

## METHODS

No statistical methods were used to predetermine sample size. The experiments were not randomized and investigators were not blinded to allocation during experiments and outcome assessment.

**Construct design, protein expression and labelling.** An N-terminally truncated *S. cerevisiae* cytoplasmic dynein gene (*DYN1*) encoding amino acids 1219–4093 (predicted molecular mass 331 kDa; referred to as Dyn) was used as a template for mutagenesis. Constructs were prepared by gene synthesis of the stalk region. DNA fragments were inserted into the genome of haploid yeast cells by homologous recombination to replace a URA3 cassette (Extended Data Fig. 2). *S. cerevisiae* strains were received from R. Vale (UCSF) and were not authenticated or tested for mycoplasma contamination. A ZZ affinity tag and a TEV protease cleavage site were inserted into the N terminus for purification and a DHA tag was inserted at the N- or C termini for labelling<sup>4</sup> (Extended Data Table 1b). The constructs were purified by binding the cell lysate to IgG beads and cleaving the protein from the beads with Tev protease<sup>4</sup>. Motors were labelled with 10  $\mu$ M fluorescent dyes functionalized with alkyl chloride when bound to IgG beads, and the excess dye was removed before Tev cleavage.

**Electron microscopy sample preparation.** Lyophilized porcine brain tubulin (cytoskeleton) was resuspended to 10 mg ml<sup>-1</sup> in MES-MT buffer (30 mM MES pH 6.5, 70 mM NaCl, 1 mM MgCl<sub>2</sub>, 1 mM DTT) and aliquoted. For polymerization, aliquots were diluted twofold in MES-MT buffer supplemented with 6 mM GTP (Sigma), followed by incubation at 37°C for 90 min. A further twofold dilution in MES-MT buffer supplemented with 20  $\mu$ M taxol was made, and the microtubules were left at room temperature overnight. Monomeric Dyn or Dyn<sub>RK+7hep</sub> was diluted fivefold into cold BRB10 (10 mM PIPES pH7.0, 1 mM EGTA, 1 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1% Tween-20), and concentrated to the original volume in an Amicon 100 MWCO 0.5-ml centrifugal concentrator. Complete buffer exchange was achieved through two further cycles of dilution and concentration, resulting in a total dilution factor of 125. Microtubules were pelleted at 20,000 r.c.f. for 10 min and resuspended in room temperature BRB10. Three minutes before grid freezing, a mixture containing 1  $\mu$ M microtubule and 150 nM dynein was made up in room temperature BRB10. Then, 4  $\mu$ l of this sample was applied to Quantifoil Au300 R1.2/1.3 grids held in a FEI Vitrobot III chamber set to 100% humidity, 22°C. Following 4–4.5 s blotting, the grid was plunged into liquid ethane and stored in liquid nitrogen until imaging.

**Electron microscopy imaging and data analysis.** Grids were loaded into a Gatan 626 cryo-holder and imaged in an FEI F20 TEM operating at 200 kV, equipped with a Falcon II detector reading out a single integrated average. Images were semi-automatically acquired with EPU, at a defocus of  $-4 \mu$ m, a flux of  $50 \text{ e } \text{\AA}^{-2} \text{ s}^{-1}$ , an exposure of 1.5 s and a pixel size of  $2.06 \text{ \AA}^2$ . Analysis of dynein on microtubules was performed as previously described<sup>14</sup>. Contrast transfer function was determined using GCTF, and micrographs were phase-flipped accordingly in Relion. Microtubule polarity was determined in FIJI<sup>27</sup> (Extended Data Fig. 5c–g), and the microtubules were boxed out into new images such that their plus end pointed towards the right of the image. The microtubule was duplicated and reflected through the long axis to ensure all unique particles were on the top edge. Monomeric dyneins were picked manually in Relion, and centred at the point at which the stalk reaches the top edge of the microtubule. Particles were disregarded if the kink at the base of the stalk was not observed, or if any neighbouring particles overlapped. Two-dimensional classification into a single class each (for Dyn and Dyn<sub>RK+7hep</sub>) aligned the particles to each other. The angle of the stalk relative to the long axis of the microtubule, and stalk length, was measured for each particle in FIJI.

