

The mechanochemical cycle of reactive full-length human dynein 1

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Pengxin Chai^{1,5}, Jun Yang^{1,5}, Indigo C. Geohring², Steven M. Markus^{2,6}✉, Yue Wang^{1,3,4,6}✉ & Kai Zhang^{1,6}✉

Dynein-driven cargo transport has a pivotal role in diverse cellular activities, central to which is dynein's mechanochemical cycle. Here, we performed a systematic cryo-electron microscopic investigation of the conformational landscape of full-length human dynein 1 in reaction, in various nucleotide conditions, on and off microtubules. Our approach reveals over 40 high-resolution structures, categorized into eight states, providing a dynamic and comprehensive view of dynein throughout its mechanochemical cycle. The described intermediate states reveal mechanistic insights into dynein function, including a 'backdoor' phosphate release model that coordinates linker straightening, how microtubule binding enhances adenosine triphosphatase activity through a two-way communication mechanism and the crosstalk mechanism between AAA1 and the regulatory AAA3 site. Our findings also lead to a revised model for the force-generating powerstroke and reveal means by which dynein exhibits unidirectional stepping. These results improve our understanding of dynein and provide a more complete model of its mechanochemical cycle.

Dyneins are large cytoskeletal motor complexes that move toward the minus ends of microtubules (MTs)¹. Cytoplasmic dynein 1 is the sole cytoplasmic isoform that drives retrograde intracellular cargo transport², organelle positioning³ and cellular morphogenesis⁴, while dynein 2 and axonemal dyneins mediate intraflagellar transport⁵ and ciliary motility⁶. Unlike the kinesin family that encompasses 45 different types of predominantly plus-end-directed motors in humans⁷, a single cytoplasmic dynein 1 transports diverse cellular cargos². This is enabled by the retrograde transport machinery comprising dynein, dynactin and a cargo adaptor (DDA complex)^{8–15}, regulated by cofactors such as LIS1 (refs. 16–23) and Ndel1 (refs. 24–27). Mutations in the genes encoding dynein and its cofactors have been implicated in many human diseases, including neurodegenerative²⁸ and neurodevelopmental disorders^{29–31} and viral infections³².

The dynein complex consists of two heavy chains (DHCs) and several accessory chains. Each DHC has a tail domain for binding

accessory chains, dynactin and adaptors and a motor domain essential for motility^{2,33–35}. The motor domain includes an AAA+ (adenosine triphosphatase (ATPase) associated with cellular activities) ring with six nonidentical AAA+ domains, a linker, a stalk coiled coil (stalk-CC), buttress, an MT-binding domain (MTBD) at the stalk tip and a C-terminal domain (CTD). It possesses ATPase activity and is responsible for converting the chemical energy in ATP into mechanical processes, collectively termed the mechanochemical cycle³³.

The dynein mechanochemical cycle underlies all dynein-mediated cellular functions. Current understanding of this cycle primarily stems from biochemical^{36–39}, biophysical^{40–43} and structural studies^{33,44–49} on motor domain fragments. Within the motor domain, AAA1 is the primary ATP hydrolysis site^{37,39}. Alternating closed and open conformations of the AAA1 and motor ring drive force generation and motility^{33,50}. The linker domain can change from a bent to a straight conformation in a nucleotide-dependent manner, which has been deemed responsible

¹Department of Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, USA. ²Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, USA. ³Present address: Innovation Center for Brain Medical Sciences, The Ministry of Education of the People's Republic of China, Huazhong University of Science and Technology, Wuhan, China. ⁴Department of Pathophysiology, School of Basic Medicine, Huazhong University of Science and Technology, Wuhan, China. ⁵These authors contributed equally: Pengxin Chai, Jun Yang. ⁶These authors jointly supervised this work: Steven M. Markus, Yue Wang, Kai Zhang ✉ e-mail: steven.markus@colostate.edu; yuew@hust.edu.cn; jack.zhang@yale.edu

for the force-generating ‘powerstroke’ (refs. 38,42,44,51,52). When straight, the linker docks onto a conserved site of AAA5 (refs. 47,53). Notably, a recent study on outer-arm dynein (OAD) revealed an alternate docking site near AAA4 (‘post 2’, compared to the classical ‘post 1’) ⁵⁴ but whether dynein 1 samples this state and its role in the cycle remain unclear. Kinetic and structural evidence suggests that phosphate (Pi) release from AAA1, coupled with AAA+ ring opening and MT binding, triggers linker straightening ^{33,38,42,50}. However, the mechanism of Pi release, through a minimal conformational change (‘molecular backdoor’) like myosin ⁵⁵ and actin ⁵⁶ or complete pocket opening, remains unresolved, leaving the relationship among Pi release, pocket opening and linker straightening poorly defined ³³.

Unlike kinesin and myosin, which bind their tracks through ATP hydrolysis domains ^{55,57}, dynein’s MTBD is 24 nm from AAA1 (ref. 34). The nucleotide states and conformations of AAA1 regulate MTBD’s affinity for MTs ^{44,46,47}. Meanwhile, the MTs provide a track for the unidirectional stepping of dynein ^{41,43,58} and stimulate the AAA1 ATPase activity ^{46,59}. Communication between AAA1 and the MTBD or MT is mediated by the buttress ^{46,48} and the 15-nm-long stalk-CC ^{60–62}. Coupled conformational changes in the buttress stalk modulate stalk-CC registries, altering MT-binding affinities ^{40,63–67}. The buttress stalk’s conformational changes are regulated by AAA1 pocket conformations ³³ and MT binding ^{61,68,69}. However, because of the limited structural information available for dynein on and off MTs, the precise mechanism of communication between dynein and MTs through the buttress stalk remains unclear. Therefore, how the dynein mechanochemical cycle is accelerated by MTs ⁷⁰ and how dynein achieves unidirectional motility along MTs ⁵⁸ require further investigation.

Previous studies elucidated the structures of truncated dynein ^{44,46–49}, full-length ‘phi’ dynein 1 (ref. 10), similar to the Greek letter Φ ⁷¹, phi dynein 2 (ref. 72), the OAD–MT complex ⁵⁴ and the DDA–MT ^{15,23} complex in specific nucleotide conditions. However, these approaches, often limited by resolution or structural dynamics (for example, use of nonhydrolyzable ATP), provide only partial insights into the mechanochemical cycle. To address these limitations, we captured the complete conformational landscape of full-length human dynein 1 progressing through its natural mechanochemical cycle by imaging dynein in ATP and other nucleotide conditions. Additionally, we imaged reconstituted dynein–MT complexes to obtain structural information on MT-bound dyneins. In total, we obtained over 40 high-resolution (2.2–3.6 Å) motor domain structures from the full-length dynein, revealing crucial intermediates, allowing us to perform comprehensive structural analyses in combination with functional assays to advance our understanding of the dynein mechanochemical cycle.

Results

Structural determination of human dynein 1 off and on MTs

To explore the conformational landscape of dynein throughout its mechanochemical cycle (‘in reaction’), we purified full-length human dynein 1 from insect cells (which contained a roughly equal mixture of phi and open states) and prepared cryo-electron microscopy (cryo-EM) grids in the presence of 5 mM ATP-Mg²⁺ (Fig. 1a). We collected a large cryo-EM dataset and performed extensive *in silico* three-dimensional (3D) classification followed by high-resolution refinement (Extended Data Figs. 1 and 2 and Supplementary Table 1). We obtained high-resolution cryo-EM maps of full-length phi dynein (Fig. 1c and Supplementary Video 1), with the motor domain at an average resolution of 2.2 Å and the majority of the tail region at a resolution better than 4 Å. This substantially improved resolution compared to the previous study ¹⁰ allowed us to build a full atomic model for phi dynein. Moreover, we observed distinct conformational states of the open motor domain, revealing snapshots of reactive intermediates during the dynein mechanochemical cycle (Fig. 1d). The high-resolution structure of the motor domain provides unprecedented details, including holes within aromatic side chains, water molecules and ions interacting with the nucleotide (Fig. 1f).

High-resolution structural determination of MT-bound dynein has been a major challenge. To address this, micrograph-based MT signal subtraction methods were developed ^{15,73,74}, leading to the successful reconstruction of OAD–MT in the apo condition and DDA–MT in the adenyllyl imidodiphosphate (AMPPNP) condition ^{15,23,54}. To capture complete MT-bound dynein 1 structures, we reconstituted full-length dynein 1 on MTs in apo, adenosine diphosphate (ADP) and AMPPNP conditions, all of which increase dynein affinity for MTs ⁷⁵ (Fig. 1b, Extended Data Fig. 3 and Supplementary Table 2). We obtained a structure of full-length MT-bound dynein at a resolution of 10 Å (Fig. 1e). Focused classification and refinement of the individual motor domains resulted in four distinct MT-bound motor domain structures at 2.9–3.6 Å (Fig. 1e and Supplementary Video 2).

Overview of conformational landscape of MT-unbound dynein

On the basis of four structural features (AAA1 pocket conformations, AAA3 pocket conformations, linker position and buttress stalk conformation), we classified the motor domain structures resolved from phi and open dynein into ten distinct categories. Focusing on AAA1 and overall AAA+ ring arrangements, these structures were further merged into eight major states (states 1–8), representing distinct points of the motor domain during the mechanochemical cycle (Fig. 2a). The state sequence was determined by AAA1 pocket dynamics, nucleotide-bound states and previous dynein models ³³, despite the absence of time resolution in our cryo-EM analysis. Among the eight states, similar structures only in states 1, 2, 7 and 8 have been reported previously (Supplementary Data 1).

In states 1–4, the motor domains feature a closed AAA1 pocket and low MT-binding affinity, as indicated by the buttress and stalk conformations ³³. State 1 (phi motor) was determined at the highest resolution (2.2 Å), enabling precise modeling of ADP, magnesium and water at the active site (Fig. 2b,c). States 2–4, resolved at 2.8–3.0 Å, also exhibit clear ADP-Mg²⁺ density in the AAA1 pocket (Extended Data Fig. 2). However, because these structures were solved in the presence of ATP, the possibility of a traveling Pi cannot be ruled out because of weak, unresolved densities. Among all these states, AAA3 adopts an open conformation with ADP bound. Unlike the autoinhibited state 1, states 2–4 are actively engaged in the cycle exhibiting distinct linker conformations. Specifically, state 2 possesses a bent linker that is stably docked between AAA2 and AAA3, similar to state 1 and previously reported motor structures before the powerstroke (Protein Data Bank (PDB) 4RH7 (ref. 44), 5NUG (ref. 10), 6RLA (ref. 72) and 8PQY (ref. 23)). State 4 has an overall straight (with an apparently straight hinge helix) but undocked linker, suggesting a powerstroke intermediate. Notably, previous models would have precluded the existence of this state ^{44,50}. State 3 represents an average of the transient conformations between states 2 and 4, with a bent hinge helix and flexible linker.

States 5–7, with open AAA1 pockets bound to ADP, dominate 60% of the active ATP cycle, underscoring that ADP release is rate limiting ⁷⁶. Variations in pocket opening correlate with distinct buttress stalk conformations (Fig. 2d), aligning with models linking AAA1 dynamics to MT affinity ^{33,44,46,47}. In these states, the AAA3 pocket is primarily ATP bound and adopts a closed conformation (Extended Data Fig. 2), indicating that AAA3 only undergoes nucleotide exchange when AAA1 is in an open state. State 7 features a straight and stably docked linker at the post 2 position ⁵⁴ and a kinked buttress indicative of high MT affinity ²¹. The AAA1 pocket and AAA+ ring conformation in this state resemble dynein structures with high MT-binding affinity in both previous structures (PDB 7Z8G (ref. 15), 8FCY (ref. 21), 4W8F (ref. 49), 8PQV (ref. 23) and 3VKG (ref. 46)) and the present study. States 5 and 6 represent intermediates with semi-open AAA1 pockets, straight and undocked linkers and intermediate MT-binding affinity. The stalk-CC in these states is likely undergoing helix sliding ⁴⁰ or local melting ⁷⁷, adopting a semi-α registry ⁶¹ or γ registry ⁵³.

State 8, which features a nucleotide-free AAA1 pocket, is transient in excess ATP but can be captured in the apo condition. AAA1’s

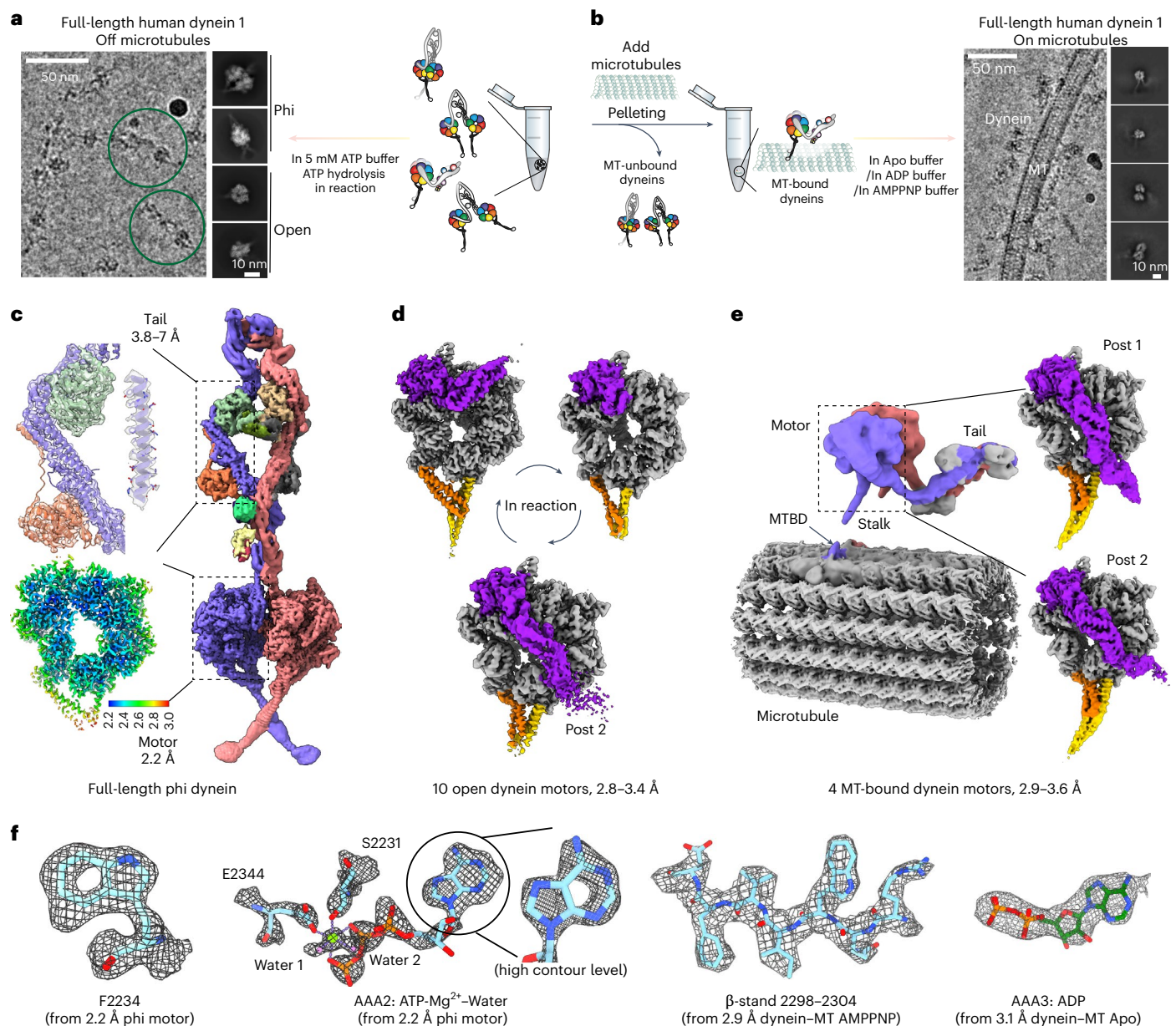


Fig. 1 | Cryo-EM analysis of full-length human dynein 1 off and on MTs.

a,b, Schematic of cryo-EM pipeline for determination of dynein structures in reaction with ATP without MTs (**a**) or with MTs in different nucleotide conditions (**b**). **c**, Cryo-EM reconstruction of phi dynein with the 3.8–7.0-Å tail region and 2.2-Å motor domain. **d**, Representative cryo-EM reconstructions of the motor domain

in different states from open dynein. The motor ring, linker, buttress and stalk are colored gray, purple, orange and yellow, respectively. **e**, Cryo-EM map of full-length two-headed dynein bound to MTs in ADP conditions at 10-Å resolution, with locally refined motor domains at 2.9–3.6-Å resolution. **f**, High-resolution features are shown by the representative density of amino acid residues and ligands.

large and small subunits are widely separated, allowing ADP release (Fig. 2d), similar to the yeast apo motor domain (PDB 4AKI)⁴⁷ and apo OAD–MT structures (PDB 7K58 and 7K5B (ref. 54)). AAA3 has ADP bound even in the apo condition. The straight linker is docked at the post 1 position, with a high-MT-affinity buttress stalk conformation.

Together, the conformational landscape of full-length dynein 1 in ATP conditions reveals a wide range of states, underscoring the benefits of our approach, offering insights into the mechanochemistry of full-length dynein.

