Intraflagellar transport dynein is autoinhibited by trapping of its mechanical and track-binding elements

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Cilia are multifunctional organelles that are constructed using intraflagellar transport (IFT) of cargo to and from their tip. It is widely held that the retrograde IFT motor, dynein-2, must be controlled in order to reach the ciliary tip and then unleashed to power the return journey. However, the mechanism is unknown. Here, we systematically define the mechanochemistry of human dynein-2 motors as monomers, dimers, and multimotor assemblies with kinesin-II. Combining these data with insights from single-particle EM, we discover that dynein-2 dimers are intrinsically autoinhibited. Inhibition is mediated by trapping dynein-2’s mechanical ‘linker’ and ‘stalk’ domains within a novel motor–motor interface. We find that linker-mediated inhibition enables efficient transport of dynein-2 by kinesin-II in vitro. These results suggest a conserved mechanism for autoregulation among dimeric dyneins, which is exploited as a switch for dynein-2’s recycling activity during IFT.

Indeed, unlike cytoplasmic transport, for which detailed models are emerging for some cargo types, relatively little is known about the motor mechanisms involved in IFT. Notably, IFT differs from cytoplasmic transport in several striking ways. First, dynein-2 and kinesin-II are integrated into long (~220 nm) linear arrays termed ‘IFT trains’ containing multiple copies of each motor, in contrast to the small groups of membrane-bound motors typical of cytoplasmic transport. Second, rather than stochastic back-and-forth switches in direction characteristic of cytoplasmic transport, the activity of dynein-2 and kinesin-II is highly coordinated: IFT trains travel to the tip of the cilium (kinesin direction) then return to the base (dynein direction) in an apparently deterministic fashion. Finally, rather than involving conventional regulators of cytoplasmic dynein-1 motility, such as dynactin, Lis1, and Ndc80, the factors controlling dynein-2 motility are mysterious.

The nature of dynein-2 motility itself is also unclear, a situation compounded by the challenges of working with the large dynein-2 mechanoenzyme in vitro. Rapid retrograde IFT has been observed in living cells with velocities ranging from 140 to 7,400 nm/s. However, in vitro assays with a partial dynein-2 complex yielded slow movement at ~70 nm/s, raising the question of how fast movement is elicited in vivo.

Structural information is available for dynein-2’s motor domain in monomeric form, revealing a classic dynein subdomain organization (Fig. 1a). The catalytic core is a ring of six AAA+ modules, of which AAA1 is the main ATPase site. Microtubule binding occurs at the tip of an antiparallel coiled-coil ‘stalk’. A C-terminal domain (CTD) lies on one face of the ring. Opposite, lies dynein’s mechanical element, the ‘linker’ domain, which arches from AAA1 over the ring and connects to the dimerizing N-terminal tail. The linker undergoes a nucleotide-dependent power stroke, involving a bent-to-straight transition, which is thought to transmit force between the head and tail domains of the motor.

A major question in structural cell biology is how micrometer-scaled organelles are constructed and maintained. Cilia, also known as eukaryotic flagella, are multifunctional organelles that emanate from almost all cell types in the human body and many other eukaryotes. Nonmotile cilia serve as ‘signaling antennae’ in processes as diverse as morphogenesis, mechanosensation, and olfaction. Motile cilia beat with a wave-like motion essential for numerous propulsive functions, and severe congenital disorders are caused by defects in cilia, igniting interest in the mechanisms of ciliary assembly.

During assembly, cilia elongate from their distal tip. IFT is a bidirectional transport system that moves ciliary components synthesized in the cytoplasm to the tip of the cilium, and returns products to the cell body. Cargoes of IFT include both structural components, such as tubulin, and functional components, such as G-protein-coupled receptors.

IFT is powered by ATP-fueled motors that move along the outer surface of the microtubule doublets. The principal motor driving anterograde movement to the ciliary tip is heterotrimeric kinesin-II (Kif3 in humans), augmented by a homodimeric kinesin-II in some cilia. Kinesin-II motility is beginning to be understood in detail. Universally, retrograde IFT is driven by dynein-2, also known as dynein-1b or IFT dynein, which is the subject of this study. Dynein-2 is a dimer of two ~0.5-MDa heavy chains, each of which possesses a motor domain, together with several associated chains (Fig. 1a). Its closest relative is cytoplasmic dynein-1, the major motor driving transport to microtubule minus ends in the cytoplasm. Despite rapid progress in dynein-1 research, advances in defining dynein-2 composition, and regulation of dynein-2 in IFT are poorly understood.

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the two motor domains and to cargo.\textsuperscript{31,35–37} It is widely held that dynein-2 force generation must be regulated in time and space in order for the complex to move as a passenger to the ciliary tip, before powering return transport in the opposite direction\textsuperscript{1}. How dynein-2 mechanochemistry is regulated is unknown.

In this work, we systematically define the motile properties of human dynein-2 motor domains as monomers, dimers, and multimotor assemblies with kinesin-II. Integrating these data with structural insights from EM, we find that dynein-2 dimers are intrinsically autoinhibited. Rather than involving the CTD, which was previously implicated in dynein-1 autoregulation,\textsuperscript{38} inhibition is mediated by trapping of dynein-2’s mechanical linker and track-binding stalk within a novel motor–motor interface. Inhibited dynein-2 dimers are efficiently transported by kinesin-II \textit{in vitro}, whereas disrupting linker-mediated interaction activates dynein-2 and retards anterograde transport. These findings suggest a conserved basis for autoregulation among dimeric dynesins, which is co-opted as a switch for dynein-2’s recycling activity in IFT.

