The AAA3 domain of cytoplasmic dynein acts as a switch to facilitate microtubule release

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Cytoplasmic dynein is an AAA+ motor responsible for intracellular cargo transport and force generation along microtubules (MTs). Unlike kinesin and myosin, dynein contains multiple ATPase subunits, with AAA1 serving as the primary catalytic site. ATPase activity at AAA3 is also essential for robust motility, but its role in dynein's mechanochemical cycle remains unclear. Here, we introduced transient pauses in *Saccharomyces cerevisiae* dynein motility by using a slowly hydrolyzing ATP analog. Analysis of pausing behavior revealed that AAA3 hydrolyzes nucleotide an order of magnitude more slowly than AAA1, and the two sites do not coordinate. ATPase mutations to AAA3 abolish the ability of dynein to modulate MT release. Nucleotide hydrolysis at AAA3 lifts this 'MT gate' to allow fast motility. These results suggest that AAA3 acts as a switch that repurposes cytoplasmic dynein for fast cargo transport and MT-anchoring tasks in cells.

Eukaryotic cells use ATP-driven molecular motors to transport intracellular cargos along cytoskeletal tracks. A primary system for these processes is the MT transport network, in which plus end– and minus end–directed transport are driven by kinesin and cytoplasmic dynein, respectively. Although cells use a variety of kinesins for specific tasks, they typically have only a single cytoplasmic dynein heavy chain–encoding gene (*DHC1*). The motor domain consists of a pseudohexameric ring with six nonidentical AAA domains (AAA1–AAA6). Between AAA4 and AAA5, there protrudes a 15-nm coiled-coil stalk bearing a small globular MT-binding domain (MTBD) (Fig. 1a). The two rings are connected by an N-terminal linker and are dimerized through the tail domain.

In *Saccharomyces cerevisiae*, the AAA3 domain of cytoplasmic dynein acts as a switch to facilitate microtubule release by using a slowly hydrolyzing ATP analog. Assigning roles to each AAA+ site is complicated by the unique sequence and structure of each site in dynein: only AAA1–AAA4 can bind nucleotide, whereas AAA5 and AAA6 do not have the conserved Walker A and B motifs required for ATP binding and hydrolysis. The kinetics of dynein stepping are coupled to ATP hydrolysis at AAA1, and this hydrolysis is strictly required for dynein motility. ATP binding at AAA1 induces linker undocking, also referred to as the 'priming stroke.' Upon ATP hydrolysis at AAA1, dynein rebinds to the MT and docks its linker via a force-generating 'power stroke.' The roles of the other AAA domains in dynein motility are not well understood. Mutations that abrogate ATP binding or hydrolysis at AAA2 and AAA4 have a minimal effect on the velocity of dynein, instead affecting only the processivity. In contrast, ATPase mutations at AAA3 reduce motor velocity and MT-stimulated ATPase activity by an order of magnitude and result in defects in AAA1-generated linker swing. These mutants strongly localize to MTs in vivo and slowly release from MTs in the presence of ATP in vitro. The ATP binding and hydrolysis loops of AAA3 are conserved in cytoplasmic dyneins but not in dyneins responsible for intraflagellar transport (IFT) or axonemal bending (Supplementary Fig. 1), thus suggesting that ATPase activity at AAA3 may be critical for repurposing cytoplasmic dynein for different cellular roles. However, repurposing is not necessary for axonemal or IFT dyneins.

Although these studies suggest that an intact AAA3 domain is required for robust motility, the precise role of the AAA3 site in dynein's mechanochemical cycle remains unknown. One possibility...
is that the dynein stepping mechanism involves two coordinated nucleotide-hydrolysis cycles at AAA1 and AAA3. This would require two ATP molecules to be hydrolyzed per step. Another possibility is that each step requires a single ATP hydrolysis at AAA1 (ref. 8) and that AAA3 acts as a switch to turn allosteric communication within the ring on or off depending on the nucleotide state. It has also remained unclear why ATPase mutations to AAA2 slow down the catalytic activity and stepping rate of the motor by an order of magnitude. These questions cannot be addressed through mutagenesis alone because disrupting the ATPase activity of AAA3 also affects the ATPase of AAA1 (refs. 15, 18).