Effect of nucleotide conditions on dynein conformations

Given the range of conformations we observed for the dynein motor, we investigated whether incubating dynein with different nucleotides

and analogs would lock the motor into a single conformation, as suggested by prior cryo-EM and crystallographic studies^{15,21,44,46,47,49,54,78}. To test this, full-length dynein (purified in 0.1 mM ATP) was incubated with 5 mM AMPPNP, ATP-Vi or ADP or in the apo condition, followed by cryo-EM classification (Extended Data Figs. 4 and 5 and Supplementary Tables 3–7). Surprisingly, multiple conformational states were observed across all conditions (Fig. 2e and Extended Data Fig. 6), likely because of dynein's ability to adopt the phi state in all conditions¹⁰. Upon exiting the phi state, which likely occurs stochastically, dynein transitions through various states before ADP release and nucleotide analog binding. Notably, in the AMPPNP condition, AAA1 remains ADP bound rather than adopting an AMPPNP-bound state (Fig. 2e). Further details on conformations and nucleotide states are described in Supplementary Note 1.

Identification of a ‘backdoor’ for Pi release

States 1–4 in the 5 mM ATP condition feature closed AAA1 pockets with linkers in bent to straight states (Fig. 2a). Similar linker states were observed with closed AAA1 pockets in ATP-Vi and AMPPNP conditions (Fig. 2e). Because AAA1 is proposed to govern linker positioning^{38,42,44,51,79}, these observations suggest that structural differences in AAA1 may exist but are not evident from a simple large-scale assessment of its open or closed state. Detailed analysis revealed that the sensor I loop, critical for AAA+ ATPase activity⁸⁰ and proposed to facilitate Pi release through a backdoor mechanism³³, adopts multiple conformations in the ATP condition (Fig. 3a). In ATP-Vi (or 5 mM AMPPNP with 2 mM Mg²⁺) conditions, the loop maintains the same conformation in closed AAA1 pockets (states 1, 2 and 4) (Fig. 3a). Furthermore, the sensor I loop becomes disordered when the AAA1 pocket is open (Fig. 3b), as seen in states 7 and 8, suggesting that its role is specific to the closed state.

Close inspection of the cryo-EM densities reveals notable conformational changes in two sensor I loop residues, N2019 and Y2022 (Fig. 3c). In the ATP-Vi and AMPPNP (low Mg²⁺) conditions, both residues orient toward the AAA1 active site (‘inward’), contacting the AAA1 Walker A motif and AAA2L. In the ATP condition, these residues exhibit distinct conformations. In both states 1 and 2, N2019 points away from the active site (‘outward’), while Y2022 adopts multiple conformations according to the flexible cryo-EM density. In state 3, absent in ATP-Vi and AMPPNP conditions, N2019 also points outward and Y2022 adopts two conformations. In state 4, the sensor I loop exhibits stable density and both residues adopt inward conformations. Thus, despite the closed AAA1 pocket for all these states, there indeed exist small-scale, residue-level changes that very likely coordinate dynein mechanochemistry.

To investigate the role of dynamic sensor I loop residues, we compared the local environment across different states (Fig. 3d). This revealed that, when both N2019 and Y2022 point outward, a channel forms, creating a possible escape route for Pi release. This observation supports the notion of a molecular backdoor³³, in which the coordinated rearrangement of residues in the sensor I loop can establish a closed or open backdoor during the Pi release process (Fig. 3e and Supplementary Video 3). To further test this model, we performed focused classification of the AAA1 active site for the phi motor (state 1) in ATP and captured what may be Pi at two different points during its release (arrows in Fig. 3f). Notably, the sensor I loop residues, including N2019 and Y2022, are highly conserved across dynein 1 in different species and among dynein types (for example, axonemal dyneins and dynein 2; Fig. 3g), suggesting that they are crucial for dynein mechanochemistry.

The dynamic nature of Y2022 suggests a critical role in mediating Pi release. To assess the importance of this residue in dynein function, we used a well-characterized GST-dimerized yeast dynein motor construct⁴³ and substituted the corresponding residue (Y1902) to either phenylalanine or alanine. While Y1902F exhibited ATPase activity comparable to the wild type, Y1902A showed defects in both basal

and MT-stimulated ATPase activity (Fig. 3h). MTs only marginally stimulated Y1902A ATPase activity (20%, compared to 3.4-fold and 3.1-fold for the wild type and Y1902F mutant, respectively), indicating uncoupling between MT binding and ATPase activity. Unlike human dynein^{12,14}, this minimal GST–dynein fragment is processive without the addition of dynactin or adaptor proteins, permitting the assessment of motility without complicating factors⁴³. Single-molecule assays revealed that, unlike Y1902F, the Y1902A mutant exhibits significant motility defects, as apparent from the reduced fraction of active motors and the much lower velocity for those motors that exhibit processive runs (Fig. 3i,j). Intriguingly, a large proportion of the Y1902A motors (84.4%) appear to exhibit bidirectional diffusive behavior along MTs (red arrows in Fig. 3i), further suggesting uncoupling of MT binding and ATPase activity. These functional data highlight the importance of this tyrosine in the catalytic and motility behaviors of dynein.

In light of this backdoor model for Pi release, we can now more accurately assign the nucleotide state in different closed AAA1 pockets. While stable Pi density is not definitively resolved, weak densities and conformations of residues N2019 and Y2022 provide clues about the presence of a traveling Pi (Fig. 3k and Extended Data Fig. 7). These results suggest that the AAA1 pockets in states 1–3 in the 5 mM ATP condition contain ADP-Mg²⁺ with a traveling Pi, whereas state 4 contains only ADP in the closed AAA1 pocket.

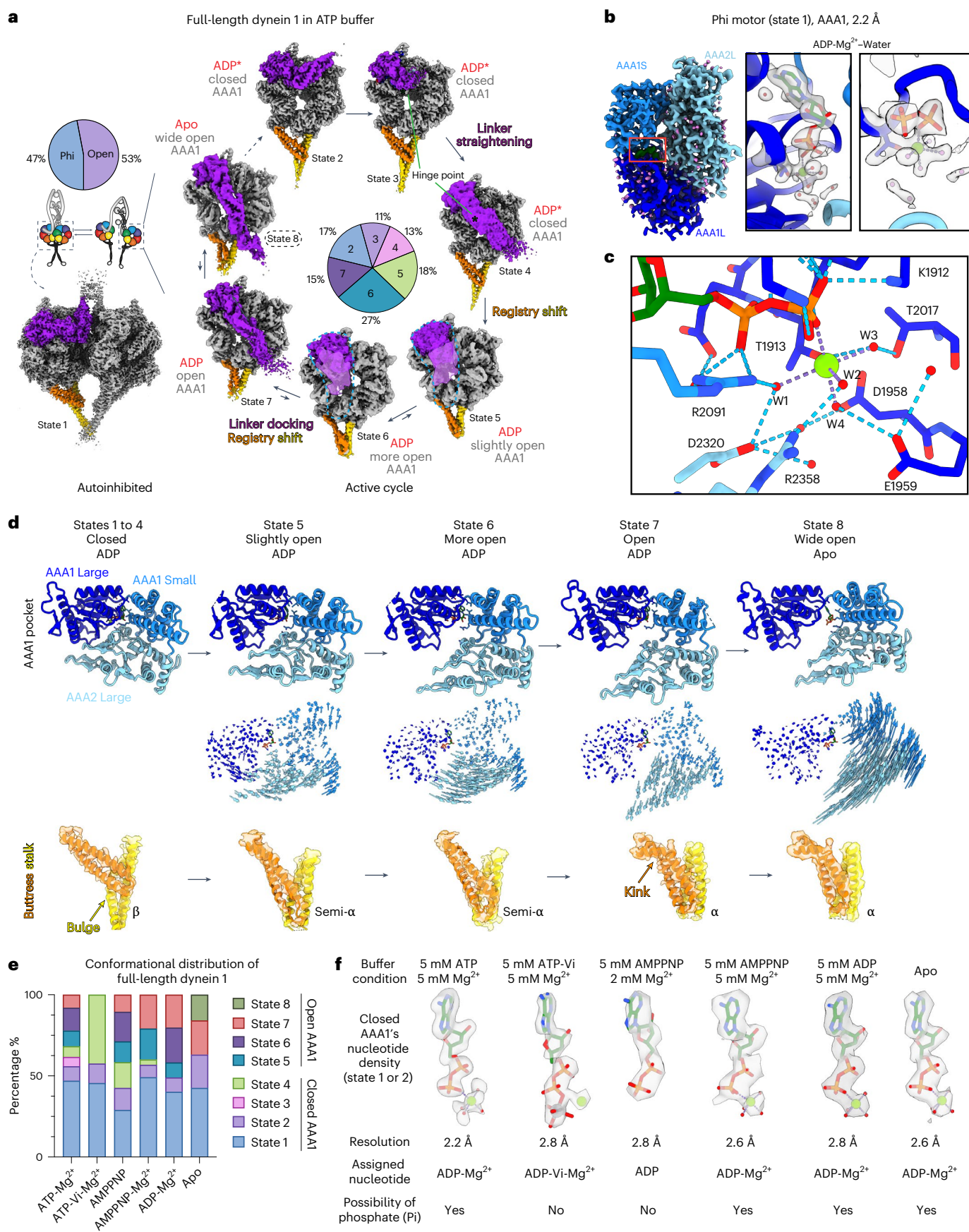
The Pi release signal triggers linker straightening

The backdoor Pi release mechanism prompted us to revisit the ATP turnover in phi and open dynein. Our data confirm that phi dynein is capable of hydrolyzing ATP, as evidenced by the clear density of ADP-Mg²⁺ and the presence of Vi in ATP-Vi conditions (Fig. 2b). Additionally, the dynamic conformation of the sensor I loop suggests that Pi is in the process of escaping the pocket (Fig. 3). However, because of the autoinhibited state of the motor domains, ATP hydrolysis and Pi release do not lead to a change in linker conformation. In open, uninhibited dynein, states 2 and 3 also exhibit dynamic sensor I loop conformations indicative of a traveling Pi. Unlike phi dynein, state 3 features a linker in a transitional conformation between bent and straight, suggesting a correlation between sensor I loop dynamics and linker position during Pi release. After Pi is released through the backdoor mechanism in state 4, as indicated by the stable conformations of residues N2019 and Y2022, the linker adopts a straight conformation. This observation aligns with previous kinetic measurements showing that the linker straightens following Pi release⁴².

On the basis of the Pi-free but closed state of the AAA1 pocket in state 4, we propose that Pi release must occur before rather than after AAA1 pocket opening. Our analysis also suggests a potential signal propagation pathway, linking Pi backdoor release and sensor I loop dynamics to linker straightening (Supplementary Note 2). Notably, linker straightening in our current model is not equivalent to the definition of the powerstroke in canonical models, as state 4 clearly corresponds to a low-MT-affinity conformation.

Fig. 2 | The conformational landscape of MT-unbound dynein motor domains in reaction. **a**, Eight major states of the dynein motor domain during the catalytic cycle divided into autoinhibited states (left) and states in the active cycle (right). Arrows indicate the proposed catalytic pathway supported by the literature and apparent AAA+ ring conformations. Small arrows show the possibility of reverse pathways. Percentages indicate the proportion of particles in each state. The apo AAA1 pocket state (state 8) is present at a very low proportion in the presence of ATP but is highly represented in apo conditions (the structure obtained in the apo condition is shown). The linkers from states 4 and 6 are low-pass-filtered to 12 Å and shown at low contours. Only one representative subclass in each state is shown. The color scheme is consistent with Fig. 1d. **b**, Cryo-EM map of AAA1 pocket from 2.2-Å phi motors. AAA1L, AAA1S and AAA2L are colored blue, light blue and sky blue, respectively. Right, carved transparent gray density is

displayed for ADP (green), Mg²⁺ (light green) and water molecules (violet). **c**, Residue side chains involved in hydrogen-bonding networks (dashed lines) at the AAA1 active site are displayed and colored by heteroatom. **d**, Cartoon models showing the AAA1 dynamics and the coupled buttress stalk conformational states. The isolated AAA1 pockets from different states are aligned on AAA1L. Vector maps depicting pairwise Cα interatomic distances between the AAA1 domains of adjacent states depict the 3D trajectories during the pocket-opening process. The buttress stalk regions are highlighted to show how the stalk-CC registries are coupled to motor ring states. **e**, Plot depicting the proportions of the various motor domain conformations from full-length dynein 1 in different nucleotide conditions. **f**, EM densities for nucleotides bound to the closed AAA1 pockets in indicated nucleotide conditions.



The communication between AAA1 pocket dynamics and the MTBD

Prior studies showed that AAA1 pocket dynamics regulate MTBD–MT affinity through stalk-CC registry changes³³. However, the mechanism underlying this long-range (~24 nm) communication remains unclear. Our dynein structures reveal pathways for AAA-to-MTBD signal propagation (Fig. 4a,b and Supplementary Video 4). The propagation of AAA1 pocket conformational changes through the motor ring is described in detail in Supplementary Note 3. In summary, signal transmission occurs in two phases (Fig. 4c,d): first, through AAA6, the CTD, AAA5 and the buttress to the stalk-CC (adopting the semi- α registry); next, through AAA2, AAA3 and AAA4 to fully establish the stalk-CC α registry.

We demonstrate that AAA1 transmits conformational signals through the buttress stalk region, altering stalk-CC registry to modulate MTBD–MT affinity. Conversely, locking the stalk-CC in specific registries perturbs dynein's ATPase activity, motility and tension sensing, supporting bidirectional signaling between MTBD and the AAA+ ring^{40,53} (Fig. 4e). To probe the structural basis of reverse communication, we analyzed a stalk-CC mutant locked in the α registry²¹ (mimicking MT-bound states; Fig. 4f and Supplementary Table 8). In the presence of ATP, this mutant adopts an open ADP-bound AAA1 pocket and a kinked buttress conformation (Fig. 4g), resembling wild-type dynein in state 7. Even with ATP-Vi, the AAA1 pocket remains ADP bound and open, consistent with the lack of Vi-dependent photocleavage (a readout for Vi binding)²¹. This suggests that α registry locking forces AAA1 into an open conformation. Taken together, these findings provide a structural basis for a comprehensive understanding of the two-way communication between AAA1 and the MTBD or MT.

MT-bound dynein structures reveal that MTs accelerate ADP release

To gain insight into the role of MTs in dynein mechanochemical cycle, we obtained four distinct structures of MT-bound dynein motor domains (Fig. 5a), with AAA1 in either apo or ADP-bound states, all of which possess stably docked linkers and a kinked buttress. In nucleotide-free buffer, AAA1 is in the apo state (AAA3 and AAA4 are in the ADP-bound state), with the linker in the post 1 position. In ADP conditions, AAA1, AAA3 and AAA4 bind ADP and the linker is docked at post 1 or 2. In AMPPNP conditions, AAA3 and AAA4 bind AMPPNP while AAA1 remains ADP bound and the linker is exclusively docked at post 1.

To assess the impact of MT binding on dynein motors, we compared these structures to the MT-unbound structures described above (Extended Data Fig. 8). Strikingly, three of the MT-bound motor domain structures in the apo and ADP conditions closely match the MT-unbound dynein structures (C α root-mean-square deviation (r.m.s.d.) < 0.3 Å with states 7 or 8). MT-bound dynein incubated in AMPPNP-containing buffer exhibits a 2.3–5.1-Å C α r.m.s.d. in comparison to the MT-unbound motor domains, with the closest match being to state 7. These findings indicate that the dynein motor domain can

naturally sample the MT-bound state even in the absence of MTs. However, it is noteworthy that the MT-bound motors exhibit more stable cryo-EM densities in the buttress stalk and linker regions compared to their MT-unbound counterparts, suggesting that MT binding can further coordinate the buttress stalk conformational change and linker docking during the dynein stepping cycle⁵³. In addition, the structures in states 5 and 6 with intermediate open pockets are no longer found in MT-bound motors, providing additional evidence that MT binding indeed stabilizes the stalk-CC registry, AAA+ ring conformation and linker docking.

It has been proposed that ADP release is the rate-limiting step in dynein's ATPase cycle²⁶ and that MTs accelerate this process⁷⁰. In agreement with this model, we found that approximately 45% of MT-unbound dynein motors exhibit ADP-bound intermediate AAA1 open pockets with their stalk-CC in the semi- α registry (states 5 and 6; Fig. 2a). MT binding shifts these two intermediates to state 7 by inducing the stalk-CC to adopt the α registry⁶¹ (Fig. 5b), promoting further motor ring opening and AAA1 pocket expansion for ADP release. MT binding also induces the adoption of the 'wide open' state of the AAA1 pocket, as observed in state 8, which allows ADP release. Supporting this, nucleotide-free buffer conditions lead to the identification of both states 7 (ADP-bound AAA1) and 8 (apo AAA1) for MT-unbound dynein but only state 8 is observed in MT-bound dynein (Fig. 5c). These results explain that two limiting steps, stalk-CC registry change⁶⁸ and ADP release⁷⁶, are strictly coupled and simultaneously stimulated by MT binding. Moreover, our evidence for a Pi backdoor release mechanism in low-MT-binding-affinity states supports a previous model that MT binding does not affect the Pi release step⁷⁰.