**RESULTS**

**Mechanochemistry of the dynein-2 motor domain**

A foundation for understanding the mechanisms of other cytoskeletal motors has been a robust source of purified protein. To dissect the action and regulation of dynein-2, we used insect cells to express a human monomeric dynein-2 motor domain\textsuperscript{31} (Dyn\textsubscript{2motor}) with a SNAP\textsubscript{f} tag at its amino terminus (Fig. 1B). The SNAP\textsubscript{f} tag enabled covalent labeling of Dyn\textsubscript{2motor} with a variety of ligands (bright fluorophores, biotin, or DNA oligonucleotides), which we used for single-molecule visualization, surface immobilization, and attachment of the motor to DNA origami structures, respectively. Dyn\textsubscript{2motor} was separated from excess ligand using size-exclusion chromatography (Fig. 2A), yielding purified (Fig. 2B) monomeric protein (Fig. 2C).

To determine the motile properties of Dyn\textsubscript{2motor}, we biotinylated it and attached it to a neutravidin-coated surface. Upon addition of microtubules and ATP, Dyn\textsubscript{2motor} powered fast gliding of microtubules as visualized by TIRF microscopy (Fig. 2D and Supplementary Video 1). Notably, the velocity of microtubule gliding varied depending on the surface density of Dyn\textsubscript{2motor}. Movement was rapid (524.0 ± 7.5 nm/s; ± standard error of the fit, here and elsewhere unless specified) at high input concentrations of Dyn\textsubscript{2motor} (>20 nM), and decreased gradually as the surface density was lowered (Fig. 2E). This concentration relationship indicates that fast microtubule movement is a property of Dyn\textsubscript{2motor} ensembles. Indeed, at intermediate Dyn\textsubscript{2motor} concentrations, longer microtubules were translocated faster than short ones (Supplementary Fig. 1A, B), suggesting that the number of motors interacting per microtubule is the critical parameter influencing velocity.

Enzymatically, Dyn\textsubscript{2motor} hydrolyzed an average of 1.3 ± 0.1 ATPs per second. This basal rate was stimulated by microtubules to yield a k\textsubscript{cat} of 48.8 ± 0.9 ATPs per second (Fig. 2F). The continuous and rapid microtubule motion driven by Dyn\textsubscript{2motor} (Fig. 2D) differs from the erratic and slower (~70 nm/s) motility reported for a partial dynein-2 complex purified from HEK cells.\textsuperscript{39} The absolute velocity and concentration dependence of microtubule movement varied with buffer type (Supplementary Fig. 1C), a phenomenon that is likely to have contributed to the slower velocities reported earlier.\textsuperscript{30,31} Together, these data reveal that Dyn\textsubscript{2motor} powers fast microtubule movement in monomer ensembles.

**Dimerization inhibits dynein-2 ATPase and motility**

We next tested if motor domains within dynein-2 dimers influence another’s activity. Cytoplasmic dynesins have shown great plasticity as dimers, retaining motor function when the N-terminal tail is replaced with a variety of dimerizing moieties,\textsuperscript{39–44} such as glutathione S-transferase (GST), coiled coils, FRB–FKBP, and DNA. We therefore dimerized Dyn\textsubscript{2motor} using GST (Fig. 1B), enabling comparison to well-studied GST cytoplasmic dynein-1 constructs. As expected, GST-Dyn\textsubscript{2motor} was a stable dimer (Fig. 3A–C).

Strikingly, dimerization inhibited, rather than stimulated, the motility and ATPase activity of Dyn\textsubscript{2motor}. The maximal rate of microtubule gliding was reduced to 242.1 ± 4.2 nm/s, while the concentration dependence of movement remained similar (Fig. 3D). In ATPase assays, the basal rate of hydrolysis by GST-Dyn\textsubscript{2motor} was 0.9 ± 0.1 ATPs per second. GST-Dyn\textsubscript{2motor} ATPase was only minimally activated by microtubules, saturating at a maximal rate of 1.9 ± 0.3 ATPs per second (Fig. 3E). Thus, dimerization perturbs the response of Dyn\textsubscript{2motor} to its allosteric activator, the microtubule. Control experiments revealed that GST per se is not responsible for the repressed motility of GST-Dyn\textsubscript{2motor} (below). These results indicate that GST-Dyn\textsubscript{2motor} behavior arises from interactions between its two motor domains.

Imaging at the single-molecule level revealed that GST-Dyn\textsubscript{2motor} bound transiently to microtubules in the presence of ATP, typically dwelling for less than a second per encounter, without undergoing measurable movement (Fig. 3F). This behavior differs from the robust...
Dyn2 motor domains power fast microtubule gliding. (a) Size-exclusion chromatogram of Dyn2motor and schematic of the construct. V₀, void volume. (b) SDS-PAGE of Dyn2motor after the final purification step. (c) Negative stain EM of Dyn2motor monomers in ATP. (d) Time sequence of microtubule translocation by surface immobolized Dyn2motor at different input concentrations. See also Supplementary Video 1. (e) Plot of mean microtubule gliding velocity (± s.d.) at different Dyn2motor concentrations. Number of microtubules analyzed per experiment: 20 nm (46), 200 nM (53). Fitted values (± standard error of the fit): Vₘₐₓ = 524.0 ± 7.5 nm/s, f = 0.3 ± 0.01. (f) Microtubule-stimulated ATPase activity of Dyn2motor. Experiments were carried out in triplicate; mean values (± s.d.) are shown. Fitted values (± standard error of the fit): kₐₜₐₜ = 4.8 ± 0.9 s⁻¹, kₕₐₜₐₜ = 1.3 ± 0.1 s⁻¹, Kₘ(MT) = 13.5 ± 7.5 µM. Source data for e,f are available online.