To define the role of AAA3 in dynein's mechanochemical cycle, we studied the motility and catalytic activity of native and mutant dynein motors by using optical-trap single-molecule fluorescence and bulk enzymology. We show that a functional AAA3 site is required for AAA1-dependent control of the MT attachment and detachment cycle. In AAA3 mutants, impaired release gates ATP hydrolysis at AAA1, and this gate is lifted with added salt. We analyzed coordination between the two sites by addition of the slowly hydrolyzable ATP analog ATPγS. ATPγS induced pauses in dynein motility, and the frequency of pausing increased with ATPγS concentration. Analysis of the pausing behavior revealed that ATPγS must bind to one site on each monomer to completely pause motility. In contrast to that of wild-type (WT) dynein, the motility of a dynein mutant unable to bind ATP to the AAA3 site was insensitive to ATPγS, thus indicating that pausing is initiated by ATPγS binding to only the AAA3 sites and not to AAA1. A dynein construct with only one AAA3 site takes many successive fast steps between long pauses in the presence of equimolar ATP and ATPγS. These results showed that AAA3 does not hydrolyze ATP in concert with AAA1 during the mechanochemical cycle. Instead, when bound to a nucleotide in a posthydrolysis state, it completes an allosteric circuit that allows AAA1 to modulate MT affinity. We propose that the AAA3 site acts as a 'switch' that converts cytoplasmic dynein from transporting to anchoring modes of motility.

RESULTS
AAA3 mutants remain tightly attached to MTs
ATPase mutations to dynein's AAA3 domain induce strong attachment to the MT and dramatically reduce motility. From these results, we hypothesized that AAA3 is required for AAA1-directed MT release. To test this hypothesis, we characterized how mutations to the AAA3 site affect nucleotide-dependent release of dynein from MTs. Specifically, we expected monomeric dyneins bearing ATPase mutations to AAA3 to show no ATP-dependent increase in release rate from the MT, comparable to WT monomers in the absence of nucleotide.

We used point mutations to the Walker A (K-A) and Walker B (E-Q) motifs of the AAA1 and AAA3 sites of an N-terminally truncated 331-kDa S. cerevisiae dynein monomer (referred to as Dyn1331kD)8. To estimate the release rate of monomers from MTs, we labeled these motors with a tetramethylrhodamine (TMR) dye at the N terminus and bound them to surface-immobilized MTs. We measured nucleotide-dependent MT affinities by quantifying changes in total fluorescence signal for each MT after nucleotide addition29. In agreement with results of biochemical assays on Dictostelium dynein18, WT monomers did not release in the absence of nucleotide or in the presence of the nonhydrolyzable ATP analog adenylylimidodiphosphate (AMP-PNP). Upon addition of 2 mM ATP, a majority (60.5%) of WT monomers released from MTs. Whereas AAA1E-Q (E1849Q) monomers released from MTs similarly (66%) to WT upon ATP addition, AAA1K-A (K1802A) showed minimal release, in agreement with the previous finding that ATP binding, not hydrolysis, at AAA1 triggers MT release14. In comparison to WT, only ~20% of AAA3K-A and AAA3E-Q monomers released from MTs after ATP addition (Fig. 1c). These results showed that both ATP binding and ATP hydrolysis mutations to AAA3 abolish nucleotide-dependent MT release of dynein, results consistent with blocked communication from AAA1 to the MT.