Post 2 linker docking mode biases dynein minus-end stepping

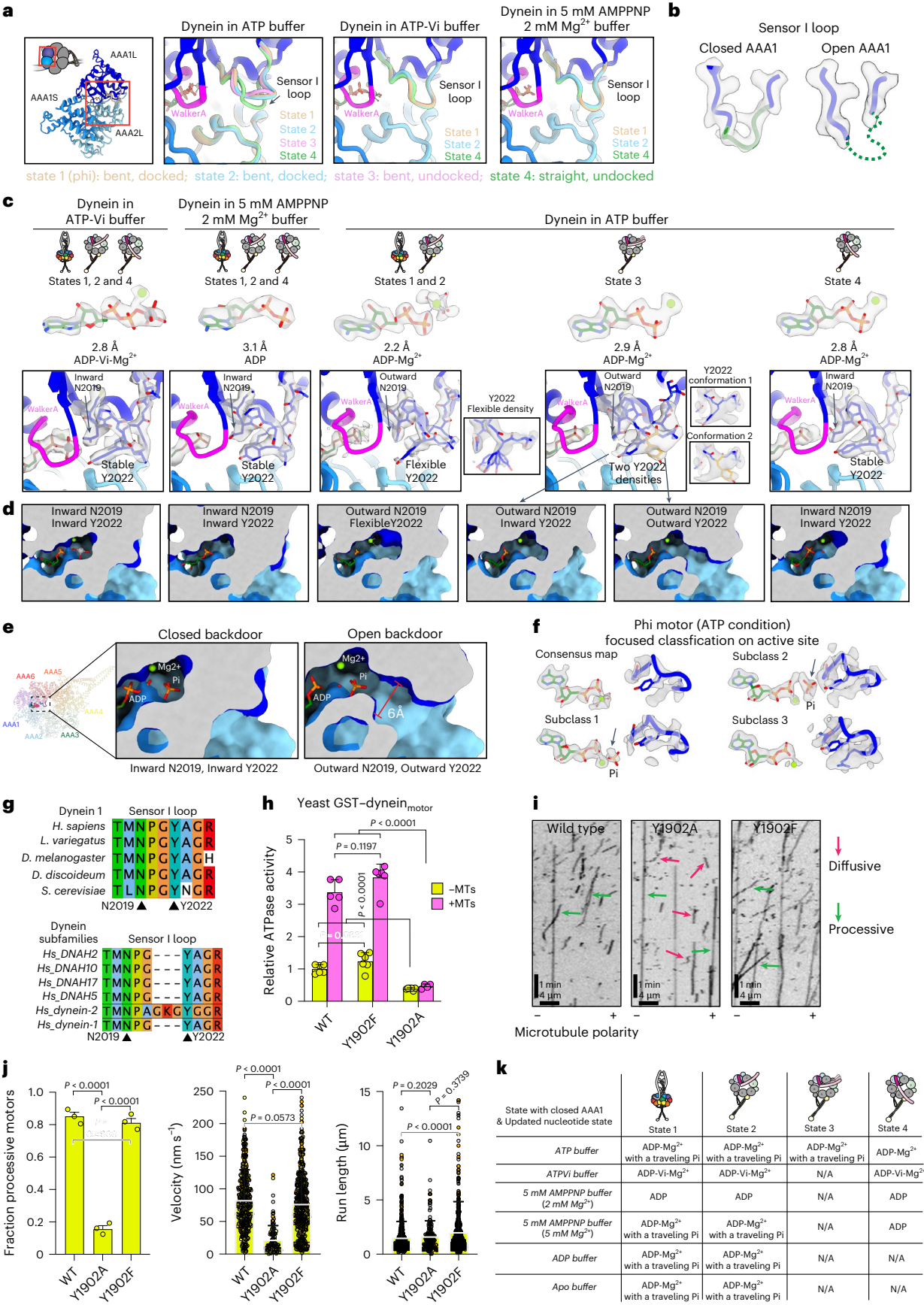
The linker swing vector model has been proposed to explain the mechanism of dynein unidirectional motility^{52,81,82}. However, which step in the mechanochemical cycle provides the net minus-end-directed bias remains unknown. We analyzed the different linker–ring interaction interfaces (Extended Data Fig. 9a) and found that the two different linker docking modes (post 1 and post 2) may directly influence dynein stepping, consistent with previous findings in OAD⁵⁴. The presence of a post 2 linker position in dynein 1 further supports the conservation of this docking mode (Extended Data Fig. 9b–e), suggesting that it has a critical role in dynein stepping.

On the basis of previous OAD–MT structures, it was proposed that dynein in the post 2 mode positions its MTBD 8 nm forward relative to dynein in the post 1 mode (Fig. 5d). To investigate the role of these docking modes, we examined how linker position correlates with stepping behavior (Fig. 5e and Supplementary Video 5). When AAA1 is in the apo state, the dynein motor binds to MTs in post 1, representing the starting position (0 nm; Fig. 5e (i)). The prepowerstroke state is induced upon ATP binding and/or hydrolysis at AAA1, leading to dynein detachment from MTs²³ (Fig. 5e (ii)). Pi backdoor release triggers linker

Fig. 3 | The release of Pi from AAA1 through a molecular backdoor mechanism.

a, Comparison of molecular models of closed AAA1 pockets in different states and nucleotide conditions. The sensor I loop exhibits conformational heterogeneity when dynein is in the 5 mM ATP condition. **b**, Cryo-EM densities of sensor I loop in closed and open AAA1 pockets. **c**, Structural comparison of the bound nucleotide and the sensor I loop in closed AAA1 states. Transparent cryo-EM densities of the sensor I loop are displayed in isosurface mode. The conformations of two residues, N2019 and Y2022, are highlighted. **d**, A slice through the structures of the AAA1 pocket near the nucleotide-binding site, shown in surface representation. **e**, Backdoor model for Pi release from dynein AAA1 controlled by N2019 and Y2022. **f**, High-resolution 3D classification of the active site in the phi motor, showing the potential Pi group in various states of escape. **g**, Conservation analysis of the sensor I loop. **h**, Plot (mean $\pm$ s.d., along with individual data points) depicting relative ATPase rates (in the absence or presence of 2 μ M MTs) for artificially dimerized yeast dynein motor domain

fragments (from left to right, $n = 6/5, 6/4$ and $6/6$ independent replicates from assays minus/plus MTs). Y1902 in yeast dynein corresponds to Y2022 in human dynein. **i,j**, Representative kymographs (i) and plots (j; mean $\pm$ s.d., along with individual data points) depicting indicated motility parameters determined from a single-molecule motility assay (from left to right, $n = 508/597, n = 148/880$ and $n = 679/837$ processive/nonprocessive dynein molecules from three independent experiments). MT polarity was determined with a wild-type dynein fragment (Methods). **P** values in **h** were calculated using an ordinary one-way analysis of variance (ANOVA) followed by a Tukey's multiple-comparison test. **P** values in **j** were calculated using a Kruskal–Wallis ANOVA followed by a Dunn's multiple-comparison test (for run length) or an ANOVA followed by a Dunnett's multiple-comparison test (for velocity). **k**, Summary of the updated nucleotide state among different closed AAA1 conformations. Note that the presence of a traveling Pi is based on the sensor I loop conformations. N/A, not applicable.



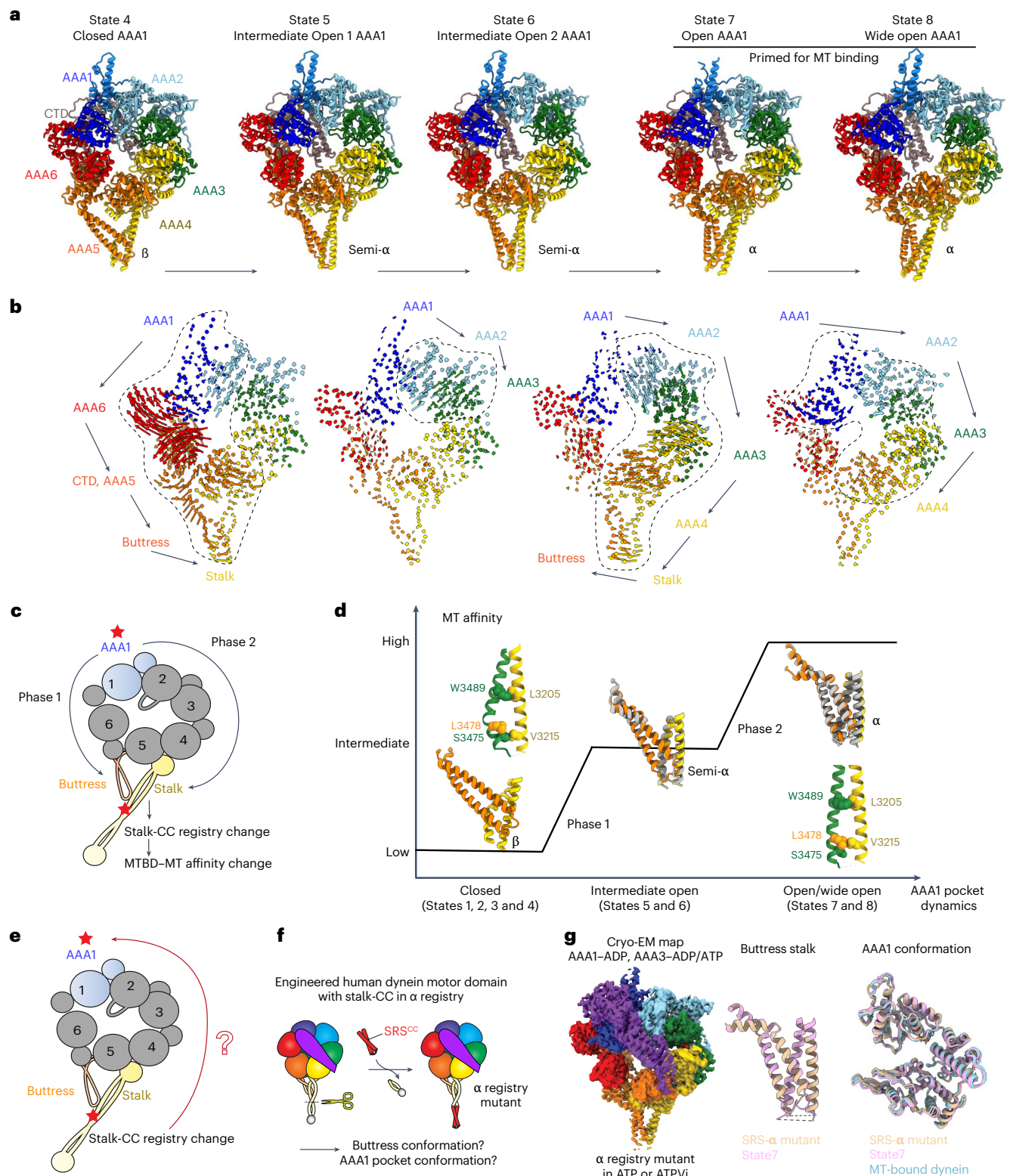


Fig. 4 | The mechanism for two-way communication between AAA1 and the MTBD. a, Cartoon models illustrating the overall motor ring conformations from different states, each with varying degrees of the AAA1 pocket opening. The linker domain is hidden for better visibility of the motor ring states. **b**, Adjacent models were aligned using the entire motor domain and then vector maps were generated that depict pairwise Cα interatomic distances (for every third residue) between the AAA domains of adjacent states. The length of the lines is proportional to the calculated interatomic distances. The major movements and directions are highlighted by dashed areas and arrows. **c**, The cartoon model

illustrates the signal propagation directions from AAA1 to the stalk-CC in two distinct phases. **d**, Changes in dynein-MT-binding affinity (y axis), as assessed by buttress stalk conformations, as a consequence of AAA1 pocket conformations in the different states (x axis). **e**, Cartoon model illustrating the communication from the MTBD to AAA1 through the stalk-CC. **f**, Cartoon model depicting the design of the α registry-locked dynein motor domain mutant. **g**, Cryo-EM reconstructions of the α registry mutant in ATP or ATP-Vi conditions. In both conditions, the motor domains exhibit only open AAA1 pockets. As expected, the buttress stalk conformation is consistent with a high MT-binding affinity.

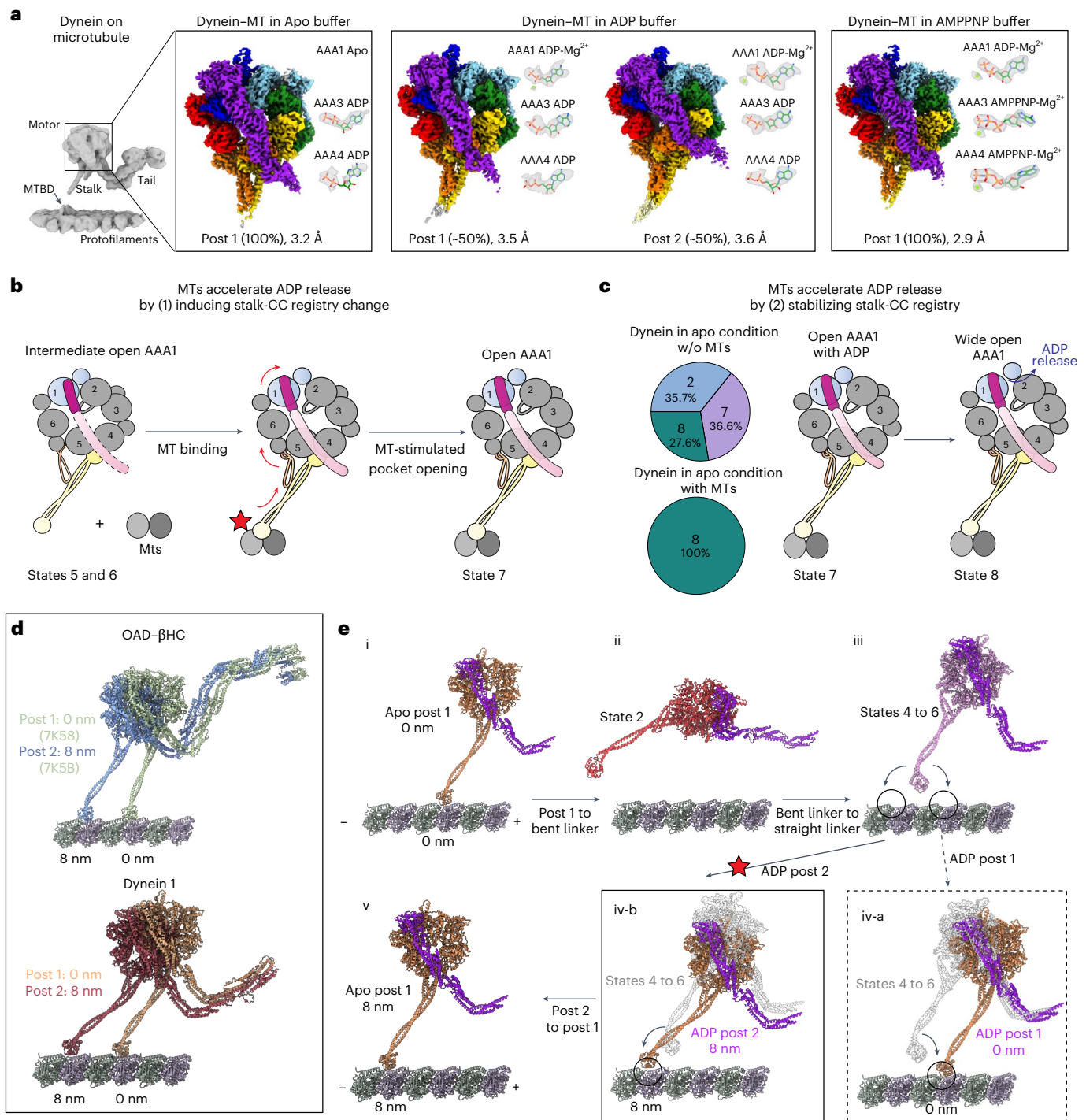


Fig. 5 | The mechanism for ATPase stimulation by MTs and dynein's unidirectional stepping along MTs. **a**, High-resolution cryo-EM maps of MT-bound dynein motor domains in different nucleotide conditions. **b, c**, Cartoon model depicts the mechanism of dynein mechanochemical cycle acceleration by MTs. MT binding rapidly induces the stalk-CC registry from the semi- α to α registry, thereby forcing the AAA1 pocket to adopt an open state (**b**). MT binding, by stabilizing the stalk-CC registry, allows the AAA1 pocket to adopt a wide open state, which promotes ADP release. Pie graphs show the distribution of dynein in the apo condition with or without MTs (**c**). **d**, Dynein adopting the post 2 linker docking mode intrinsically favors a forward step according to previous OAD-MT structures (PDB 7K58 and 7K5B (ref. 54)). Aligning dyneins by their N termini (to the right of the models in the picture) show that the post 2 dynein motor for both

OAD and dynein 1 adopts a forward-stepping pattern with respect to the post 1 dyneins. **e**, Proposed mechanism for dynein unidirectional stepping along MTs. (i) Dynein is bound to MTs in the apo state in post 1 mode (after completing a step). (ii) Upon ATP binding and/or hydrolysis, dynein unbinds MTs with a bent linker. (iii) Following Pi release from AAA1, dynein with a straight but undocked linker (states 4–6) has low to intermediate MT-binding affinity and is searching for a new tubulin-binding site. (iv-b) The preferred post 2 mode in ATP conditions (denoted by a star) creates a net bias toward the minus end of MTs and allows the MTBD to take an 8-nm forward step. Note that adopting a post 1 mode upon MT rebinding (iv-a) leads to a 0-nm step. The cartoon model from state 4 (from (iii)) is shown in gray in (iv-a) and (iv-b) for comparison. (v) Switching from a post 2 to a post 1 mode and ADP release leads to an 8-nm forward movement of the entire dynein complex.