**Figure 2** Monomeric dynein-2 motor domains power fast microtubule gliding. (a) Size-exclusion chromatogram of Dyn2motor and schematic of the construct. V₀, void volume. (b) SDS-PAGE of Dyn2motor after the final purification step. (c) Negative stain EM of Dyn2motor monomers in ATP. (d) Time sequence of microtubule translocation by surface immobile Dyn2motor at different input concentrations. See also Supplementary Video 1. (e) Plot of mean microtubule gliding velocity (± s.d.) at different Dyn2motor concentrations. Number of microtubules analyzed per experiment: 20 nm (46), 200 nM (53). Fitted values (± standard error of the fit): Vₘₐₓ = 524.0 ± 7.5 nm/s, f = 0.3 ± 0.01. (f) Microtubule-stimulated ATPase activity of Dyn2motor. Experiments were carried out in triplicate; mean values (± s.d.) are shown. Fitted values (± standard error of the fit): kₐₜₐₜ = 4.8 ± 0.9 s⁻¹, kₕₐₜₐₜ = 1.3 ± 0.1 s⁻¹, Kₘ(MT) = 13.5 ± 7.5 µM. Source data for e,f are available online.

**Figure 3** Dimerization inhibits dynein-2 motor domains. (a) Size-exclusion chromatogram of GST-Dyn2motor and schematic of the construct. For comparison, a normalized monomeric Dyn2motor trace is shown (dashed gray line). V₀, void volume. (b) SDS-PAGE of GST-Dyn2motor after the final purification step. (c) Negative stain EM of GST-Dyn2motor dimers in ATP. (d) Plot of mean microtubule gliding velocity (± s.d.) at different GST-Dyn2motor concentrations (Dyn2motor values from Fig. 2e are plotted in gray for comparison). Number of microtubules analyzed per concentration: 0.5 nM (33), 2 nM (24), 5 nM (38), 10 nM (45), 20 nM (48), 200 nM (48). Fitted values (± standard error of the fit): Vₘₐₓ = 242.1 ± 4.2 nm/s, f = 0.2 ± 0.01. (e) Microtubule-stimulated ATPase activity of GST-Dyn2motor (Dyn2motor values from Fig. 2f plotted in gray for comparison). Experiments were carried out in triplicate; mean values (± s.d.) are shown. Fitted values (± standard error of the fit): kₐₜₐₜ = 1.9 ± 0.3 s⁻¹, kₕₐₜₐₜ = 0.9 ± 0.1 s⁻¹, Kₘ(MT) = 4.8 ± 4.2 µM. (f) Single-molecule behavior of GST-Dyn2motor. Left, kymograph showing encounters of TMR-labeled GST-Dyn2motor with the microtubule in 1 mM ATP. Middle, quantification of microtubule landing rate. Green line; mean (± s.e.m.) from 18 landing rates, calculated from 941 landing events. Right, histogram of GST-Dyn2motor dwell times on the microtubule, and single-exponential-decay fit (green). Number of dwell times, 927. t, average dwell time (decay constant⁻¹) ± standard error of the fit. Source data for d-f are available online.

**Figure 4** 2D stacking of GST-Dyn2motor with the microtubule in 1 mM ATP. Middle, quantification of microtubule landing rate. Green line; mean (± s.e.m.) from 18 landing rates, calculated from 941 landing events. Right, histogram of GST-Dyn2motor dwell times on the microtubule, and single-exponential-decay fit (green). Number of dwell times, 927. t, average dwell time (decay constant⁻¹) ± standard error of the fit. Source data for d-f are available online.

processivity of *S. cerevisiae* dynein-1 GST dimers, being more akin to that of dynein-1 from mammal. In summary, we conclude that pairing two dynein-2 motor domains substantially inhibits their enzymatic and microtubule gliding activities.

**Dynein-2 dimers adopt a stacked conformation**

To gain insight into how the two dynein-2 motor domains influence each other’s activity, we examined the structure of GST-Dyn2motor using single-particle negative stain EM. In the presence of ATP, GST-Dyn2motor molecules displayed a distinctive ‘stacked’ conformation (Fig. 4a,b), in which the AAA+ rings of the two motor domains are closely apposed (Supplementary Fig. 2c). This appearance recalls the compact “phi particle” shape observed for cytoplasmic dynein-1, which is a putatively inhibited state (Supplementary Fig. 3d). Protruding from dynein-2’s AAA+ rings, the coiled-coil stalk and globular microtubule-binding domain at its tip are visible (Fig. 4a). Invariantly, the stalks cross each other at a fixed angle, intersecting at a distance one-third along their length (Supplementary Video 2).

Given the inherent flexibility of dynein’s stalk, these observations indicate an interaction between the coiled coils at the crossing site.

Stacking of GST-Dyn2motor was nucleotide dependent. In the absence of nucleotide, or in ADP-conditions, the AAA+ rings were separated, exhibiting a wide range of motor–motor distances (Fig. 4b, Supplementary Fig. 2a,c and Supplementary Video 2), in contrast to the sharp distribution of low motor–motor distances characteristic of stacking in ATP conditions. Stacking was also observed in the ADP(Vi) (vanadate) state, mimicking the ADP.Pi state (Fig. 4b and Supplementary Fig. 2a,c). The linker—the main mechanical element of dynein—is straight in the no-nucleotide and ADP states, but bent in the ATP and ADP.Vi states, raising the possibility that stacking is promoted by a bent linker.