To quantify the MT affinity of AAA3 mutants in comparison to WT, we measured the effect of applied force on the release rates of dynein monomers by using optical trapping. We attached monomeric dyneins to polylysine beads from their C termini by using a short DNA tether. We then moved the motor-coated beads ±125 nm between two positions above the MT in a square wave pattern. Stochastic binding and release of dynein from MTs allowed measurement of release rates over a range of applied forces (1–12 pN) (Fig. 1d). In the absence of ATP (apo), individual WT monomers released from MTs with 7.7 s−1 pN−1 when force was applied toward the minus end, whereas release toward the plus end was slower (0.04 s−1 pN−1)29.
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**Figure 2** Dyn1 motility is gated by MT release in the absence of an active AAA3 site. (a) Schematic of Dyn1331KD dimerized through GST. (b,c) Kymographs of TMR-labeled WT (b) and AAA3K-A (c) walking along axonemes at 1 mM ATP. (d,e) Single-molecule velocities of WT (d) and AAA3K-A (e) at 1 mM ATP and 0–100 mM KCl. Error bars, s.e.m. Number of technical replicates (individual molecules) in d: 0 mm, 105; 20 mm, 125; 40 mM, 104; 75 mM, 142; 100 mM, 123. Number of technical replicates in e: 0 mM, 70; 20 mM, 88; 40 mM, 71; 75 mM, 92; 100 mM, 92; 150 mM, 102; 200 mM, 103; 250 mM, 42. (f,g) MT-stimulated ATPase activity of WT (f) and AAA3K-A (g) as a function of added KCl. Error bars, s.e.m. (n = 3 technical replicates).

(1d and Supplementary Fig. 2). Addition of 1 mM ATP increased the release rate of WT monomers by an order of magnitude. In contrast, the MT release rates of AAA3K-A (K2424A) and AAA3G-Q (E2488Q) at 2 mM ATP (1.7 s⁻¹ pN⁻¹ and 0.04 s⁻¹ pN⁻¹ toward the MT minus end and plus end, respectively) were both similar to those of WT monomers in the apo state (Fig. 1e and Supplementary Fig. 2). Therefore, AAA3 mutants remain strongly attached to MTs in the presence of ATP and can be removed from the MT only by force, similarly to WT in the apo state. Because ATP binding to AAA1 is required for MT release in WT dynein¹⁸,²³, our results suggest that communication from AAA1 to the MT requires a functional AAA3 domain.

**AAA3 lifts an MT gate to allow robust motility**

We next investigated the effects of alterations in MT affinity on the motility of a dynein dimer. The reduced motility and ATPase activity of AAA3 ATPase mutants may be related to the inability of AAA1 to control detachment from MTs without an active AAA3 site, such that impaired MT release slows down ATP hydrolysis at AAA1. Alternatively, AAA1 may require hydrolysis at AAA3 to proceed through its mechanochemical cycle. To distinguish between these possibilities, we reduced the MT affinity of dynein motors by increasing the salt (KCl) concentration and measured the velocity and MT-stimulated ATPase rates of WT and AAA3 mutants. If MT detachment rate limiting in AAA3 mutants, we would expect both the velocity and MT-stimulated ATPase activity to increase with added salt. If hydrolysis at AAA3 were required for the cycle to proceed, we would not have expected addition of salt to substantially affect motility.

To measure single-molecule velocity, we artificially dimerized Dyn1331KD monomers with a glutathione S-transferase (GST) tag. This construct has nearly identical motility properties to those of full-length dynein⁸,⁹. We recorded the velocity and run length of TMR-labeled motors along surface-immobilized axonemes at 0–200 mM KCl, using total internal reflection fluorescence (TIRF) microscopy (Fig. 2a–c). WT moved at 120 nm s⁻¹ without added salt, and its velocity remained largely unaffected with up to 100 mM added salt (Fig. 2d). In contrast, the velocity of AAA3K-A increased substantially with added salt. At 200 mM KCl, the velocity (42 nm s⁻¹) was three times greater than that in the no-salt condition (14 nm s⁻¹) (Fig. 2e). Run lengths for both WT and AAA3K-A decreased with added salt, consistently with reduced MT affinity (Supplementary Fig. 3).

To test whether increased MT release could speed up the AAA1 hydrolysis cycle in the absence of AAA3 activity, we measured the catalytic rate constant ($k_{cat}$) of WT and AAA3K-A at saturating MT concentrations and variable salt concentration. At 150 mM KCl, the $k_{cat}$ of AAA3K-A increased by 70%, whereas the $k_{cat}$ of WT decreased slightly (25%) (Fig. 2f,g). Addition of 100 mM salt increased the Michaelis-Menten constant ($K_m$) for WT by an order of magnitude for WT and AAA3K-A, results consistent with reduced MT affinity (Supplementary Table 1). We note that the velocity and ATP hydrolysis rate of AAA3K-A was lower than that of WT at high salt. Full recovery in speed and ATPase activity may require proper timing of strong and weak MT affinity states orchestrated with the force-generation cycle, which cannot be achieved with salt titrations.