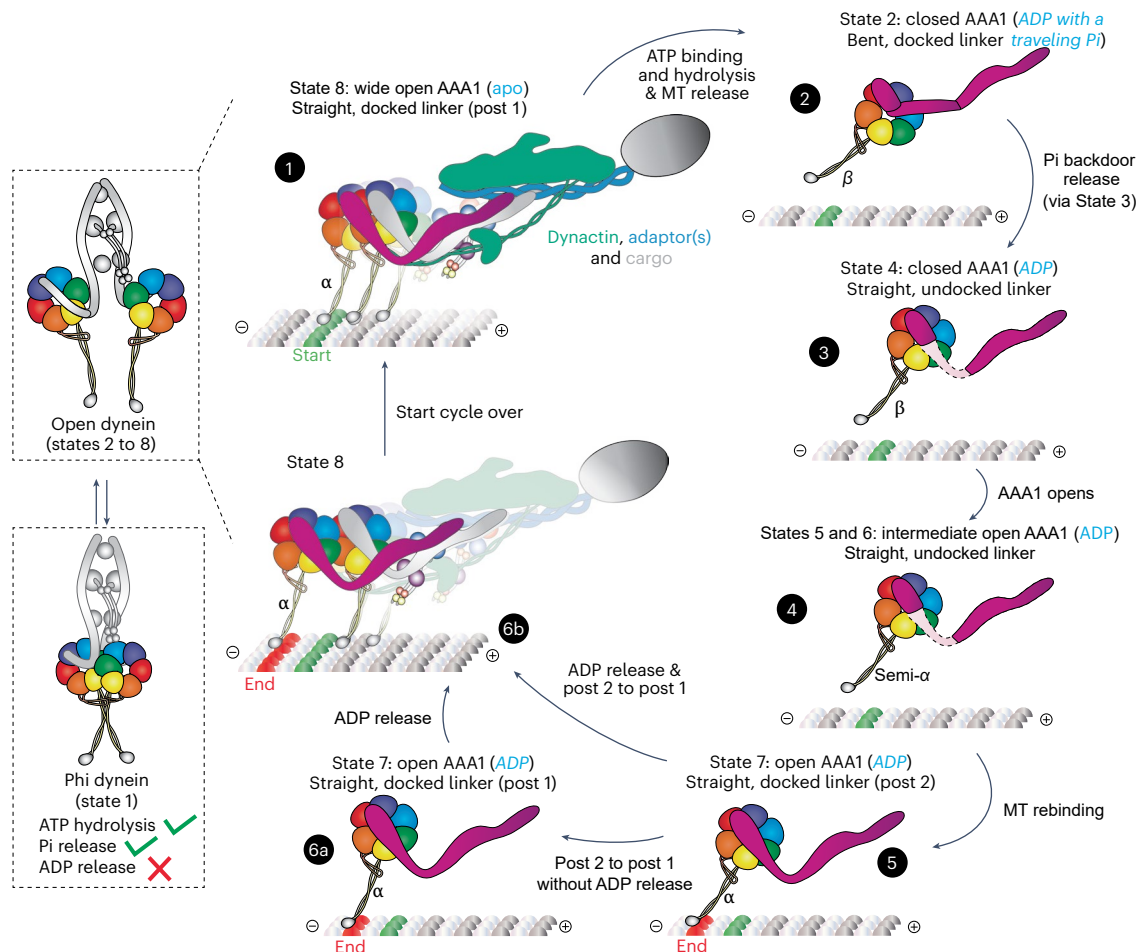


Fig. 6 | Model for the mechanochemical cycle of full-length human dynein 1.

The motor domains in phi dynein adopt an autoinhibited state and are capable of ATP hydrolysis and Pi release but not ADP release. (1) The mechanochemical cycle of the active motor states starts with the apo AAA1 state. (2) ATP binding and/or hydrolysis induces pocket closure and triggers MT release. The linker changes from a straight, stably docked configuration to a bent, stably docked

conformation. (3) Release of Pi through the backdoor channel triggers the straightening of the linker. (4) A semi-α registry of the stalk-CC helps dynein for MT rebinding. (5) Linker docked at post 2 site provides a net bias toward the minus end of MTs and dynein rebinds to MTs. (6a,6b) The linker docking mode changes from post 2 to post 1 and ADP is released, thus completing the cycle.

straightening, likely causing the AAA+ ring to rotate relative to the tail rather than pulling it forward, as dynein is in a low-MT-binding-affinity state⁸² (Fig. 5e (iii)). Following AAA1 pocket opening, the motor can rebound to MTs with an ADP-bound open pocket. If the motor domain adopts the post 1 mode, the MTBD returns to its original position without any forward stepping (Fig. 5e (iv-a)). By contrast, the motor domain in post 2 mode generates a net minus-end-directed bias, allowing the dynein MTBD to bind 8 nm forward (Fig. 5e (iv-b)). Supporting this model, the MT-unbound motors in state 7 (ADP-bound open AAA1) primarily adopt the post 2 mode in the presence of ATP (Fig. 2a). Lastly, the transition from ADP post 2 to ADP post 1 or apo post 1 results in an 8-nm translocation of the cargo-binding tail domain (Fig. 5e (v)).

AAA1–AAA3 crosstalk is revealed in motor domain structures

Our comprehensive collection of dynein structural states reveals that the regulatory AAA3 site can adopt both closed (ATP-bound or AMPPNP-bound) and open (ADP-bound) conformations. Previous studies showed that AAA3 acts as a ‘switch’ to allosterically regulate AAA1 activity and the dynein cycle^{49,59,83}. To uncover the key structural principles governing inter-AAA communication, we conducted a structural survey combined with statistical analysis of all available dynein 1 motor domain structures, including 43 structures from this study and 13 from previous studies (Extended Data Fig. 10 and Supplementary Data 2).

Detailed analysis, described in Supplementary Note 4, revealed a mutual inhibition model between AAA1 and AAA3. In this model, closure of either pocket (through ATP, AMPPNP or ADP-Vi binding) inhibits ADP release from the opposing pocket and locks it in the open conformation.

Discussion

Technical advances to explore the dynein mechanochemistry

In this work, we demonstrate that our improved cryo-EM pipeline (Fig. 1) enables the identification of the conformational landscape of full-length human dynein 1 in reaction with ATP, unlike previous studies that mostly relied on truncations, mutants or specific nucleotide conditions^{44,46,47,49}. Our pipeline not only captures distinct states but also resolves them at high resolutions, including the 2.2-Å phi motor domain structure, enabling detailed atomic-level analysis at the reactive center (Fig. 2). By systematically examining dynein structures off and on MTs, we provide a comprehensive view of its mechanochemical cycle (Figs. 2–5). The numerous intermediate states reported in this work, which are technically challenging to capture using conventional approaches, substantially expand our understanding of the dynein conformational landscape.

The release of Pi through a molecular backdoor mechanism

We present structural and functional evidence for a backdoor Pi release mechanism, involving subtle rearrangements of the AAA1 sensor I loop

(Fig. 3). In the presence of ATP, two key residues (N2019 and Y2022) in the sensor I loop adopt various conformations across different states. We propose that N2019 senses Pi cleavage while Y2022 gates Pi release. This is supported by N2019's close interaction with the vanadate group in the ADP-Vi-bound state (<3 Å). ATP cleavage forces N2019 outward to accommodate Pi, while Y2022's conformational flexibility creates a gated exit for Pi. Once the Pi is fully released, both residues stably contact the Walker A motif, completing this process to allow subsequent pocket opening.

Our data also indicate that sensor I loop conformational changes transmit Pi release signals to linker straightening for open dynein (Supplementary Note 2). This finding explains why both bent and straight linkers are observed in the presence of ATP-Vi for human dynein 1 (this study), yeast dynein 1 (ref. 49), dynein 2 (ref. 44) and axonemal dynein c (refs. 51,79). As Pi is in the process of releasing through the backdoor, vanadate can attack the active site while the linker remains in multiple intermediate states, thereby stabilizing dynein in a closed motor ring state with either a bent or straight linker.

Dynein conformation distribution under various conditions

Our findings reveal that nucleotide conditions influence the conformational distribution of the motor domain from full-length dynein (Fig. 2 and Supplementary Note 1). A recent structure of the DDA-LIS1 complex bound to MTs in the presence of AMPPNP revealed a pre-powerstroke state motor domain bound to LIS1 (ref. 23). The AAA1 pocket binds ADP with a closed conformation, which is speculated to be induced by LIS1 (ref. 23). Our results demonstrate that this motor state (state 2), without LIS1 binding, can be directly observed under ATP, ADP, AMPPNP and even apo conditions in the absence of LIS1 (Extended Data Fig. 4), indicating that this is an intrinsic property of the dynein motor domain. However, we cannot rule out that the presence of LIS1 may stabilize and enrich this state in the context of an MT-bound DDA complex²³. In the future, by comparing the conformational landscape of dynein with LIS1 or other regulators to the many structures reported in this study, the impacts of regulators on dynein can be better characterized.

Model for full-length human dynein 1 mechanochemical cycle

Our work greatly improves our understanding of dynein's mechanochemical cycle (Fig. 6). We find that the motor domain from the auto-inhibited phi dynein is competent for ATP hydrolysis and Pi release. However, the closed AAA1 pocket in phi dynein sterically hinders ADP release, as the Pi release channel is not sufficiently large to accommodate ADP release (Fig. 3). The motor domains engaged in the mechanochemical cycle are predominantly in ADP-bound states, consistent with ADP release being the rate-limiting step and with the fact that dynein only possesses basal ATPase levels off MTs^{70,76}. By comparing the conformational landscapes for dynein on and off MTs, we propose a model to explain its mechanochemical cycle with MTs. Note that dynein can still progress through this cycle when unbound from MTs (Fig. 2).

(1) ATP binding to the apo AAA1 state (state 8) and/or subsequent hydrolysis induces pocket closure, triggering a stalk-CC registry shift from α to β and a transition of the linker from straight to bent³³. (2) Following ATP hydrolysis, the sensor I loop facilitates Pi release from AAA1 through a molecular backdoor mechanism. After the release of Pi, the linker is straightened but not stably docked on the closed motor ring. (3) The ADP-bound AAA1 pocket gradually transitions from closed to intermediate and open states. Throughout this process, the buttress and stalk respond to the pocket-opening signal across the motor ring, resulting in semi- α and α registry stalk-CC configurations. Meanwhile, the straight linker first docks at the post 2 position, allowing the 8-nm forward stepping of dynein and initial translocation of cargo (less than 8 nm). (4) Further widening of the AAA1 pocket ensures ADP release and dynein further pulls its cargo by 8 nm by changing docking modes from post 2 to post 1, completing the current cycle. The powerstroke

process diverges substantially from previous models^{33,34,84,85}, revealing a more active role for the linker, as detailed in Supplementary Note 5.

It is important to note that our current model centers on the AAA1. While we identified crosstalk between AAA1 and AAA3, our cryo-EM data do not yet conclusively resolve the ongoing debate regarding whether the hydrolysis of two ATP molecules at both AAA1 and AAA3 is required⁸⁶ or whether only one ATP at AAA1 is hydrolyzed per dynein step^{87,88}. Furthermore, this model focuses solely on a single motor domain. However, our full-length dynein structures also offer insights into potential coordination between the two motor domains both on and off MTs, a topic that will be explored in future studies incorporating intramolecular tension^{41,53,89}.

Our model, supported by previous structural studies and over 40 high-resolution structures in this study, sheds light on many long-standing questions in the field³³. Our approach to determining the conformational landscape of dynein in reaction revealed critical mechanistic details that were previously inaccessible because of prior methods favoring conformational homogeneity. Beyond generating testable hypotheses (through mutagenesis and functional assays), our high-resolution intermediates offer a structural foundation for computational simulation⁹⁰ and protein engineering⁹¹. Moreover, our high-resolution dynamic cryo-EM analysis establishes a paradigm that can be extended to gain deeper mechanistic insight into the entire dynein transport machinery, including DDA with or without cargo/load on MTs^{15,23,92} and its regulation by cofactors such as LIS1 (ref. 20) and Ndel1 (ref. 27).

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at <https://doi.org/10.1038/s41594-025-01543-3>.

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Methods

Purification of human full-length dynein 1 and dynein motor domain

The plasmid encoding full-length human dynein 1 was graciously provided by the A. Carter lab (Addgene, plasmid 11903). Both full-length dynein 1 and the α -registry-locked motor domain were expressed in Sf9 insect cells (American Type Culture Collection) using recombinant baculovirus generated by transfecting DH10MultiBac-derived Bacmid (Geneva Biotech) with Cellfectin II (Gibco). Sf9 cells (2.5 million cells per ml) were infected with 28 ml of P2 virus per 1.4 L of culture and incubated for 75 h. Cells were harvested by centrifugation (1,000 rcf, 15 min, 4 °C), flash-frozen in liquid nitrogen and stored at -80 °C. Thawed pellets (1.4 L of culture) were resuspended in 100 ml of lysis buffer (50 mM HEPES pH 7.2, 100 mM NaCl, 1 mM DTT, 0.1 mM ATP and 10% glycerol) with two Roche protease inhibitor tablets and 2 mM PMSF and then lysed by Dounce homogenization (15–25 strokes on ice). Lysates were clarified by ultracentrifugation (371,000 rcf, Ti70 rotor, 1 h, 4 °C) and incubated with 3 ml of IgG Sepharose beads (Cytiva) for 3 h at 4 °C. Beads were washed with 200 ml of lysis buffer followed by 200 ml of TEV buffer (50 mM Tris-HCl pH 7.4, 148 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 10% glycerol, 0.1 mM ATP and 1 mM DTT). TEV protease (400 μ g) was added, and the mixture was incubated overnight at 4 °C. The eluate was concentrated using a 100-kDa Amicon Ultra-15 filter (Millipore) and then purified by size-exclusion chromatography (TSKgel G4000SWXL 7.8/300 column) in GF150 buffer (25 mM HEPES pH 7.2, 150 mM KCl, 1 mM MgCl₂, 5 mM DTT and 0.1 mM ATP). Peak fractions were pooled and concentrated to 2.5–3 mg ml⁻¹ for cryo-EM grid preparation. Protein quality was verified by negative-staining EM (Extended Data Fig. 1).

Purification of yeast dynein motors

Purification of yeast dynein (wild type and mutants; ZZ-2 $\times$ TEV-6 $\times$ His-GFP-3 $\times$ HA-GST-dynein_{MOTOR}-HALO, all under the control of the galactose-inducible promoter, *GAL1p*) was performed as previously described^{17,93}. Briefly, yeast strains were generated with plasmids encoding wild-type or mutant dyneins integrated into the yeast genome (after digestion with *Apal*) as previously described^{17,93}. The strains were grown in YPA supplemented with 2% galactose, harvested, washed with cold water and then resuspended in a small volume of water. Cell pellets were flash-frozen in liquid nitrogen and lysed using a coffee grinder (Hamilton Beach). Lysates were mixed with 4 $\times$ lysis buffer (final 1 $\times$: 30 mM HEPES pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate, 0.2 mM EGTA, 1 mM DTT, 0.1 mM Mg-ATP and 0.5 mM Pefabloc SC) and clarified by centrifugation (310,000g, 1 h). The supernatant was then bound to IgG Sepharose 6 fast flow resin (Cytiva) for 2–4 h at 4 °C, which was subsequently washed three times in 5 ml of lysis buffer and twice in TEV buffer (50 mM Tris pH 8.0, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 0.005% Triton X-100, 10% glycerol, 1 mM DTT, 0.1 mM Mg-ATP and 0.5 mM Pefabloc SC). To fluorescently label the proteins, the bead-bound protein was incubated with either 10 μ M JFX646-HaloTag or 10 μ M JFX549-HaloTag for 30–60 min at 4 °C. The resin was then washed four more times in TEV digest buffer and then incubated in TEV buffer supplemented with TEV protease overnight at 4 °C. Following TEV digest, the protein-containing supernatant was collected using a spin filtration device, aliquoted, flash-frozen in liquid nitrogen and stored at -80 °C.

MT preparation

Porcine tubulin, either obtained from Cytoskeleton or purified in-house, was resuspended in MT buffer (25 mM MES, 70 mM NaCl, 1 mM MgCl₂, 1 mM EGTA and 1 mM DTT, pH 6.5) to a final concentration of 10 mg ml⁻¹, flash-frozen and stored at -80 °C. MTs were polymerized using a previously described protocol¹⁵. In brief, tubulin was diluted in MT buffer supplemented with 3 mM guanosine triphosphate. The mixture was incubated on ice for 5 min and subsequently transferred

to a 37 °C incubator for 1 h. Following polymerization, 5 μ M paclitaxel (MilliporeSigma) was added to the mixture. The MTs were then pelleted (20,000 rcf for 8 min at room temperature) and resuspended in MT buffer containing 5 μ M paclitaxel.

ATPase measurements

Basal and MT-stimulated ATPase activities were measured using the EnzChek Pi assay kit (Invitrogen). Assays were performed in motility buffer (30 mM HEPES pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA and 10% glycerol) supplemented with 1 mM Mg-ATP, with or without 2 μ M taxol-stabilized MTs and 5 nM 6 $\times$ His-GST-dynein_{MOTOR} (wild type or mutants). Reactions were initiated with the addition of dynein and the absorbance at 360 nm was monitored by plate reader (BMG LabTech CLARIOstar Plus) for at least 20 min. Background Pi release levels (presumably from MTs) for each reaction were measured for 2–5 min before addition of dynein motors. Relative ATPase levels were determined by fitting linear regressions to the time-resolved absorbance values and determining the slope from each. Data shown are from 4–6 independent replicates from two independent protein preparations.