Stacking of GST-Dyn2motor in ATP was almost entirely abolished by the addition of 500 mM salt (Fig. 4b and Supplementary Fig. 2a,c), suggesting that the stacking interface has an electrostatic component and is comparatively weak. In line with a weak interaction, spontaneous stacking between Dyn2motor monomers was not observed at the nanomolar concentrations of our EM experiments (Fig. 2c). However, after dimerization, which holds the Dyn2motor protomers at a high local concentration >0.2 mM (derived from their maximum separation of
30 nm in unstacked molecules), the large majority of molecules are stacked, with only a small subset showing large motor–motor separation (Supplementary Fig. 2c). These data indicate that (1) in ATP conditions, the majority of dynein-2 motor domains adopt a stacked conformation, and (2) stacking is metastable and may be influenced by external cues.

The linker and stalk are trapped at the stacking interface

We next determined the spatial arrangement of the two dynein-2 motor domains in the stacked configuration, exploiting our EM data and the availability of a crystal structure of a monomeric dynein-2 motor construct31. Although dynein-2 has only been crystallized as a monomer, it is in the ADP.Vi state. As the linker domain is bent in this state, we predicted it to be capable of forming the stacking interface (Fig. 4b).

Moreover, while monomeric dynein-2 motors do not stack spontaneously in solution, the high effective concentrations during crystallization might promote formation of the stacking interface, analogous to the high local concentration within GST-Dyn2motor dimers. We therefore searched the crystal lattice, and found pairs of dynein-2 motors that quantitatively resemble the stacked molecules we observed in isolated GST-Dyn2motor dimers (Fig. 4c, Supplementary Fig. 3c and Supplementary Video 3).

The major interface between the paired dynein-2 motors involves the linker domains (Fig. 4c). It features a cluster of side chain contacts between the linkers at their distal tips, and an interaction between the fourth AAA+ module (AAA4) in the ring and the linker near its hinge. Additional inter-motor contacts occur between the AAA5 modules at their helix 2 and β-hairpin insert, and between the coiled-coil stalks as they cross, consistent with our EM analysis. Contacts are detailed in Supplementary Figure 3b. The CTDs are located opposite to the interface, and do not interact. This architecture contrasts with the existing model of autoinhibition in dynein-1, which evokes an interaction between motors through their CTDs38 (Supplementary Fig. 3a).

To test if linker-mediated interaction is responsible for the stacked dynein-2 conformation observed by EM, we mutated to alanine three charged or polar amino acids at the linker–AAA4 interface (Fig. 5a,b). Specifically, we targeted linker residues predicted to hydrogen bond with AAA4 (D1406, Q1407, and R1410). The resulting construct (GST-Dyn2motor(DQR)mutant) remained dimerized, but stacking was almost completely abolished (Figs. 4b and 5c and Supplementary Fig. 2b). The motors displayed a wide range of separations, with a distribution similar to that of the parental wild-type construct in no-nucleotide or ADP conditions (Supplementary Fig. 2c). Quantitative comparison shows that linker-mediated stacking not only matches the dynein-2 EM data (Supplementary Fig. 2c), but also provides a similarly strong match to class averages of the dynein-1 holozymen phi particle (Supplementary Fig. 3d). Thus, the stacked arrangement of dynein-2 motor domains in GST-Dyn2motor is strikingly similar to that of dynein-1 motor domains natively dimerized by the tail. These data indicate that the linker-mediated interaction may be ancient and conserved, predating the ancestor of dynein-1 and dynein-2. We conclude that paired dynein-2 motor domains stack via an interface involving their main mechanical element—the linker domain.

Untrapping dynein-2’s linker and stalk rescues ATPase and motility

Inspection of Dyn2motor stacking indicates incompatibility with dynein motility at three levels (Fig. 4d). First, the linker—dynein’s mechanical amplifier—is not free to move, being trapped in the bent conformation at the interface between the motor domains. The linker’s critical docking site at AAA5 is also directly occluded36. Second, the two microtubule-binding domains point in opposite directions, meaning that they could not engage the microtubule simultaneously, as proposed36. Finally, consistent with our EM analysis, the coiled-coil stalks interact at their crossing point. This contact is likely to restrict intra-coiled-coil helix sliding in the stalk, which canonically mediates communication between dynein’s ATPase and microtubule-binding sites, and is essential for mechanochemistry48,49.

If linker-mediated stacking is the basis for inhibition in GST-Dyn2motor dimers, then disrupting the stacking interface should
residues are indicated. To form intermolecular hydrogen bonds crucial for stacking based upon elevated to a maximal rate 4.1 times on the microtubule, and single-exponential-decay fit (red). Number error of the fit): out in triplicate; mean values from 956 landing events. Data for GST-Dyn2 motor from Q1407

\[ \text{basal} = 1.5 \pm 0.1 \text{ s}^{-1}, \ K_m(\text{MT}) = 3.9 \pm 1.2 \text{ µM}. \]

\[ k_{\text{off}} = 4.1 \pm 0.2 \text{ s}^{-1}, \ k_{\text{on}} = 1.5 \pm 0.1 \text{ s}^{-1}, \ V_{\text{max}} = 562.2 \pm 4.7 \text{ nm/s}, \ t = 4.0 \pm 0.01. \]

\[ t = 0.10 \pm 0.002 \text{ s} \text{.} \]