These results demonstrate why AAA3 mutants have dramatically reduced velocity and ATPase activity. ATP bound at AAA1 cannot directly release from the MT without an active AAA3 domain. In AAA3K-A mutants, MT release becomes rate limiting and gates hydrolysis at AAA1, to result in slow motility.

**Single-molecule inhibition of dynein motility**

We next turned our attention to the question of whether the AAA3 and AAA1 sites coordinate directly to drive MT release²⁷. Alternatively, AAA3 may serve as switch that allows AAA1 to communicate with the MTBD and does not coordinate with AAA1 (refs. 15,19,26) (Fig. 3a). Because mutations to the active site of AAA3 also affect the ATPase activity of AAA1, analysis of AAA1 and AAA3 mutants was not sufficient to address this question. We performed single-molecule enzyme-inhibition experiments, using a slowly hydrolyzable ATP analog, ATPγS³⁰,³¹. Binding of ATPγS, which mimics the ATP-bound state, to a motor stalled the nucleotide hydrolysis cycle and induced a pause in motility³². Analysis of pause frequency and duration provided insight into coordination among multiple ATPase sites²⁸,³⁰,³¹. To measure motility in the presence of ATPγS, we labeled WT dynein with a bright quantum dot (QD) and tracked it at nanometer resolution for several minutes in the presence of saturating (1 mM) ATP and variable (0–100 µM) ATPγS³⁰. In the absence of ATPγS, the motors moved at a nearly constant velocity of 120 nm s⁻¹. The addition of 2–100 µM ATPγS reduced the velocity of the motors (Supplementary Movie 1). Traces of individual motors revealed that the slowing was due to transient pauses in motility (Fig. 3b). As the concentration of ATPγS increased, both the frequency and duration (Supplementary Fig. 4) of pausing increased.

The pause density (PD), defined as the number of pauses per unit length traveled by the motor, is related to the mole fraction of inhibitor-bound motors²⁸,³⁰. To calculate PD, we measured the residence time of individual motors within 50-nm bins along the MT axis...
Figure 3 Single-molecule enzyme inhibition of dynein by ATP$_7$S. (a) AAA3 may facilitate MT release either by directly coordinating its ATP hydrolysis cycle with that of AAA1 (left and middle) or by controlling an allosteric switch that allows AAA1 to communicate with the MTBD (right). (b) Representative traces of WT dynein motility at 1 mM ATP and varying amounts of ATP$_7$S, showing increased frequency of pauses at higher ATP$_7$S concentrations. Number of technical replicates: 0 µM, 246; 1 µM, 120; 2 µM, 188; 4 µM, 232; 6 µM, 170; 8 µM, 179; 12 µM, 144; 20 µM, 140; 40 µM, 231; 100 µM, 135. (c) Residence-time histogram of a single motor for each 50-nm distance traveled along its path. Peaks indicate locations in which the motor experiences a prolonged pause. (d) Cumulative residence-time histogram of 120 traces, fit to biexponential decay (red curve). The areas under the red and yellow curves represent the slow and fast populations, respectively. (e) PD (blue circles) as a function of ATP$_7$S concentration. Red curve represents a fit to the Hill equation. (f) Velocity of WT dynein as a function of ATP concentration (black circles). Data fit well ($R^2 = 0.996$) to Michaelis-Menten kinetics (blue curve) and deviate away ($R^2 = 0.72$) from the Hill equation with Hill coefficient $n = 2$ (red dashed curve). Error bars, s.d. Number of traces of single motors: 1,000 µM, 127; 500 µM, 72; 200 µM, 93; 100 µM, 97; 50 µM, 61; 30 µM, 7; 20 µM, 86; 10 µM, 85; 5 µM, 66; 2 µM, 74; 1 µM, 101; 0.5 µM, 24; 0.2 µM, 20.

(3c and Supplementary Note). A residence-time histogram fit well to a biexponential decay ($R^2 > 0.97$ for each ATP$_7$S concentration tested), revealing two distinct populations: fast, with a short residence time ($0.374 \pm 0.004$ s, mean $\pm$ 95% confidence interval (CI)), and slow, with a longer residence time ($3.18 \pm 0.45$ s, Fig. 3d and Supplementary Fig. 4). We calculated the PD from the ratio of the slow population to the entire population (Supplementary Fig. 5).