Single-molecule motility assays

Single-molecule motility assays were performed as previously described^{17,25} with minor modifications. Flow chambers were constructed using plasma-cleaned, silanized coverslips and slides separated by double-sided adhesive tape. Chambers were coated with anti-tubulin antibodies (YL1/2, 8 μ g ml⁻¹; Accurate Chemical and Scientific Corporation) and blocked with 1% Pluronic F-127 (Fisher Scientific). Taxol-stabilized MTs assembled from unlabeled porcine tubulin (Cytoskeleton) were introduced into the chamber. After incubation for 2–5 min, the chamber was washed with motility buffer supplemented with 20 μ M taxol and 1 mM DTT. Dynein, diluted in the same oxygen-scavenging motility buffer, was added to the chamber. Total internal reflection fluorescence (TIRF) microscopy imaging was performed immediately using a Nikon Ti-2E microscope equipped with an iLAS2 RING TIRF system, a 1.49 numerical aperture $\times$ 100 TIRF objective, a Ti2-SS-E motorized stage, piezo Z control and an iXon LIFE 897 electron-multiplying charge-coupled device camera (Andor). Lasers (561 or 640 nm; LUN-F 3, Nikon) and a multipass quad filter cube (C-TIRF for 405/488/561/638 nm; Chroma) with emission filters (600/50 nm and 700/75 nm; Chroma) were used. To determine MT polarity (because of the diffusive or bidirectional behavior of the Y1902A mutant), wild-type GST-dynein_{MOTOR}-Halo^{JF549} (diluted in oxygen-scavenging motility buffer) was introduced into the imaging chamber immediately after acquiring a video of the mutant GST-dynein_{MOTOR}-Halo^{JF646}. To image nonfluorescent MTs, we used interference reflection microscopy, as previously described⁹⁴. Images of dynein motors were acquired at 2-s intervals for 8 min. Velocity and run length values were determined from kymographs generated using the MultipleKymograph plugin for Fiji/ImageJ. Data shown are from three independent replicates from two independent protein preparations.

Cryo-EM sample and grid preparation for dynein unbound from MTs

For AMPPNP, ATP-Vi and ADP conditions, dynein was diluted to 2.0 mg ml⁻¹ in GF150 buffer (25 mM HEPES pH 7.2, 150 mM KCl, 1 mM MgCl₂, 5 mM DTT and 0.1 mM ATP) and incubated with 5 mM ATP-Vi, AMPPNP or ADP for 1 h on ice before vitrification (Extended Data Fig. 4). Dynein in ATP conditions was incubated with 5 mM ATP and vitrified immediately. For apo conditions, dynein was diluted into GF150 buffer (without ATP), concentrated using an Amicon concentrator (three cycles, $\sim$ 8,000-fold ATP dilution) and adjusted to 2 mg ml⁻¹. Samples were applied to Quantifoil grids (R2/1300-mesh gold or R2/200-mesh), incubated for 5 s at 4 °C and 100% humidity (Vitrobot Mark IV), blotted for 3–5 s and plunge-frozen in liquid ethane. The cryo-EM sample preparation of the α -registry-locked motor domain

in ATP or ATP-Vi conditions was performed as previously described²¹, using graphene-oxide-coated grids with a sample concentration of 0.1 mg ml⁻¹ and similar vitrification parameters.

Cryo-EM sample and grid preparation for dynein bound to MTs
For dynein–MT complexes (Extended Data Fig. 2), dynein in apo buffer was mixed with MTs in MT-binding buffer (30 mM HEPES pH 7.2, 60 mM KCl, 1 mM EGTA, 5 mM MgSO₄, 1 mM DTT and 5 μM paclitaxel) at room temperature. Complexes were pelleted (20,000 rcf, 8 min) and resuspension volume (optimized by negative-staining EM) was adjusted for cryo-EM. For ADP and AMPPNP conditions, the buffer was supplemented with 5 mM ADP or AMPPNP. A 3.5-μl sample was applied to Quantifoil grids (R2/1, 300-mesh gold), incubated for 4 s, blotted for 3–6 s and plunge-frozen in liquid ethane using a Vitrobot Mark IV (FEI) at 95% humidity and 22 °C.

Cryo-EM data collection

All grids were screened using a Glacios microscope (Yale Science Hill Cryo-EM facility). Final datasets were collected at three facilities: the Yale Science Hill Cryo-EM facility (Glacios microscope, 300 keV, K3 detector), the Yale West Campus Cryo-EM facility (Krios microscope, 300 keV, K3 detector, Bioquantum energy filter) and the Laboratory for BioMolecular Structure (LBMS) at Brookhaven National Laboratory (Krios microscope, 300 keV, K3 detector, Bioquantum energy filter).

Automatic data collection was facilitated by SerialEM⁹⁵ or EPU software. For MT-unbound dynein, 33,302 (ATP), 6,016 (ATP-Vi), 7,488 (AMPPNP-low Mg²⁺), 8,355 (AMPPNP-high Mg²⁺), 5,920 (ADP) and 5,884 (Apo) videos were acquired. For MT-bound dynein, 9,935 (Apo-MT), 11,828 (ADP-MT) and 5,690 (AMPPNP-MT) videos were collected. For the α-registry-locked human dynein 1 motor domain, 9,498 (ATP) and 2,466 (ATP-Vi) videos were collected. Detailed parameters are in Supplementary Tables 1–8.

Cryo-EM image processing for dynein unbound from MTs

Preprocessing steps, including motion correction, contrast transfer fraction (CTF) estimation and particle picking, were conducted either in cryoSPARC Live⁹⁶ or with an in-house script using MotionCor2 (ref. 97), GCTF⁹⁸ and Gautomatch. Cryo-EM scripts for real-time data transfer and on-the-fly preprocessing are available on GitHub (<https://github.com/JackZhang-Lab>).

For the dynein–ATP off MT dataset (Extended Data Figs. 1 and 2), the blob picker was used to select motor domains in phi or open conformations, with the cross-correlation score set to 0.01 to enable sampling of all possible motors during the picking step. A total of 8,534,500 particles were extracted using a box size of 400 pixels, subsequently binned to 200 pixels (pixel size: 1.664 Å) for faster computation. From this pool, a subset of 640,000 particles underwent subsequent rounds of two-dimensional (2D) classification, ultimately yielding 164,658 high-quality particles selected for ab initio reconstruction (six classes). During this phase, phi motor domains and several single motor domains were identified. Following the initial model acquisition, all original particles were used for subsequent heterogeneous refinement to prevent the loss of motor particles during 2D classification. The original particles were divided into four subsets, with each subset subjected to heterogeneous refinement (12 classes). This initial heterogeneous refinement process was iterated three times, with updated references provided each time, successfully identifying phi motors, single motor domains with bent or straight linkers and several junk classes.

Each motor class from four subsets was merged for additional heterogeneous refinement or 3D classification without alignment. This step further explored the dynamics of the motor domain. For example, state 4, characterized by a straight linker, was initially obscured within the state 2 class because of the closed motor ring conformation. Similarly, states 5–7 were initially grouped into a single class and later separated during subsequent heterogeneous refinement. The second-level

heterogeneous refinement was conducted three times, with updated references provided each time. Further classification of individual classes ceased to reveal further conformations because of limited particle numbers and achievable resolution. An increase in dataset size may unveil additional intermediate or transient conformations.

Following homogeneous refinement, most classes achieved resolutions at the Nyquist limit of 3.3 Å. Subsequently, particles were re-extracted at the full box size of 400 pixels (0.832 Å). For high-resolution refinement, two rounds of global and local CTF refinement followed by local refinement were performed. The resolution of single motor domains from open dynein reached 2.9–3.6 Å, while phi motor domains achieved 2.6 Å using C₂ symmetry. To further enhance the resolution of phi motor domains, symmetry expansion of the phi motor domain was executed to account for intermotor domain dynamics. Particles were recentered on one of the motor domains and re-extracted using a box size of 640 pixels to incorporate delocalized high-resolution information. After localized refinement and another round of global and local CTF refinement, the phi motor domain achieved 2.25-Å resolution.

For full-length phi dynein reconstruction, two sets of particles were considered: (1) initial refinement-derived junk particles, refined against the dynein tail map (EMD-3703)¹⁰ and junk references to identify tail-containing classes; (2) phi motor particles recentered on the tail and re-extracted. Merged particles underwent heterogeneous refinement targeting the tail, yielding classes with clear tail features. Selected classes were re-extracted (1,024-pixel box, binned to 256 pixels) and refined to 6.8 Å. To enhance tail resolution, particles were recentered, masked and locally refined, achieving 4.3 Å overall (tail) with regional variability. Focused masks for the N-terminal dimerization domain (NDD), middle and neck regions improved local resolution (middle, 3.8 Å side-chain density; NDD and neck, 6.5 Å secondary structure). Localized refined maps were fitted to the consensus map and stitched together in ChimeraX⁹⁹ using the ‘volume maximum’ command to create a composite map of full-length human phi dynein. The resolutions of all maps were estimated by Fourier shell correlation calculations^{100,101} embedded in cryoSPARC. Local resolution analysis¹⁰² was performed in cryoSPARC.

Datasets of dynein under ATP-Vi, AMPPNP, ADP and apo conditions underwent processing using the same procedures. The simple flow-chart and map quality are summarized in Extended Data Figs. 4 and 5.

Cryo-EM image processing of dynein bound to MTs

For cryo-EM image processing of dynein–MT datasets, we used a pipeline previously developed for MT tracing and MT signal subtraction (Extended Data Figs. 3 and 5)^{73,74}. Initially, MT particles were detected using template matching with a distance cutoff of 8 nm, with a relatively low cross-correlation score set to include MT particles with low contrast or located at crossover locations. Following three rounds of 2D classification, false-picked MT particles, including carbon edges and miscentered MTs, were rejected. The metadata of the selected particles were converted to RELION style using ‘csparc2star.py’ in pyem (<https://zenodo.org/records/3576630>) and the coordinates were recentered and subjected to ‘multicurve fitting’. After MT signal subtraction, original micrographs were replaced by MT signal subtracted micrographs.

Subsequently, the blob picker was used to detect particles in these new micrographs, which were then extracted using a box size covering the single motor domain. A 2D classification was used to remove junk particles and residual MT particles, after which the selected particles underwent ab initio reconstruction. All original particles were used for initial heterogeneous refinement. The 3D classification-based particle sorting greatly aided in identifying dynein particles with low signal-to-noise ratios because of residual MT signal influence. After several rounds of sorting, classes with clear features were selected and subjected to local, global and local CTF refinement. The resolution of individual motor domains ranged from 2.9 Å to 3.6 Å.

For the reconstruction of full-length dynein bound to MT, particles were re-extracted using a large box size covering two motor domains.

The particles were directly reconstructed into a volume featuring the weak density of the second motor. Subsequently, this map was low-pass-filtered to 60 Å for heterogeneous refinement, where some classes exhibited enhanced density for the second motor domain. We then manually created a two-headed dynein map. This map, together with previous reconstructions, was used for next rounds of heterogeneous refinement. Ultimately, three main states were predominantly observed for each dynein–MT dataset: (1) two stable and parallel heads, (2) one stable leading head and (3) one stable trailing head. Limited particle numbers and resolution precluded detection of motors with differing step sizes in dynein–MT datasets.

Additionally, we attempted to reconstruct dynein MTBD–MT by reverting the MT signal using the original micrographs. Unlike prior out-arm dynein–MT structures with defined tubulin density⁵⁴, the dynein 1–MT protofilament exhibited smeared density. Furthermore, subsequent 3D classification without alignment failed to generate a relatively stable stalk–MTBD–protofilament class because of the limited number of particles. These limitations precluded us from performing localized refinement on the MTBD–MT region.

Model building and refinement

For model building and refinement, we used two previously reported human dynein motor structures (PDB 5NUG (ref. 10) and 7Z8G (ref. 15)) as initial models for different states of dynein in ATP. Individual domains (linker, AAA large and AAA small) were rigidly docked into the cryo-EM map in UCSF ChimeraX. Subsequently, Namdinator¹⁰³, a molecular dynamics flexible fitting tool, was used to further refine the model into the cryo-EM map. Manual inspection and adjustments of the model were carried out in Coot (version 0.9.5)^{104,105}.

For water model building in the phi motor, the maps underwent sharpening using two methods: (1) different *B*-factor sharpening with local filtering based on local resolution in cryoSPARC and (2) density modification with phenix.resolve_cryo_em¹⁰⁶ using only maps as input. The processed maps were upsampled with a pixel size of 0.416 Å and cropped to a box size of 400 pixels. Initial solvent water molecules were placed using phenix.douse, resulting in approximately 2,358 automatically placed water molecules. Subsequent manual inspection in Coot led to the addition or deletion of water molecules, resulting in a final placement of 1,897 water molecules.

All models underwent iterative refinement using PHENIX real-space refinement 1.21rc1_5190 and manual rebuilding in Coot. These refined models were used as initial models for dynein in other nucleotide conditions or dynein bound to MTs. The quality of the refined models was assessed using MolProbity¹⁰⁷ integrated into PHENIX¹⁰⁶, with statistics reported in Supplementary Tables 1–8.

Vector map analysis of dynein motor domains

For vector map in Figs. 2d and 5b, we used the ‘PDBArrows’ script (<https://github.com/sami-chaaban/PDBArrows>). In brief, the isolated AAA domains or entire motor domains were aligned in ChimeraX (two structures per vector map). After alignment, the two structures were saved into the same coordinate system. PDBArrows was used to generate sequences of the two structures for multiple-sequence alignment using ClustalW. Even though the two structures are both human dynein, this step is necessary because one structure may be missing some residues during model building. A ‘bild’ file (a vector map of paired Cα distances) was then generated with the script using the two PDBs and sequence alignment files as inputs. The color command was manually added to bild file to color the individual domains.

Plots and molecular graphics

Plots were generated using GraphPad Prism. Molecular graphics were prepared using UCSF ChimeraX⁹⁹. Figures were generated using Adobe illustrator.

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

Models and cryo-EM maps were deposited to the PDB and EM Data Bank as outlined below.

Dynein in ATP buffer: PDB 9BLY and EMD-44681 (phi particle dynein), PDB 9BLZ and EMD-44682 (state 1), PDB 9BM0 and EMD-44683 (state 2), PDB 9BM1 and EMD-44684 (state 3), PDB 9BM2 and EMD-44685 (state 4), PDB 9BM3 and EMD-44686 (state 5a), PDB 9BM4 and EMD-44687 (state 5b), PDB 9BM5 and EMD-44688 (state 6), PDB 9BM6 and EMD-44689 (state 7a, post 2), PDB 9BM7 and EMD-44690 (state 7b, post 2) and PDB 9BM8 and EMD-44691 (state 7c, post 2).

Dynein in ATP–Vi buffer: PDB 9BMF and EMD-44697 (state 1), PDB 9BMG and EMD-44698 (state 2) and PDB 9BMH and EMD-44699 (state 4).

Dynein in 5 mM AMPPNP buffer with 2 mM Mg²⁺: PDB 9BMJ and EMD-44701 (state 1), PDB 9BML and EMD-44702 (state 2), PDB 9BMM and EMD-44703 (state 4), PDB 9BMN and EMD-44704 (state 5), PDB 9BMO and EMD-44705 (state 6) and PDB 9BMP and EMD-44706 (state 7, post 2).

Dynein in 5 mM AMPPNP buffer with 5 mM Mg²⁺: PDB 9DH5 and EMD-46856 (state 1), PDB 9DH6 and EMD-46857 (state 2), PDB 9DH7 and EMD-46858 (state 4), PDB 9DH8 and EMD-46859 (state 5), PDB 9DH9 and EMD-46860 (state 7, post 2) and PDB 9DHA and EMD-46861 (state 7, post 1).

Dynein in ADP buffer: PDB 9BMR and EMD-44708 (state 1), PDB 9BMS and EMD-44709 (state 2), PDB 9BMT and EMD-44710 (state 5), PDB 9BMU and EMD-44711 (state 6), PDB 9BMV and EMD-44712 (state 7, post 1) and PDB 9BMW and EMD-44714 (state 7, post 2).

Dynein in apo buffer: PDB 9BMY and EMD-44715 (state 1), PDB 9BMZ and EMD-44716 (state 2), PDB 9BNO and EMD-44717 (state 7, post 2) and PDB 9BNI and EMD-44718 (state 8, post 1).

Dynein bound to MTs: PDB 9BMA and EMD-44693 (apo, post 1), PDB 9BMB and EMD-44694 (ADP, post 1), PDB 9BMC and EMD-44695 (ADP, post 2) and PDB 9BMD and EMD-44696 (AMPPNP, post 1).

Dynein motor domain α registry mutant in ATP buffer: PDB 9BN3 and EMD-44720 (class 1) and PDB 9BN4 and EMD-44721 (class 2).

Dynein motor domain α registry mutant in ATP–Vi buffer: PDB 9BN5 and EMD-44722 (class 1) and PDB 9BN6 and EMD-44723 (class 2).

Previously reported atomic models used in this study (for model building and structural analysis) were obtained from the PDB under accession codes 5NUG, 7K58, 7K5B, 8FD6 and 7MGM. Additional data and materials can be obtained from the corresponding authors upon request. Source data are provided with this paper.