Figure 5 Untrapping dynein-2 dimers rescues their motility. (a) Depiction of amino acids within the linker and AAA4 module of dynein-2 predicted to form intermolecular hydrogen bonds crucial for stacking based upon the model of Figure 4c. (b) Amino acid conservation of residues shown in a among dynein-1 and dynein-2 sequences. Pairs of interacting residues are indicated. (c) EM class averages of GST-Dyn2motor and GST-Dyn2(DQR)motor in ATP conditions. Scale bar, 10 nm. (d) Microtubule-stimulated ATPase activity of GST-Dyn2(DQR)motor (GST-Dyn2motor values from Fig. 3e plotted in gray for comparison). Experiments were carried out in triplicate; mean values ± s.d. are shown. Fitted values (± standard error of the fit): \( k_{\text{off}} = 4.1 \pm 0.2 \text{ s}^{-1}, \ k_{\text{on}} = 1.5 \pm 0.1 \text{ s}^{-1}, \ V_{\text{max}} = 3.9 \pm 1.2 \text{ µM}. \) (e) Plot of mean microtubule gliding velocity (± s.d.) at different GST-Dyn2(DQR)motor concentrations (GST-Dyn2motor values from Fig. 3d plotted in gray for comparison). Number of microtubules analyzed per concentration: 0.2 nM (29), 0.3 nM (34), 1 nM (31), 3.5 nM (20), 5 nM (34), 10 nM (53), 20 nM (46), 200 nM (42). Fitted values (± standard error of the fit): \( V_{\text{max}} = 562.2 \pm 4.7 \text{ nm/s}, \ t = 4.0 \pm 0.01. \) (f) Left, quantification of microtubule landing rate for GST-Dyn2(DQR)motor. Red line, mean ± s.e.m. of 14 landing rates, calculated from 956 landing events. Data for GST-Dyn2motor from Figure 3f are shown for comparison (black line). Right, histogram of GST-Dyn2motor dwell times on the microtubule, and single-exponential-decay fit (red). Number of dwell times, 945. t, average dwell time (decay constant)\(^{-1}\) ± standard error of the fit. Source data for d-f are available online.

Rescue motility. Restoration of activity in a GST dimer would also demonstrate that GST per se is not responsible for inhibition. We therefore determined the motile behavior of the unstacked GST-Dyn2(DQR)motor mutant. Importantly, the DQR substitution had little or no impact on dynein-2 motility and ATPase in the context of a monomer, showing that the mutations do not intrinsically affect motor activity (Supplementary Fig. 4c,d). However, in the context of a dimer, the unstacking DQR mutations had a significant impact. The maximum velocity of microtubule gliding increased to 562.2 ± 4.7 nm/s (Fig. 5e), restoring it to the level of the Dyn2motor monomer (Fig. 2e). The ATPase of GST-Dyn2(DQR)motor was elevated to a maximal rate 4.1 ± 0.2 ATPs per second at saturating microtubule concentrations (Fig. 5d), again similar to the level of Dyn2motor. As expected from the concentration dependence of microtubule gliding (Fig. 5e), measurably processive movement by GST-Dyn2(DQR)motor single molecules was extremely rare. However, the loading rate of GST-Dyn2(DQR)motor on the microtubule was elevated relative to GST-Dyn2motor (Fig. 5f), consistent with the availability of its microtubule-binding domains. Together, these findings indicate that linker-mediated stacking is responsible for the inhibition of dynene-2 dimers; furthermore, they suggest that rupture of linker stacking activates dynene-2 to a level comparable to the isolated motor domain.

**Linker-mediated inhibition facilitates dynene-2 transport by Kif3**

What might be the role of linker-mediated stacking of dynene-2 in IFT? One possibility is that it provides the basis for dynene-2 to be targeted to the tip of the cilium by kinesin-II in a switched-off state—a phenomenon that has been long suspected but never tested or rationalized. To test this notion, we built a multimotor assembly of dynene-2 and kinesin-II. Pioneering studies in *Chlamydomonas* indicate that IFT motors are linked together in vivo by linear arrays of ~22 different proteins (the IFT–A and IFT–B complexes)\(^{50–52}\). However, IFT trains tend to fall apart during purification from cilia\(^{50}\) and vary in length\(^{26}\), so motor composition is not preserved or controlled following isolation, while recombinantly expressed IFT subcomplexes have not formed arrays, even at high concentrations\(^{52}\). Thus, native protein-based methods have been unable to systematically assemble multiple IFT motors in vitro.

To obtain control over the composition of dynene-2 and kinesin-II per assembly, we used a synthetic-biology-inspired approach, and coupled them via a programmable DNA origami chassis\(^{54}\) (Fig. 6a). For kinesin-II, we developed a Kif3 construct with a C-terminal SNAP\(_1\) tag, yielding an active motor that can be labeled with single-stranded DNA (Supplementary Fig. 5). For the chassis, we used a 12-helix DNA bundle design, in which a specified number of single-strand sequences project from one of its helices\(^{54}\). These ‘handle’ sequences serve as specific attachment sites for DNA-labeled Kif3 or dynene-2. Use of three attachment sites per motor facilitates comparison with similar cytoplasmic motor assemblies\(^{54}\). The dimensions of the DNA structure (225 nm × 14 nm) approximate that of an IFT train\(^{27}\) (Fig. 6b). We refer to these IFT motor–DNA assemblies herein as synthetic ‘trains’.

In the absence of dynene-2, trains bearing human Kif3 motors bound to and traveled along microtubules toward the plus end with a mean velocity of ~600 nm/s (Fig. 6c), similar to Kif3 from other species\(^{10}\), slightly slower than single human Kif3 motors (Supplementary Fig. 5f), and within the range of anterograde IFT velocities reported in vertebrate cilia\(^{29}\). Notably, upon addition of GST-Dyn2motor, train movement remained uniformly plus-end directed (Fig. 6c). Trains exhibited a range of speeds, with peak velocities similar to those of trains bearing Kif3 alone, and a tail of slower velocities (Fig. 6d). The latter may reflect stochastic unstacking and activation of GST-Dyn2motor (Fig. 4b, top right). The uniform train movement toward the plus end distinguishes IFT motor assemblies from those with *Sacccharomyces cerevisiae* dynein-1 and human kinesin-1, in which most events were minus-end directed or stalled in a tug-of-war\(^{54}\). These results indicate that GST-Dyn2motor provides little resistance to Kif3, resulting in transit of both motors to the plus end of the microtubule. To determine if linker-mediated inhibition facilitates GST-Dyn2motor transport as a cargo of Kif3, we replaced it with GST-Dyn2(DQR)motor—the mutant deficient in linker-mediated inhibition. In comparison to GST-Dyn2motor GST-Dyn2(DQR)motor...
severely retarded train motility. Movement remained plus-end directed, but most trains were brought to a virtual standstill, with velocities <150 nm/s (Fig. 6c,d). Thus, we conclude that linker-mediated inhibition prevents dynein-2 motors from conferring strong resistance to Kif3, enabling efficient transit of both motors to the plus end of the microtubule.