To simulate projections of the motor on a microtubule the following PDB entries were used: 3VKG<sup>10</sup> (for the stalk) and 4AI6<sup>11</sup> (for the ADP-bound state of the ring and linker). The coordinates were converted into a simulated electron density volume with the EMAN program 'pdb2mrc'. The volume was bandpass filtered between 30  $\text{\AA}$  and 500  $\text{\AA}$  using 'bfilter', and projected in different orientations in Relion.

**Fluorescence microscopy.** Assays were performed on a custom-built objective-type total internal reflection fluorescence (TIRF) microscope, equipped with Nikon Ti-Eclipse inverted microscope body, the perfect focusing system, and a 1.49 NA 100 $\times$  oil immersion objective (Nikon). The fluorophores were excited with 488 nm (for GFP and QDs) and 561 nm (for TMR and Cy3) and 633 nm (for Cy5) lasers, and the fluorescent signal was detected by the EM-CCD camera (Ixon, Andor) with an effective pixel size of 106 nm. The videos were recorded at 1 Hz. For dual colour imaging, fluorescence emission was separated into two channels on a CCD camera using Optosplit II (Cairn) image splitter.

**Microtubule-bridge assays.** Bead-motility assays on microtubule bridges were performed as previously described<sup>19</sup>. In brief, 2- $\mu$ m diameter polystyrene beads were coated with an anti-GFP antibody (Covance) using EDC-NHS crosslinking. The beads were incubated with 0.5  $\mu$ M SRS<sub>85-82</sub>-GFP<sup>23</sup> and excess protein was removed by pelleting the beads. The beads were non-specifically adsorbed

to the surface of the flow chamber and the surface was pre-blocked with 30  $\mu$ l DLBC (DLB buffer (30 mM HEPES pH 7.4, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, and 10% glycerol) supplemented with 1 mg ml<sup>-1</sup> casein). Cy3-labelled microtubules (0.015 mg ml<sup>-1</sup>) were flown into the chamber. After 10 min, unbound microtubules were removed by a 30- $\mu$ l DLBC wash. Anti-GFP antibody-coated beads (0.5- $\mu$ m diameter) were incubated on ice with 5–10 nM GFP-dynein for 10 min and flown into the chamber in the imaging buffer. The sample was placed on a bright-field microscope equipped with a Nikon Ti-E Eclipse microscope body, a Nikon 100 $\times$  1.49 NA oil immersion objective, Nikon 1.4 NA oil condenser and LED white-light illuminator (Sutter). The sample was scanned for a microtubule bridge that is longer than 10  $\mu$ m and oscillates less than 2 pixels. The spontaneous attachment of freely diffusing dynein-coated beads and their processive motility along the microtubule bridges were captured with the CMOS camera (Hamamatsu) at 10 Hz with an effective pixel size of 57 nm. Cargo beads were tracked using a Gaussian fitting algorithm in MATLAB. The helical pitch ( $\lambda$ ) was calculated from the periodicity of the  $x$ - $y$  projection of the traces between peak-to-peak positions<sup>19</sup>. The distance between the centre of the microtubule cylinder and pivoting point of dyneins at their linker domains ( $r$ ) was estimated to be 27 nm. The pitch angle was defined as  $\tan^{-1}(2\pi r/\lambda)$ . The  $z$  position of a bead was calibrated by measuring the intensity of surface immobilized 0.5- $\mu$ m beads while the microscope objective was moved  $\pm 250$  nm in the  $z$ -direction with 25-nm increments using a piezoelectric objective scanner (Physik Instrumente).