Code availability

All codes involved in general cryo-EM data processing and MT signal subtraction are publicly available from GitHub (<https://github.com/JackZhang-Lab>).

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Author contributions

K.Z. designed the study. J.Y. and Y.W. purified the full-length human proteins. J.Y., Y.W. and P.C. prepared the cryo-EM samples and collected the data. P.C., J.Y., Y.W. and K.Z. processed the images and built the PDB models. S.M.M. purified the human MT-B dynein motor domain. I.C.G. generated the yeast strains and purified the proteins from yeast. I.C.G. and S.M.M. performed and analyzed the ATPase and single-molecule assays. P.C., J.Y., Y.W., K.Z. and S.M.M. generated the figures and videos. P.C., J.Y. and K.Z. wrote the manuscript. P.C., J.Y., Y.W., K.Z. and S.M.M. edited and revised the manuscript. S.M.M. and K.Z. acquired funding.

Competing interests

The authors declare no competing interests.

Additional information

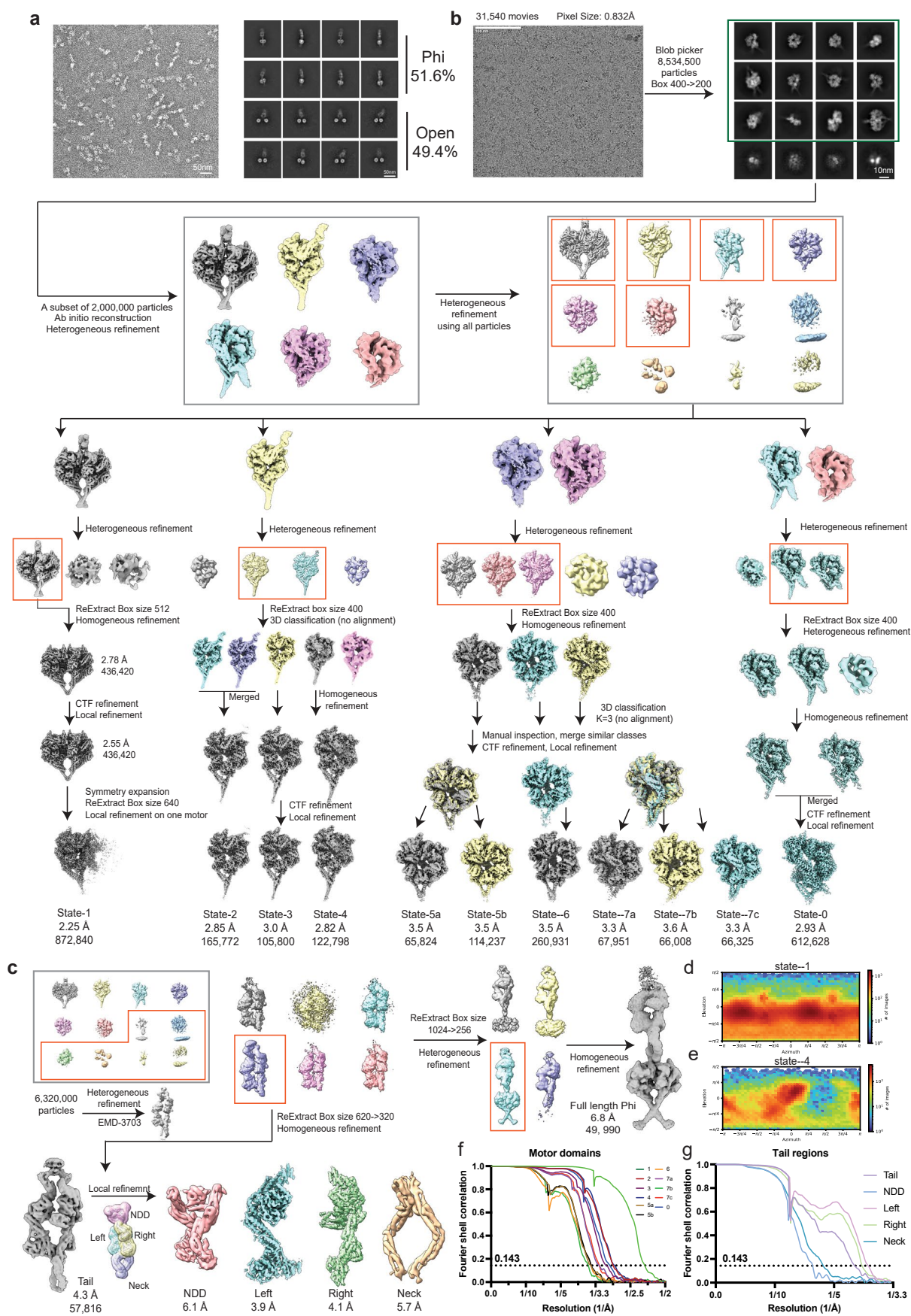
Extended data is available for this paper at <https://doi.org/10.1038/s41594-025-01543-3>.

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Correspondence and requests for materials should be addressed to Steven M. Markus, Yue Wang or Kai Zhang.

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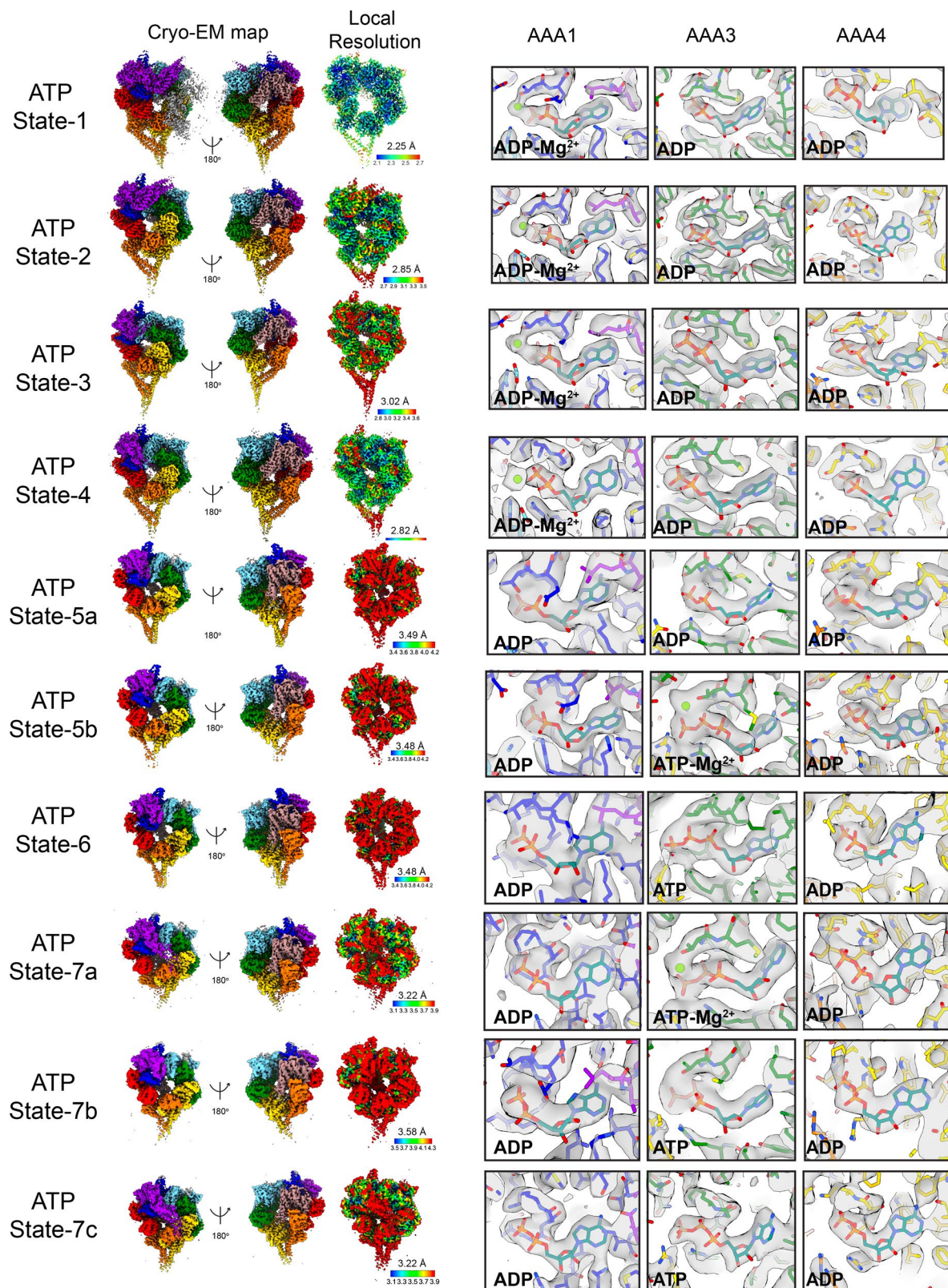
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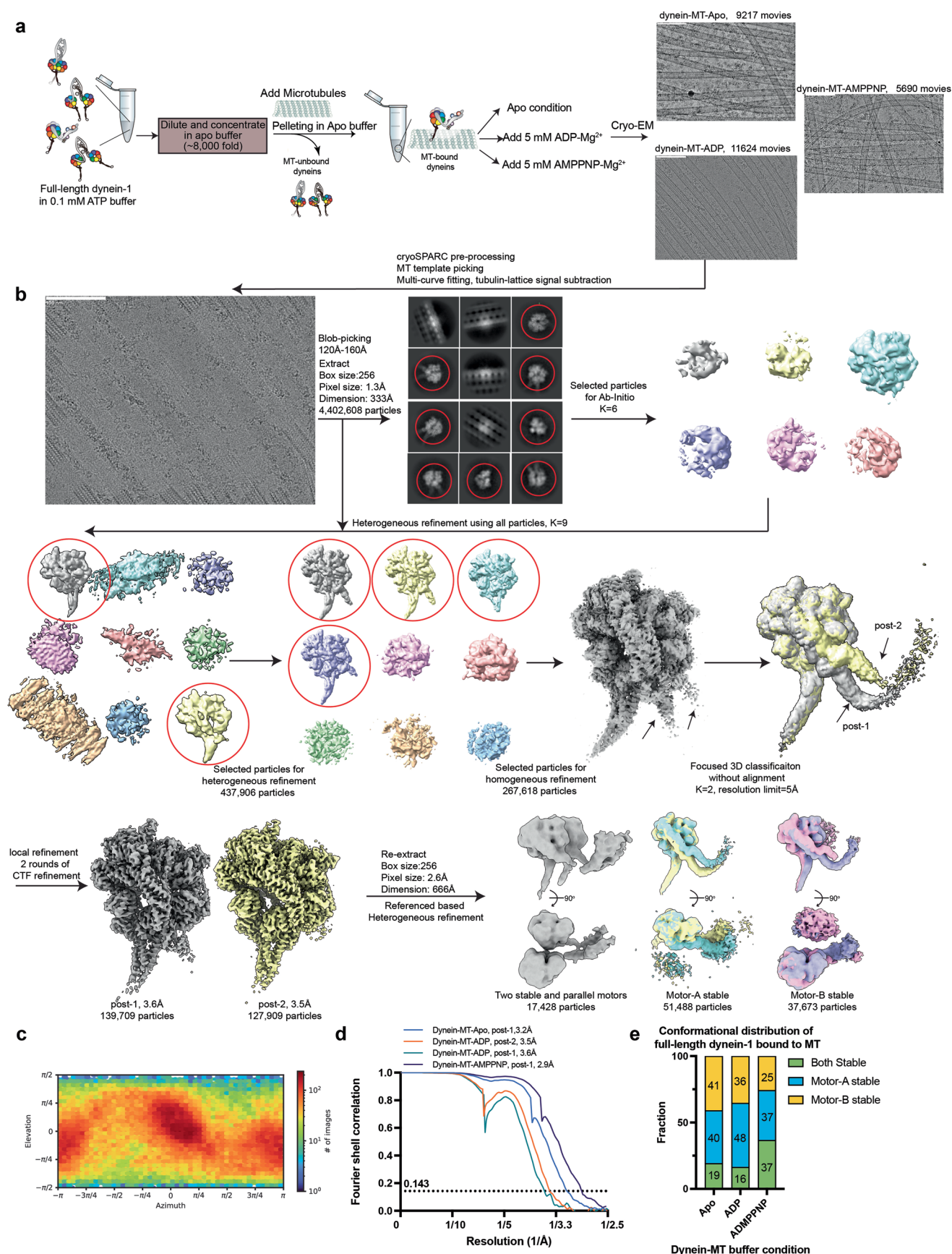
Extended Data Fig. 1 | See next page for caption.

Extended Data Fig. 1 | Cryo-EM data processing of full-length human dynein-1 in the presence of 5 mM ATP. (a) A representative negative staining micrograph (total 100 micrographs) of purified full-length human dynein-1 and corresponding 2D class averages. (b) A typical cryo-EM micrograph (total 33,302 micrographs) and the flowchart of cryo-EM image processing. (c)

Image processing of full-length phi dynein and tail region. (d, e) Orientation distribution of phi dynein and a representative state from open dynein. (f, g) The Fourier shell correlation (FSC) curves of the motor domain in different states and the tail domain. Datasets for dynein in other nucleotide conditions (ATP-Vi, AMPPNP, ADP, apo) were processed similarly.

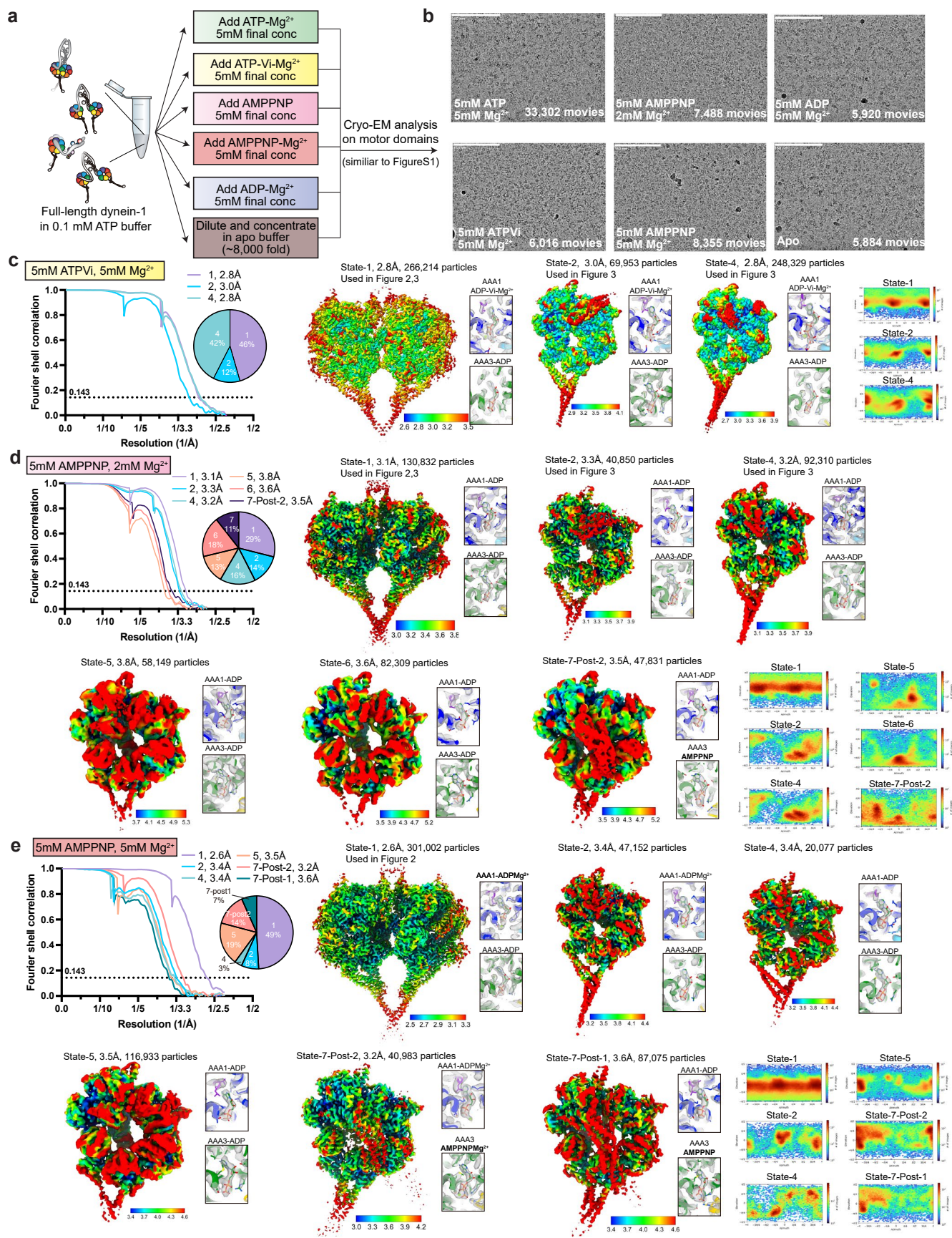


Extended Data Fig. 2 | Local resolution analysis and identification of bound nucleotides in AAA1, AAA3, and AAA4 in different states from the dynein-ATP dataset. The color scheme for the motor domain is consistent with Fig. 4.