Finally, we asked if teams of dynein-2 motors could power processive minus-end-directed movement when coupled on a train. The precise number of motors per IFT train in vivo is uncertain, with fluorescence and force-based estimates ranging between 4 and 40. We found that, in vitro, synthetic trains with three attachment sites for GST-Dyn2\textsubscript{motor} or GST-Dyn2(DQR)\textsubscript{motor} exhibited processive movements infrequently (not shown), as did assemblies with seven GST-Dyn2\textsubscript{motor} sites (Supplementary Fig. 6b). In contrast, trains with seven GST-Dyn2(DQR)\textsubscript{motor} sites displayed robust processive movement toward microtubule minus ends, with an average and maximal velocity of 334.0 ± 4.7 (± s.e.m.) and 572.1 nm/s respectively (Fig. 6e).

The mean travel distance was 3.5 ± 0.2 microns (± s.e.m.). These results suggest that when teams of dynein-2 motors are activated by untrapping their linkers and stalks, they power continuous movement over distances comparable to the length of a primary cilium.

**DISCUSSION**

Using purified human proteins, mechanochemical assays, EM, and synthetic-biology-inspired engineering, we have obtained insights into dynein-2 activity significant for understanding the molecular mechanism of IFT. First, monomeric dynein-2 motor domains are capable of driving fast microtubule gliding in vitro, approaching the velocities of retrograde IFT in vertebrate cells. Second, the motility and ATPase of dynein-2 motor domains are inhibited upon dimerization (their natural oligomeric status). Third, within dimers, dynein-2 motor domains tend to associate via an interface that traps their linker domains and crosses their microtubule-binding stalks, shutting down their activities. Fourth, mutations that disrupt this entrapment activate dynein-2 dimers, restoring their ATPase and motility while causing them to strongly resist kinesin-II.

We integrate these data and previous studies into a model for dynein-2 regulation, which enables efficient cycles of IFT (Fig. 7). We propose that following its synthesis and dimerization in the cytosol, dynein-2 intrinsically adopts the inhibited configuration. In this switched-off state, with its linkers trapped and stalks crossed, dynein-2 is loaded onto anterograde IFT trains near the base of the cilium. It is then transported as a passenger to the ciliary tip by kinesin-II. Proximal to the tip of the cilium, a localized signal activates dynein-2. A key feature is the disruption of the inhibitory interface we define, unshackling dynein-2’s mechanical and track-binding elements. This enables them to power the return transport of turnover products, IFT machinery, and signaling molecules out of the cilium, completing the IFT cycle. This model applies to the force-generating heavy chains of dynein-2. Its associated subunits may be labile, associating and dissociating from the complex, giving rise to distinct dynamics. In essence, dynein-2 is naturally in an off state.

One role of linker-mediated inhibition in dynein-2 may be to limit futile ATP hydrolysis, akin to that of other cytoskeletal motors. However, we find that the ATPase of dynein-2’s motor domain is comparatively low. Thus, we suggest that a major function of linker-mediated inhibition in IFT is to prevent interference and a tug-of-war between dynein-2 and kinesin-II, which would impair anterograde delivery of cargoes and clog the confined space between the axoneme and ciliary membrane.

Point mutations in dynein-2 are associated with Jeune asphyxiating thoracic dystrophy and short rib polydactyly. Morphologically, loss of dynein-2 function is associated with stumpy cilia with accumulations of IFT particles at their tips. Mutations that constitutively activate dynein-2, but maintain anterograde IFT train attachment, may have an even more severe impact in vivo by disrupting anterograde IFT and kinesin-II, both of which are vital for ciliogenesis.

The mechanism of dynein-2 autoinhibition identified by this work may be tuned by cellular factors. We interpret our EM data and the residual gliding and microtubule-stimulated ATPase activities of...
Our structure-guided mutagenesis shows that the linker-mediated inhibition serves as a model for all autoregulated motors, analogous to cytoplasmic dynein-1 inhibition by dynactin\(^{65,66}\). A difference between the motor-centric and train-centric IFT models is that in the former, each dynein-2 complex would need to reach the ciliary tip in an inhibited state, with its linkers trapped and stalks crossed (Fig. 7). For cytoplasmic dynein, a conserved inhibitory architecture is likely to control the motor’s many functions in vesicle trafficking, virus transport, mRNA localization, and chromosome segregation, which can be investigated using the interface residues established here.

**METHODS**

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

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**AUTHOR CONTRIBUTIONS**

K.T. and A.J.R. conducted biochemical and TIRF experiments. K.T. performed electron microscopy experiments. M.M., K.T., and A.J.R. generated and purified constructs. K.T. and A.J.R. analyzed data and wrote the paper.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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Cultures were maintained at 1–2 × 10^6 cells/ml in flasks with shaking at 100 rpm. Transfection was evaluated by monitoring YFP expression from EMBacY using growing Sf9 cells in 2 ml of medium. After 3 days of incubation, the efficiency of size-exclusion chromatography on a Superose 6 Increase 3.2/300 column using incubated at 4 °C for 2 h. Proteins were purified from excess SNAP ligand by resin and bound-proteins were collected by gentle centrifugation at 670 rotor at 337,932 g for 6 min.