To extract the binding parameters of ATP$_7$S to dynein, we fit PD as a function of ATP$_7$S concentration to a Hill equation (Fig. 3e), assuming that there were no intermediate states of the pausing behavior. The dissociation constant ($K_d$) of ATP$_7$S for WT dynein was $9.9 \pm 3.2$ µM (mean $\pm$ s.d.), indicating that analog binding at the site of inhibition was nearly saturated by 100 µM ATP. The Hill coefficient ($n$) was 1.97, indicating that two ATP$_7$S must bind to the motor to initiate a pause. The plot was not well fit by fixing the Hill coefficient to $n = 1$ ($P = 0.03$, F test, one tailed, $n = 9$ concentrations).

The fit also revealed the maximum PD ($PD_{max} = 0.011$ nm$^{-1}$) at saturating ATP$_7$S (Fig. 3e). Because the mean step size of dynein is ~8 nm, the result indicated that WT dynein takes ~11 steps between pauses at saturation. The velocity at saturation (36 nm s$^{-1}$, Supplementary Fig. 6) remained substantially faster than for AAA5 and AAA1 mutant homodimers,19,26 thus indicating that the site of inhibition is not responsible for stepping.12,21 To estimate the number of ATP hydrolysis events required for dynein stepping, we measured the velocity of WT at 200 nM–1 mM ATP in the absence of ATP$_7$S (Fig. 3f). Velocity measurements fit well to Michaelis-Menten kinetics ($K_{m(ATP)} = 46.3 \pm 6.5$ µM (mean $\pm$ 95% CI), $V_{max} = 114$ nm s$^{-1}$), thus suggesting a single hydrolysis event per step. The results excluded a model with two coordinating ATP hydrolysis sites per step.

ATP$_7$S specifically inhibits the AAA3 site

We next sought to identify which sites are inhibited by ATP$_7$S. Because a single active head is sufficient to drive dynein motility4, and two ATP$_7$S are required to generate a pause (Fig. 3), we hypothesized that one ATP$_7$S must bind to each head of the dimer during pauses. To test whether ATP$_7$S preferentially binds to the AAA3 site to induce a pause, we measured the pausing behavior of the AAA3$_{K-A}$ mutant at saturating ATP and $0$–$1,000$ µM ATP$_7$S (Fig. 4a and Supplementary Movie 2). Surprisingly, we found that the velocity and PD of these mutants were entirely unaffected by ATP$_7$S at all concentrations tested (Fig. 4b,c), results indicating that the AAA1 site is insensitive to ATP$_7$S. We conclude that ATP$_7$S specifically inhibits AAA3, and both AAA3 sites in a dimer must be inhibited to initiate a pause.

We confirmed this result in bulk, using ATPase assays at saturating ATP and varying concentrations of ATP (Fig. 4d,e and Supplementary Table 2). We measured the $K_{m(ATP)}$ and $k_{cat(ATP)}$ of WT and AAA3$_{K-A}$ in response to $0$–$40$ µM (for WT) or $0$–$1,000$ µM (for AAA3$_{K-A}$) ATP$_7$S (Supplementary Note). In agreement with the velocity measurements (Eq. 3f), $K_{m(ATP)}$ for WT was $46 \pm 5$ µM in the absence of ATP$_7$S, and it increased to $818 \pm 80$ µM at 10 µM ATP$_7$S, whereas $k_{cat(ATP)}$ remained largely unaffected ($17.0 \pm 0.5$ s$^{-1}$ versus $19.0 \pm 0.7$ s$^{-1}$, Supplementary Table 2), results consistent with competitive inhibition. By comparison, the AAA3$_{K-A}$ homodimer was largely insensitive to ATP$_7$S at concentrations of up to 1 µM, except for a modest increase in $K_{m(ATP)}$ from $38 \pm 3$ µM to $106 \pm 36$ µM. These results showed that ATP$_7$S is a strong inhibitor of the AAA3 site and not of the AAA1 site.