**Gliding assays.** To polarity-mark the microtubules, *N*-ethylmaleimide (NEM) modified tubulin was prepared by mixing 10 mg ml<sup>-1</sup> unlabelled tubulin (purified from porcine brain<sup>28</sup>) in BRB80 (80 mM PIPES pH 6.8, 1 mM EGTA, 2 mM MgCl<sub>2</sub>) with 1 mM NEM and 0.5 mM GTP for 10 min on ice. The reaction is quenched with 8 mM  $\beta$ -mercaptoethanol ( $\beta$ ME) for 30 min on ice. Brightly labelled microtubule seeds were polymerized by incubating 0.4 mg ml<sup>-1</sup> Cy3-labelled tubulin, 0.5 mg ml<sup>-1</sup> unlabelled tubulin, 1 mM GMP-CPP (Jena BioSciences) and 1 mM DTT in BRB80 for 15 min at 37°C. The 1.5  $\mu$ l seed was added to a mixture containing 0.1 mg ml<sup>-1</sup> Cy3-tubulin, 1 mg ml<sup>-1</sup> unlabelled tubulin, 1 mg ml<sup>-1</sup> NEM-modified tubulin, 1 mM GTP and 1 mM DTT in BRB80 and incubated at 37°C. Immediately after mixing, 2  $\mu$ l of 2  $\mu$ M, 20  $\mu$ M and 200  $\mu$ M taxol was added with 10 min breaks. After an additional 15-min incubation at 37°C, microtubules were pelleted over 300  $\mu$ l 30% glycerol cushion at 65,000g for 10 min. The pellet was resuspended in BRB80 with 20  $\mu$ M taxol and 1 mM DTT and stored in dark at room temperature. Polarity-marked microtubules were prepared fresh daily for the gliding assays.

For microtubule gliding assays<sup>4</sup>, rabbit monoclonal anti-GFP antibody ( $\sim 0.4 \text{ mg ml}^{-1}$ , Covance) was flown to an assay chamber and incubated for 5 min. The chamber was washed with 60  $\mu$ l of buffer DLBCT (DLBC supplemented with 20  $\mu$ M taxol). Subsequently, 10  $\mu$ l of 20 nM GFP-tagged motor in DLBCT was added to the chamber. After a 3-min incubation, unbound motor was removed by 30- $\mu$ l DLBCT wash. Then, 10  $\mu$ l of 200 nM freshly polymerized polarity-marked microtubules were flown to the chamber and allowed to bind dynein for 2 min. The chamber was washed with 100  $\mu$ l of DLBCT. Lastly, 30  $\mu$ l of imaging buffer (DLBCT supplemented with 2.5 mM PCA (protocatechuic acid), 50 nM PCD (protocatechuate-3,4-dioxygenase) and 1 mM ATP) containing the desired KCl concentration was flown to the chamber.

**Single-molecule motility assays.** Sea urchin axonemes were immobilized on a glass coverslip in a flow chamber. The chamber was washed with 50  $\mu$ l DLBC. GFP-tagged mutant dynein (0.2 nM) and TMR-tagged wild-type dynein (0.2 nM) were added into the chamber in DLBC and allowed to bind microtubules for 3 min. The chamber was then washed with 100  $\mu$ l DLBC and 20  $\mu$ l imaging buffer. Two fluorescent channels were overlaid, and the velocity and directionality of the constructs was determined with kymograph analysis using ImageJ.

For high-resolution tracking assays, 655 nm amine-labelled QDs (Invitrogen) were coated with anti-GFP antibody by using sulfo-SMCC (sulfo-succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate) as a cross-linking reagent. QDs (100 nM) were mixed with 25  $\mu$ M sulfo-SMCC ( $\sim 250$ -fold molar excess) and incubated for 1 h. Excess sulfo-SMCC was removed by using 30k MWCO spin-concentrator with 3 consecutive dilutions into DLB. Anti-GFP antibodies (0.4 mg ml<sup>-1</sup>) were reduced with 4 mM TCEP (tris (2-carboxyethyl) phosphine) for 30 min and mixed with QDs. After 1 h, excess antibody was removed by using spin filter for 3 consecutive dilutions into 25 mM sodium borate buffer pH 8.0, and concentrated to 5  $\mu$ M for storage. All reactions were performed at room temperature.

Anti-GFP antibody-labelled QDs (5 mM) were mixed with 100 nM GFP-tagged dynein with 1:1 ratio and incubated 15 min on ice. Polarity-marked and biotinylated microtubules were immobilized on the coverslips using biotinylated-BSA and streptavidin. Dynein-QD mixture was diluted 100-fold and was flown into the chamber. After a 3-min incubation, the chamber was washed with 100  $\mu$ l DLBC and 20  $\mu$ l imaging buffer containing 5–10  $\mu$ M ATP. Two fluorescent channels of Cy3-labelled microtubules and QDs were overlaid to determine the directionality