Protein purification. Protein purifications were performed at 4 °C. Frozen cell pellets from 250 ml S9 cultures were resuspended in 20 ml purification buffer (30 mM HEPES [pH 7.4], 300 mM KCl, 50 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 10% [v/v] glycerol, 1 mM DTT, 0.2 mM Mg-ATP, 1 mM PMSF) supplemented with a Complete™ EDTA-free Protease Inhibitor Cocktail (Roche). Cells were lysed using a Dounce homogenizer and 10 strokes with a small clearance pestle. Lysates were clarified by ultracentrifugation in a Type 70 Ti rotor at 183,960 g for 30 min. The supernatant was incubated for 1 h on a roller with 1 ml IgG Sepharose 6 resin (GE Healthcare) pre-washed in purification buffer. Frozen cell lysates were collected by gentle centrifugation at 670 g for 5 min, transferred into a 20 ml column, and washed with 2× 20 ml volumes of purification buffer and 1× 20 ml volume of TEV buffer (50 mM Tris [pH 7.5], 150 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 10% [v/v] glycerol, 1 mM DTT, 0.2 mM Mg-ATP). Proteins of interest were eluted by resuspending the resin in TEV buffer, adding 100 µg TEV protease, and incubating the reaction overnight on a roller. TEV-cleaved proteins were separated from the resin using an empty column, concentrated to 0.5–4 mg/ml using Amicon Ultra centrifugal filters (100 kDa cutoff), and cleared by ultracentrifugation in a TLA 100 rotor at 337,932 g for 6 min.

Protein labeling. SNAP-tagged motor proteins (2–5 µM) were labeled in 100 µl reactions with 10–20 µM SNAP ligand (SNAP-Cell TMR-Star, SNAP-Surface Alexa Fluor 647 [NEB], or SNAP-conjugated oligonucleotide). Reactions were incubated at 4 °C for 2 h. Proteins were purified from excess SNAP ligand by size-exclusion chromatography on a Superose 6 Increase 3.2/300 column using an ÄKTAmicro system (GE Healthcare), pre-equilibrated with gel filtration buffer (50 mM Tris [pH 7.5], 150 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 5% [v/v] glycerol, 1 mM DTT, 0.1 mM Mg-ATP). Fractions (100 µl) were analyzed by SDS-PAGE on 4–12% Tris-Bis gels with Sypro Red staining (Thermo Fisher Scientific), and imaged using an FLA-3000 fluorescent image analyzer (Fujifilm). Peak fractions were flash frozen under liquid nitrogen in single-use aliquots and stored at −80 °C.

DNA origami. DNA origami chassis structures consisting of a 12-helix bundle with the specified number of motor protein attachment sites were folded and purified as described54,67, with the following modifications. Oligonucleotides were purchased in 96-well format from Eurofin Genomics. Folding reactions comprised 50 nM p8064 scaffold DNA (Tilibit Nanosystems), 250 nM staple oligos, 500 nM motor attachment oligos, and 2.5 µM TMR oligo in folding buffer (5 mM Tris [pH 8.0], 1 mM EDTA, 16 mM MgCl2). After assembly on ice, reactions were transferred to a thermostated pre-heated to 65 °C. The temperature was decreased from 65 °C to 61 °C in steps of 1 °C per hour, then from 60 °C to 40 °C in steps of 1 °C per two hours. Folded chassis was purified from excess oligos and misfolded species by ultracentrifugation through a 15–45% glycerol gradient in 0.5× TBE buffer supplemented with 11 mM MgCl2. Ultracentrifugation was performed at 199,896 g for 2 h 10 min using a SW 55 rotor. Fractions containing folded structures were identified by electrophoresis using a 2% (w/v) agarose/0.5× TBE gel supplemented with 11 mM MgCl2 and SYBR Safe stain. Peak chassis-containing fractions were incubated with oligo-labeled motors for 30 min on ice (5 nM chassis, 70 nM motors), and examined immediately by TIRF microscopy. Motor attachment was verified by gel shift54,67 using a 0.7% agarose/0.5× TBE gel supplemented with 11 mM MgCl2 and 0.1% LDS.

Microtubules. Tubulin was purified and labeled with Alexa-488 or biotin as described48,49. To prepare taxol-stabilized microtubules, a mixture comprising 100 µM tubulin, 1 mM GTP, 1 mM MgCl2, 1 mM DTT and 10% DMSO was assembled in BR880 (80 mM PIPES [pH 6.9], 2 mM MgCl2, 1 mM EGTA) on ice, then incubated at 37 °C for 30 min. Following addition of an equal volume of BR880 + 40 µM taxol, the solution was incubated for a further 10 min at 37 °C, then stored at ambient temperature. For fluorescent visualization or surface attachment, 10% of Alexa-488 tubulin or biotin tubulin were included in the polymerization mixture respectively. Microtubules for ATPase assays were separated from unpolymerized tubulin by ultracentrifugation through a 60% glycerol cushion in BR880 + 20 µM taxol at 267,008 g for 30 min. Microtubule concentrations are expressed for the tubulin dimer.