Extended Data Fig. 3 | Cryo-EM data processing of full-length human dynein-1 bound to MTs. (a) Flowchart of sample preparation and typical cryo-EM micrographs of dynein bound to MTs in apo, ADP, and AMPPNP conditions. The number of micrographs is shown in the panel. **(b)** Microtubule signal subtraction

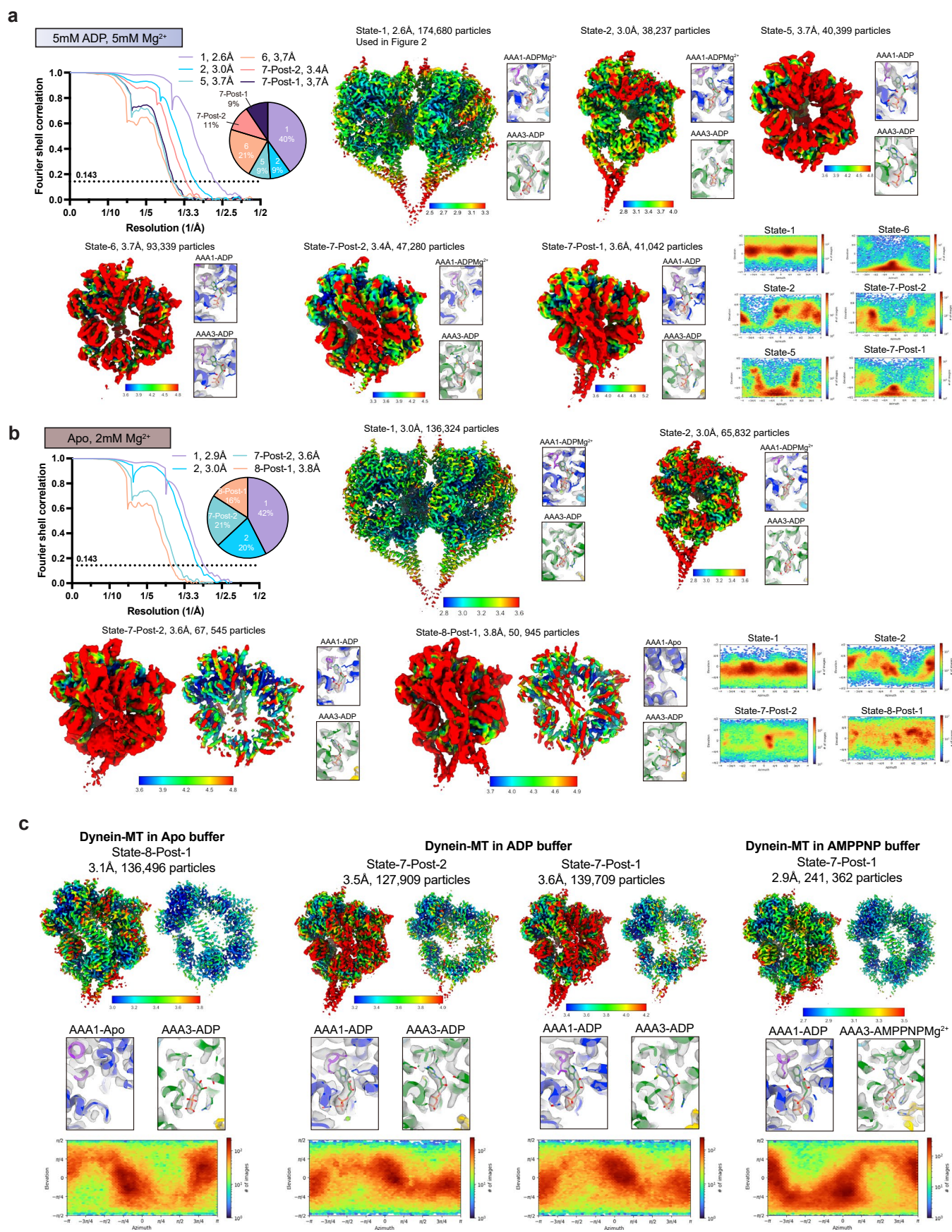
and image processing flowchart. **(c)** Orientation distribution of dynein-MT-ADP reconstruction. **(d)** FSC curves of four MT-bound motors. **(e)** Plot for the full-length dynein bound to MTs in different conformations (two stable heads, one stable trailing head, and one stable leading head).



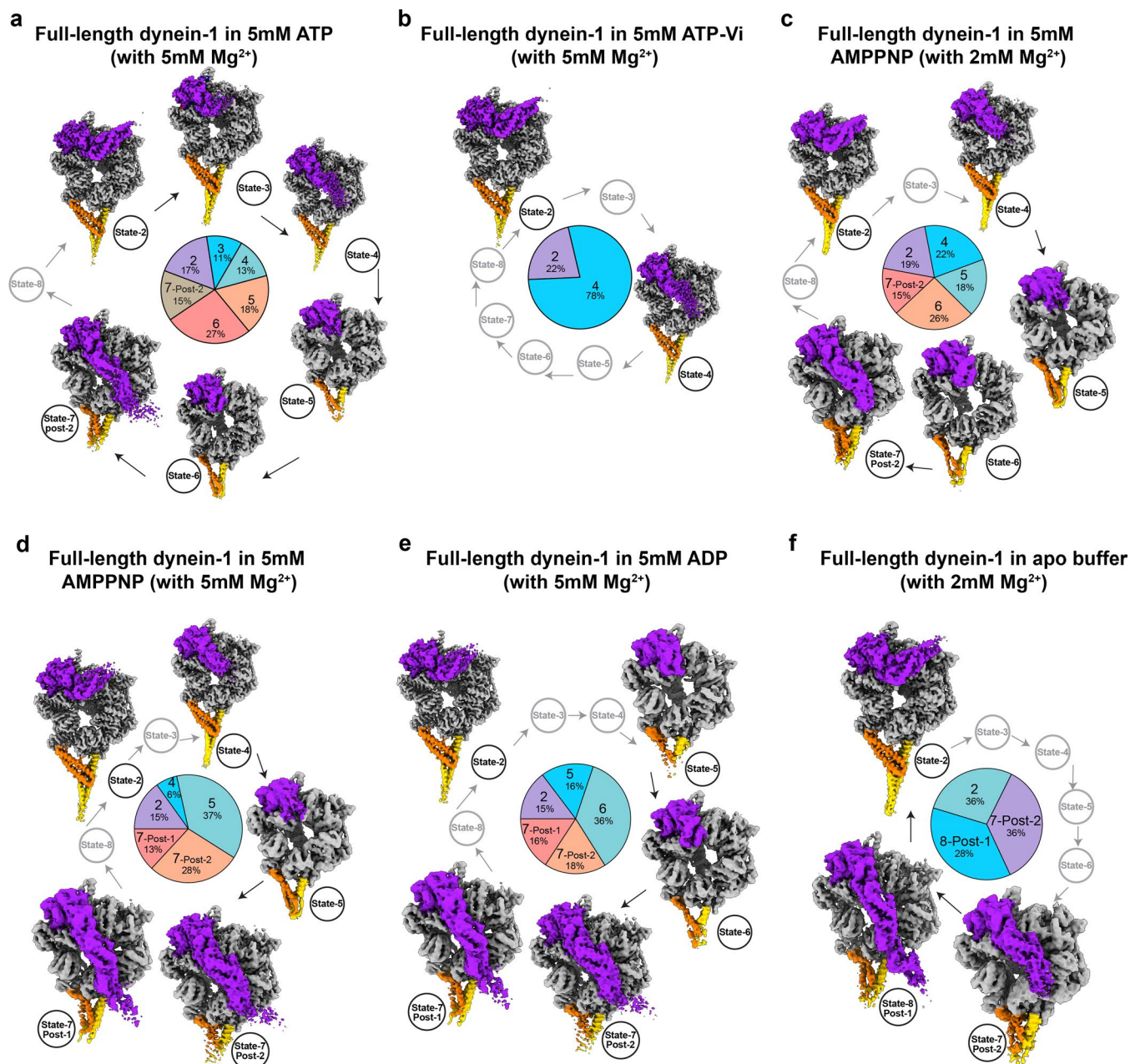
Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Cryo-EM analysis of dynein in ATP-Vi and AMPPNP conditions. (a) Cartoon schematic depicting the experimental method for cryo-EM analysis of full-length dynein in different nucleotide conditions. (b) Typical cryo-EM micrographs of dynein in different nucleotide conditions, with

total number of micrographs shown in the panel. (c, d, e) FSC curves and local resolution analysis of dynein in ATP-Vi, AMPPNP-low Mg^{2+} , and AMPPNP-high Mg^{2+} conditions.

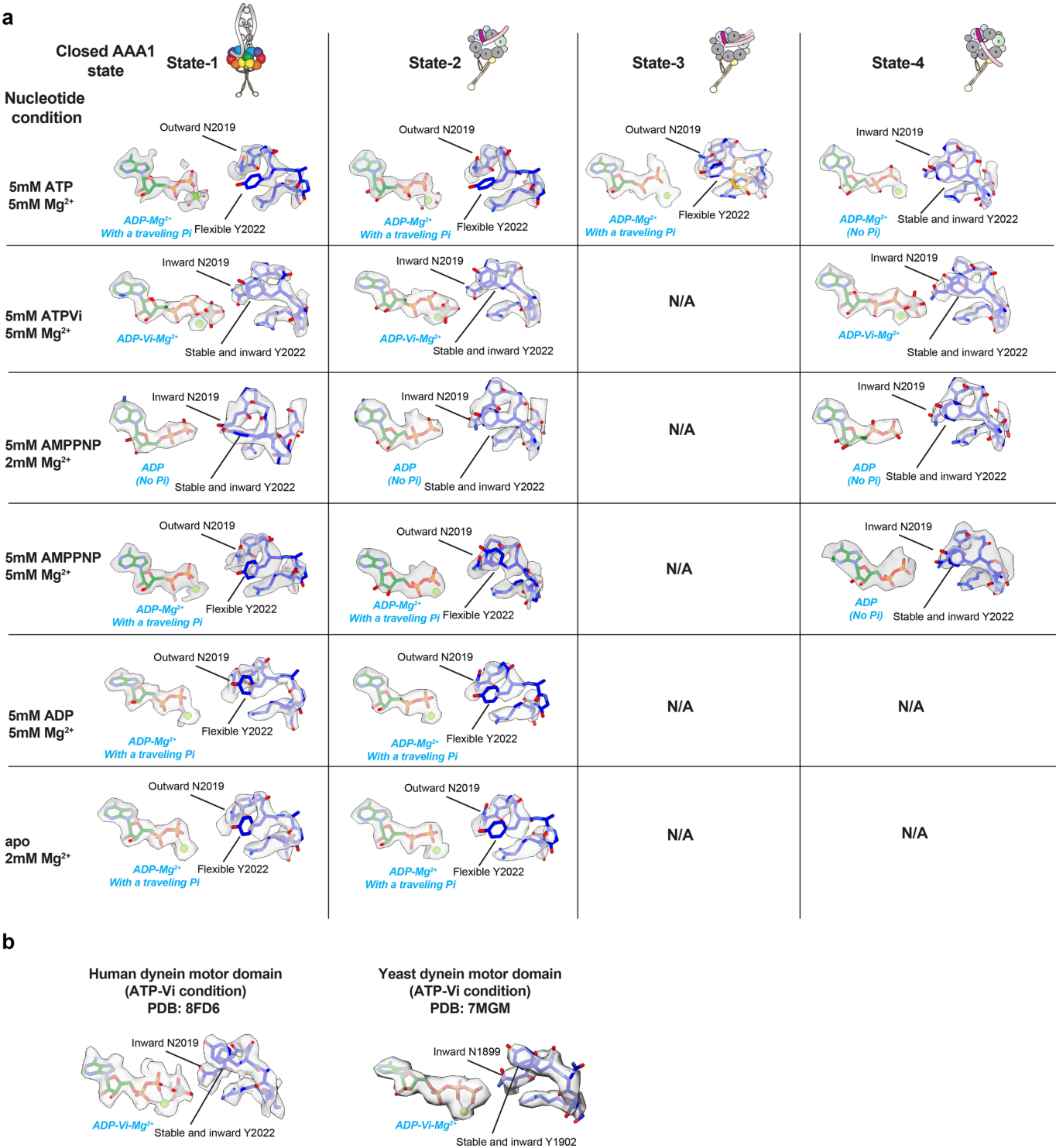


Extended Data Fig. 5 | Cryo-EM analysis of dynein in ADP and apo conditions and dynein-bound to microtubules. (a, b) FSC curves and local resolution analysis of dynein in ADP and apo conditions. (c) FSC curves and local resolution analysis of dynein-bound to microtubules.

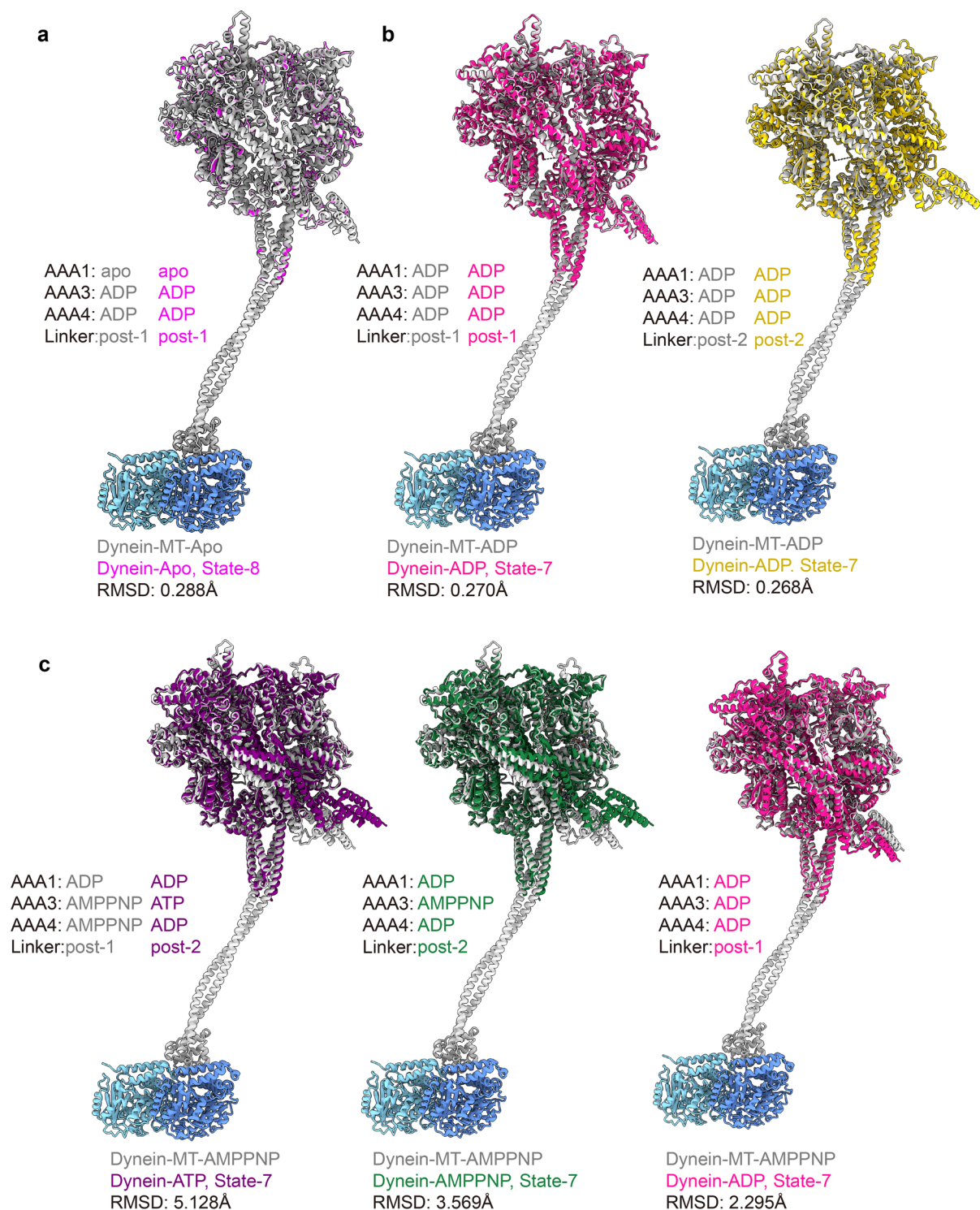


Extended Data Fig. 6 | The conformational landscapes of active cycle motor domains in different nucleotide conditions. (a-f) Conformation landscapes of motor domains in ATP, ATP-Vi, AMPPNP, ADP, and apo conditions. Arrows

indicate mechanochemical pathways. Percentages indicate the proportion of particles in each state. The missing states in each condition are shown in grey. Phi dynein is not included in this figure.

