhead represents the detachment of the motor from the microtubule after the stall. (c) The histogram of observed stalls reveals the mean stall force (mean ± s.e.m., n = 21 stalls from 5 beads).
Supplementary Figure 4  Examples of dynein-kinesin colocalizers in the presence of dynactin and BICD2N. Dynein is tagged with Alexa647 (red) and kinesin is tagged with TMR (cyan). The red and cyan channels are laterally offset by five pixels to enhance the visibility of the colocalizers. Individual channels are shown in grayscale. All microtubules are arranged with the plus ends facing towards the right. Scale bars are identical in all images.
Supplementary Figure 5 Full images of all gels shown in main figures. (a) Denaturing SDS–PAGE gel of purified dynein fractions, stained with Instant Blue. Bands corresponding to all dynein subunits can be observed. (b) Denaturing SDS–PAGE gel of purified BICD2N, stained with Instant Blue. (c) Denaturing SDS-PAGE gel of kinesin labeled with DNA. Excess DNA is removed from labeled kinesin by microtubule bind and release (MTBR). The MTBR fractions shown in lanes 10 through 14 are Pre (kinesin pre-mixed with microtubules), SN1 (supernatant from the first spin), P1 (pellet from the first spin), SN2 (supernatant from the second spin), and P2 (pellet from the second spin). (d–e) Denaturing SDS-PAGE gel of dynein labeled with DNA and Alexa647. Dynein was first labeled with ssDNA-BG-GLA-NHS at the indicated molar ratios, then labeled with an excess of Alexa647-BG-GLA-NHS. (d) Silver stain reveals the total amount of dynein, regardless of what fraction of it is labeled with ssDNA. (e) When imaged specifically for Alexa647 fluorescence, the intensities of individual bands on the same gel reveal the fraction of dyneins whose SNAPf sites are occupied by ssDNA and therefore not accessible to labeling by Alexa647-BG-GLA-NHS.
Supplementary Video Legends

**Supplementary Video 1 Human dynein exhibits minimal motility in the absence of tail-binding proteins.** GFP-labeled recombinant dynein motors interacting with surface-immobilized axonemes in the presence of 2 mM ATP. Scale bar: 5µm.

**Supplementary Video 2 Addition of dynactin and BICD2N to dynein leads to fast processive movement.** GFP-labeled dynein motors walking on surface-immobilized axonemes in the presence of a 5x molar excess of dynactin, 2x molar excess of BICD2N, and 2 mM ATP. Scale bar: 5µm.

**Supplementary Video 3 Fast motility is activated by the binding of large cargos to dynein’s tail.** Bright-field recording of dynein-driven motility of a 200 nm polystyrene bead on a surface-immobilized axoneme in the presence of 2 mM ATP. The position of the motile bead is marked with a yellow arrow for clarity. Scale bar: 5µm.

**Supplementary Video 4 Motility of a dynein-kinesin colocalizer in the absence of dynactin and BICD2N.** Two-color TIRF imaging of kinesin-TMR (shown in cyan) and dynein-Alexa647 (shown in red) on a microtubule. Colocalizers are marked with white arrows.

**Supplementary Video 5 Motility of a dynein-kinesin colocalizer in the presence of dynactin and BICD2N.** Two-color TIRF imaging of kinesin-TMR (shown in cyan) and dynein-Alexa647 (shown in red) on a microtubule. The colocalizer is marked with white arrows.