ATPase assays. ATPase assays were performed using an EnzChek phosphate assay kit (Molecular Probes) as described70,71. The final reaction comprised: 50 nM dynine-2, 0–20 µM taxol stabilized microtubules, 2 mM Mg-ATP, 200 µM 2-amino-6-mercapto-7-methyl-purine riboside, 1 U/ml purine nucleoside phosphorylase, and BR880 buffer supplemented with 1 mM DTT and 20 µM taxol. A SpectraMax Plus 384 microplate reader (Molecular Devices) was used to monitor the coupled reaction at 360 nm at 5 s intervals for 10 min. ATPase data were fit to the following equation72: 

\[
\frac{\text{obs}}{\text{cat}} = \frac{\text{cat} - k_{\text{basal}}}{\text{MT}} (K_{\text{cat}} / (\text{MT}) + [\text{MT}]) + k_{\text{basal}}.
\]

TIRF microscopy. Fluorescently-labeled molecules were visualized on an Eclipse Ti-E inverted microscope with a CFI Apo TIRF 1.49 N.A. oil objective, Perfect Focus System, H-TIRF module, LU-N4 laser unit (Nikon) and a quad band filter set (Chroma). Images were recorded with 30–100 ms exposures on an iXon DU888 Ultra EMCCD camera (Andor), controlled with NIS-Elements AR Software (Nikon).

Motility assays were assembled in flow chambers made between glass slides, biotin-PEG coverslips, and double-sided tape. For microtubule gliding assays, chambers were sequentially incubated with 1) blocking solution (0.75% Pluronic F-127, 5 µg/ml casein) for >5 min, followed by two washes with B80-TK (80 mM PIPES [pH 6.9], 2 mM MgCl2, 1 mM EGTA, 1 mM DTT); 2) 0.5 mg/ml neudrinavir for 2 min, followed by two washes with B80-TK; 3) biotinylated motor protein (0.2–200 µM) for 2 min, followed by two washes with B80-TK supplemented with 1 mg/ml casein; 4) 0.1 µM Alexa-488 microtubules in assay solution (B80-TK supplemented with 1 mg/ml casein, 1 mM Mg-ATP, 71 mM β-mercaptoethanol, 20 µM glucose, 300 µg/ml glucose oxidase, 60 µg/ml catalase).
Single-molecule assays were prepared similarly for steps 1–2, except washes were performed with B80-T buffer (80 mM PIPES [pH 6.9], 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 20 μM taxol). Then, chambers were incubated with: 3) 0.25 μM Alexa-488, biotinylated microtubules for 2 min, followed by two washes with B80-T supplemented with 1 mg/ml casein; 4) 0.1–10 nM of TMR-labeled motor protein in assay solution (B80-T with supplements as above). DNA origami experiments were performed in the same way, except a modified buffer (50 mM Tris [pH 8.0], 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, 20 μM taxol, 100 mM KC1) was used to minimize non-specific interactions between DNA and the PEG coverslip, and the final assay solution contained 30–100 pM chassis-motor complex and Kif3-Alexa647 as a marker of microtubule polarity. Velocities and durations of microtubule association were calculated from kymographs generated in FIJI. For dwell time analysis, microtubule associations ≥ 90 ms (3 frames) were analyzed. Dwell time data were fit with a single exponential decay. For microtubule gliding experiments, microtubules < 25 (3 frames) were analyzed.

Electron microscopy. Dynein samples were diluted to 50–75 nM in EM buffer (50 mM Tris [pH 7.5], 150 mM K-acetate, 2 mM Mg-acetate, 1 mM EGTA, 1 mM DTT) supplemented with 1 mM nucleotide and 500 mM KC1 as indicated. DNA origami was diluted to 7.5–10 nM in EM buffer or mixed with a sixfold molar excess of oligonucleotide labeled dynein-2 for 30 min and diluted tenfold in EM buffer. Samples were prepared for electron microscopy by either adding 4 μl of specimen directly onto a glow-discharged continuous carbon grid (Electron Microscopy Sciences) or by pipetting ~4 μl of 2% uranyl acetate, a small air gap and then ~4 μl of sample into a single tip and depositing the contents onto the EM grid. Grids were then stained in three sequential drops of 75 μl 2% uranyl acetate, blotted and air dried. For DNA origami, grids were washed in three 75 μl drops of EM buffer following sample application to remove glycerol before staining.

Micrographs were acquired using a Tecnai T12 microscope (FEI) operating at 120 kV with a tungsten electron source and a 4k × 4k CCD camera (Gatan US4000). The nominal magnification was 52,000× for DNA-origami or 67,000× for dynein specimens, giving a sampling of 2.09 and 1.64 Å/pixel at the object level respectively. Images were collected with a dose of 20–30 e⁻/Å² and a nominal defocus of ~1 μm. For dynein images, single particles were picked from the micrographs manually in RELION and CTF corrected by phase flipping using RELION and CTFIN3D software. Particles were binned by 2 for subsequent processing, windowed into 200 × 200 pixel boxes, band-pass filtered (10 Å–45 Å) and centered in Imagick.

For measurement of the motor–motor separation, images of dynein dimers were subjected to several rounds of classification and multi-reference alignment in Imagick resulting in classes containing an average of 10 images. The distance between centroid positions of the two motor domains in each class average was measured using Boxer.

For computational comparison between EM class averages of dynein and the dynein-2 crystal structure model (PDB 4RH I all steps were carried out in SPIDER. The crystal structure was low-pass filtered to 30–40 Å. The resulting maps were then projected with an angular sampling of 5.5° and a ~1 μm defocus CTF applied. The projections were aligned to each class average and scored by cross correlation to identify the best matching projection. Crystal structures were displayed using The PyMOL Molecular Graphics System (Version 1.7 Schrödinger, LLC) and UCSF Chimera software.

Data availability. Source data underlying the graphical representations used in Figures 2e,f, 3d–f, 5d–f and 6c–e are available with the paper online. Coordinates for the linker-stacking model, derived from PDB 4RH, are available as Supplementary Data Set 1. Other relevant data are available from the corresponding author upon reasonable request.

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