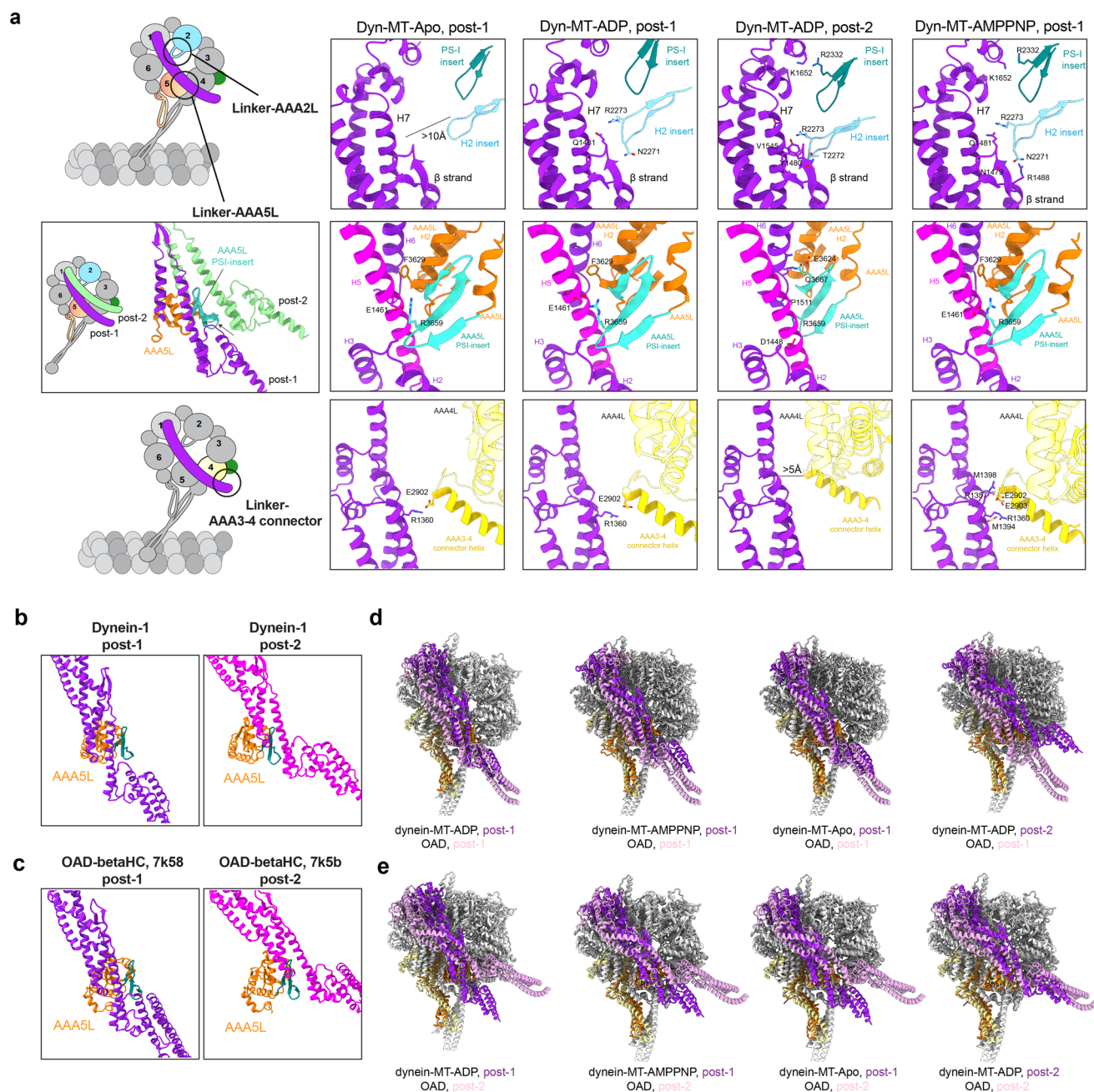


Extended Data Fig. 7 | Cryo-EM densities of the nucleotide and sensor-I loop in various closed AAA1 states. (a) From this work. **(b)** From previously published structures (PDB:8FD6 ref. 21, 7MGH ref. 78). The conformations of N2019 and Y2022 are highlighted.

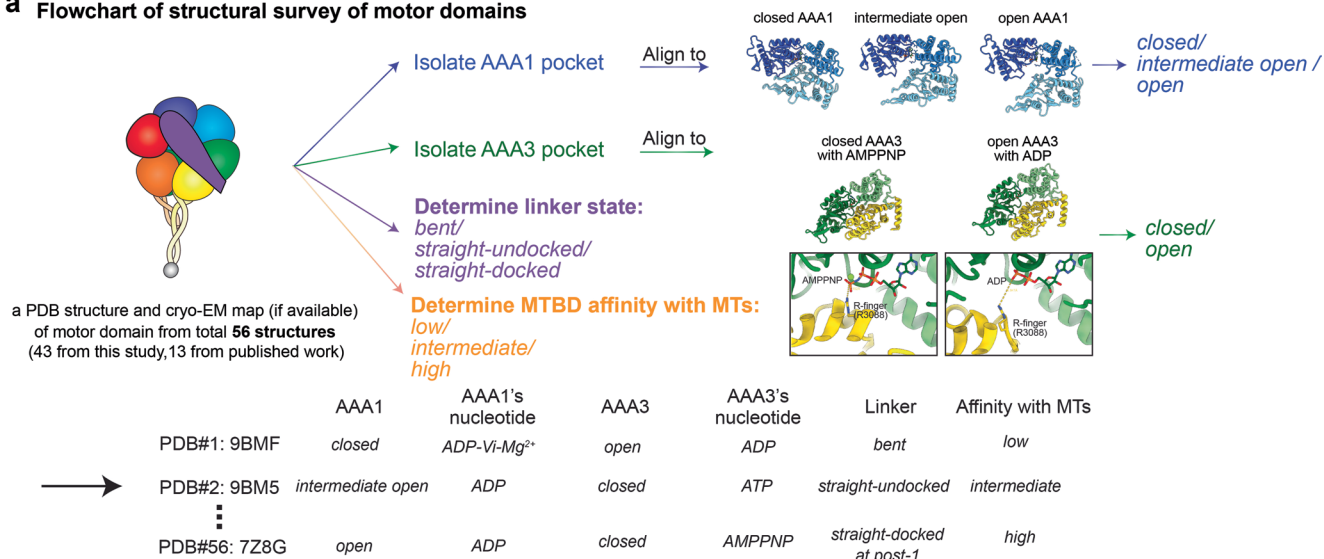
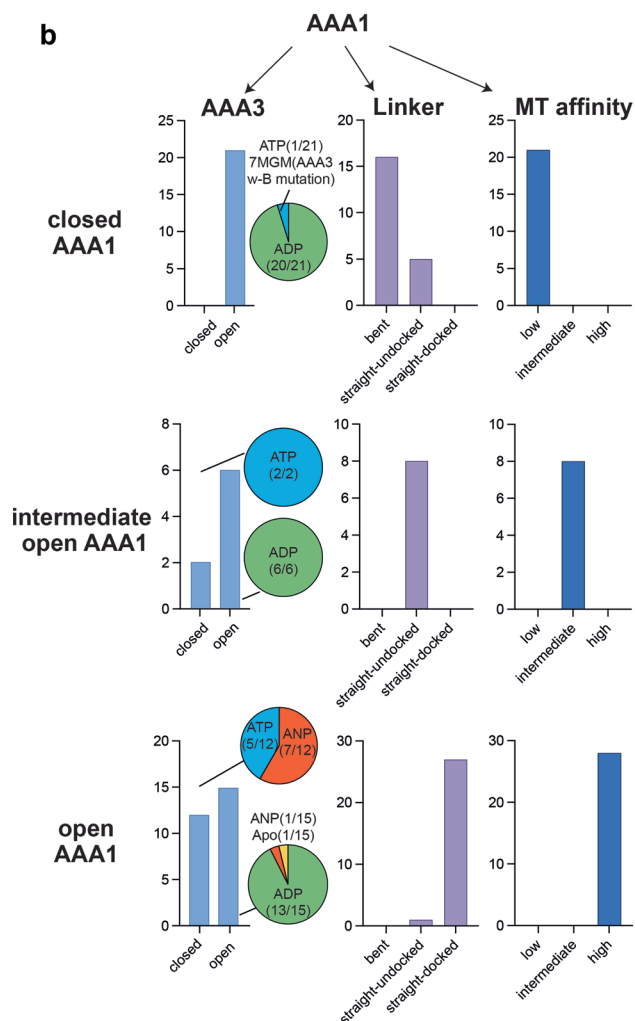
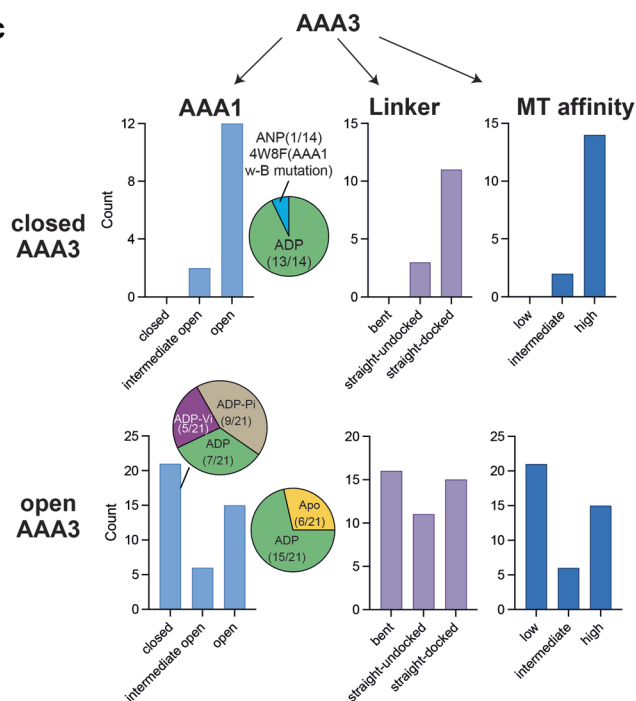
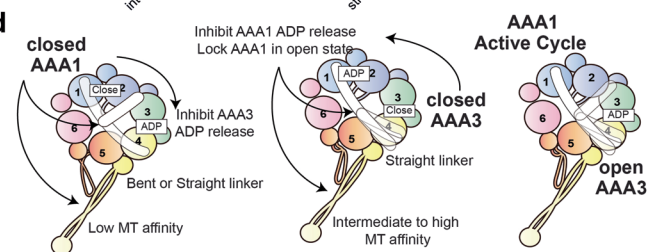


Extended Data Fig. 8 | Structural comparisons of MT-bound and -unbound dynein motors. (a) Comparison between MT-dynein in apo condition with state-8 motor domain. (b) Comparison between MT-dynein in ADP condition with state-7 motor domains. (c) Comparison between MT-dynein in AMPPNP

condition with state-7 motor domains. The nucleotide states in AAA1, AAA3, and AAA4 and the linker docking mode are listed. The MT-bound dynein motor domains (from linker to C-terminus) were used as references for structural fitting in ChimeraX.



Extended Data Fig. 9 | Systematic analysis of linker-ring interactions among four MT-bound motor domains. (a) Cartoon and molecular models showing the interaction interfaces including linker-AAA2L, linker-AAA5L, and linker-AAA3-4-connector helix. **(b-e)** Comparison of linker docking modes between dynein-1 and outer-arm dynein.

a Flowchart of structural survey of motor domains**b****c****d**

Extended Data Fig. 10 | Structural survey of motor domains and statistical analysis of crosstalk between AAA1 and AAA3. (a) Flowchart of structural survey of 56 motor domain structures. **(b)** Statistical graphs showing how AAA1

communicates to AAA3, linker and MTBD. **(c)** Statistical graphs showing how AAA3 communicates to AAA1, linker and MTBD. **(d)** Cartoon model summarizing communication between AAA1, AAA3, linker and MTBD.

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n/a Confirmed

- ☐ ☒ The exact sample size (n) for each experimental group/condition, given as a discrete number and unit of measurement
- ☐ ☒ A statement on whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
- ☐ ☒ The statistical test(s) used AND whether they are one- or two-sided
Only common tests should be described solely by name; describe more complex techniques in the Methods section.
- ☒ ☐ A description of all covariates tested
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- ☐ ☒ A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- ☐ ☒ For null hypothesis testing, the test statistic (e.g. F , t , r) with confidence intervals, effect sizes, degrees of freedom and P value noted
Give P values as exact values whenever suitable.
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Software and code

Policy information about [availability of computer code](#)

Data collection

For fluorescence microscope control: NIS Elements 5.21.03 (Build 1489)
For cryo-EM data collection and microscope control: SerialEM 4.0 and EPU 3.

Data analysis

For Cryo-EM data processing: MotionCor2, GCTF 1.06, Gautomatch 0.56, cryoSPARC Live and cryoSPARC v3.
For model building and refinement: ChimeraX 1.4, Namdinator, COOT v0.9.5, AlphaFold2 and Phenix 1.20
For fluorescence image analysis: FIJI/ImageJ 2.3.0/1.53f
For figure rendering: ChimeraX 1.4, Adobe Illustrator 27.1.1, and Graphad Prism 9.

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- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our [policy](#)

Models and cryo-EM maps have been deposited in the PDB and EMD as follows:

Dynein in ATP buffer: PDB-9BLY/EMD-44681 (phi particle dynein), PDB-9BLZ/EMD-44682 (state-1), PDB-9BM0/EMD-44683 (state-2), PDB-9BM1/EMD-44684 (state-3), PDB-9BM2/EMD-44685 (state-4), PDB-9BM3/EMD-44686 (state-5a), PDB-9BM4/EMD-44687 (state-5b), PDB-9BM5/EMD-44688 (state-6), PDB-9BM6/EMD-44689 (state-7a-post-2), PDB-9BM7/EMD-44690 (state-7b-post-2), PDB-9BM8/EMD-44691 (state-7c-post-2).

Dynein in ATP-Vi buffer: PDB-9BMF/EMD-44697 (state-1), PDB-9BMG/EMD-44698 (state-2), PDB-9BMH/EMD-44699 (state-4).

Dynein in 5 mM AMPPNP buffer with 2 mM Mg²⁺: PDB-9BMJ/EMD-44701 (state-1), PDB-9BML/EMD-44702 (state-2), PDB-9BMM/EMD-44703 (state-4), PDB-9BMN/EMD-44704 (state-5), PDB-9BMO/EMD-44705 (state-6), PDB-9BMP/EMD-44706 (state-7-post-2).

Dynein in 5 mM AMPPNP buffer with 5 mM Mg²⁺: PDB-9DH5/EMD-46856 (state-1), PDB-9DH6/EMD-46857 (state-2), PDB-9DH7/EMD-46858 (state-4), PDB-9DH8/EMD-46859 (state-5), PDB-9DH9/EMD-46860 (state-7-post-2), PDB-9DHA/EMD-46861 (state-7-post-1)

Dynein in ADP buffer: PDB-9BMR/EMD-44708 (state-1), PDB-9BMS/EMD-44709 (state-2), PDB-9BMT/EMD-44710 (state-5), PDB-9BMU/EMD-44711 (state-6), PDB-9BMV/EMD-44712 (state-7-post-1), PDB-9BMW/EMD-44714 (state-7-post-2).

Dynein in apo buffer: PDB-9BMY/EMD-44715 (state-1), PDB-9BMZ/EMD-44716 (state-2), PDB-9BN0/EMD-44717 (state-7-post-2), PDB-9BN1/EMD-44718 (state-8-post-1).

Dynein bound to microtubules: PDB-9BMA/EMD-44693 (apo-post-1), PDB-9BMB/EMD-44694 (ADP-post-1), PDB-9BMC/EMD-44695 (ADP-post-2), PDB-9BMD/EMD-44696 (AMPPNP-post-1).

Dynein motor domain alpha registry mutant in ATP buffer: PDB-9BN3/EMD-44720 (class-1), PDB-9BN4/EMD-44721 (class-2).

Dynein motor domain alpha registry mutant in ATP-Vi buffer: PDB-9BN5/EMD-44722 (class-1), PDB-9BN6/EMD-44723 (class-2).

Previously reported atomic models used in this study (for model building and structural analysis) are: PDB-5NUG, PDB-7K58, PDB-7K5b, PDB-8FD6, PDB-7MGM

Research involving human participants, their data, or biological material

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Reporting on sex and gender

n/a

Reporting on race, ethnicity, or other socially relevant groupings

n/a

Population characteristics

n/a

Recruitment

n/a

Ethics oversight

n/a

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	For cryo-EM processing, no methods were used to predetermine sample size. The size of the cryo-EM datasets was determined by the need to obtain structures with sufficient resolution for model building. The number of micrographs and particles are listed in the Methods and Extended Data.
Data exclusions	For EM averages, particles with low quality (low contrast, ice contamination) were discarded.
Replication	Biochemical assays and single molecule microscopy assays, cryo-EM sample preparation and 3D reconstruction were reproduced from at least 2 independent datasets collected from samples prepared with different protein purifications (see Figure legends for precise sample size and replicate information). All differences noted between e.g., mutant and wild-type protein or cells were replicated successfully.
Randomization	For all imaging experiments (e.g., cryo-EM), random fields-of-view were selected for imaging. For analysis of these data, all molecules from a given field-of-view were included in the analysis. For calculation of the Fourier Shell Correlation (FSC), cryo-EM particles were randomly split into two halves using the cryoSPARC software. For single molecule motility assay, the field of view is randomly selected to have a good number of microtubules for following tracing.
Blinding	Blinding is not necessary since there are no groups that need subjective analysis.

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Materials & experimental systems

n/a	Involved in the study
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☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☒	☐ Clinical data
☒	☐ Dual use research of concern
☒	☐ Plants

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Eukaryotic cell lines

Policy information about [cell lines and Sex and Gender in Research](#)

Cell line source(s)	SF9 cells (ATCC CRL-171)
Authentication	Authentication by supplier. Viability testing of SF9 cell line was performed at ATCC.
Mycoplasma contamination	Tested negative for mycoplasma contamination by manufacturer (tested using qPCR).
Commonly misidentified lines (See ICLAC register)	No commonly misidentified cell lines were used throughout this study.

Plants

Seed stocks	n/a
Novel plant genotypes	n/a
Authentication	n/a