To gain insight into the mechanism of AAA3-specific inhibition, we measured the ATP$_7$S-hydrolysis rate of WT and AAA3$_{K-A}$ in the absence of ATP (Fig. 4f). The $k_{cat(PP)}$ of $2.3 \pm 0.2$ s$^{-1}$ for AAA3$_{K-A}$ and $4.5 \pm 0.4$ s$^{-1}$ for WT dynein) was relatively fast, and the $K_{m(PP)}$ was similar for WT and AAA3$_{K-A}$ ($500 \pm 110$ µM for WT, $425 \pm 98$ µM for AAA3$_{K-A}$). These results indicated that the fast hydrolysis of ATP$_7$S at high concentrations does not arise from AAA3. This is because $k_{cat(PP)}$ was much faster than the pausing rate of WT in the
A dynein motor with a single active AAA3 site would be expected to switch between two states: a fast, uninhibited state and a slow, inhibited state. For this purpose, we used a heterodimer of a WT monomer and a chimeric protein containing a seryl-tRNA synthetase (SRS) domain fused to a dynein stalk-MTBD23,24 (referred to as SRS-WT, Fig. 5a). SRS-WT moves processively at 35% of the speed of the WT homodimer29. Because SRS-WT contains a single AAA+ ring, we expected it to switch between fast and slow states without an intermediate in the presence of ATPγS. We tracked the motility of QD-labeled SRS-WT at 1 mM ATP and 0–100 μM ATPγS. As the ATPγS concentration increased, the velocity of SRS-WT decreased, owing to frequent pauses (Fig. 5a), similarly to observations for WT (Fig. 3a). Normalized histograms of motor velocity revealed two distinct populations (Fig. 5b). The velocity of the fast state was 68 ± 31 nm s−1, whereas the motor was nearly immotile (−1.2 ± 8.2 nm s−1, mean ± s.d.) in the slow state (Fig. 5b). We observed the velocity distribution of WT at low ATPγS concentrations showed signs of more than two subpopulations (Supplementary Fig. 5).

### Amino Acid Mutations Alter Properties of the AAA3 Site

| Amino Acid | Mutant | Description |
|------------|--------|-------------|
| AAA3       | K-A    | Kinase-Activated |

The K-A mutant is insensitive to ATPγS. (b) Representative traces of QD-labeled AAA3K-A motility at 1 mM ATP and 0–1,000 μM ATPγS (n = 805 traces), in which long pauses are not visible even at 1,000 μM ATPγS. (c) Velocity of AAA3K-A as a function of ATPγS concentration. Error bars, s.e.m. of 87–161 traces. (d) PD of AAA3K-A at 0–1,000 μM ATPγS concentration. Error bars, s.e.m. of PDs calculated from 87–161 traces. (e) ATPase rates of WT (d) and AAA3K-A (e) at saturating MT concentrations. Colors represent different concentrations of ATPγS. All plotted data are from 3 technical replicates. (f) ATPγS hydrolysis rates of WT and AAA3K-A at saturating MTs in the absence of ATP, showing a weak affinity of ATPγS (K_m(AMP)) = 500 ± 110 μM for WT and 425 ± 98 μM for AAA3K-A, mean ± 95% CI) and a relatively fast turnover rate (k_cat(AMP) = 4.52 ± 0.42 s−1 for WT and 2.34 ± 0.19 s−1 for AAA3K-A, mean ± 95% CI) consistent with poor inhibition of AAA1. Number of technical replicates (individual traces) in b and c: 0 μM, 87; 10 μM, 193; 40 μM, 75; 200 μM, 141; 400 μM, 161; 1 mM, 148.
The SRS-WT heterodimer takes long runs between pauses at equimolar concentrations of ATP and ATPγS. (a–c) Representative traces of SRS-WT motility at 10:1 (a), 2:1 (b) and 1:1 (c) ratios of ATP/ATPγS, respectively. Long runs of fast motility (>15 nm s−1) are shown in red. (d–f) Velocity (d), frequency of fast runs (e) and lengths of fast runs (f) of SRS-WT motility at different ratios of ATP to ATPγS. Error bars, s.e.m.

The fast run frequencies (e) and run lengths (f) are calculated from 141, 68 and 9 fast runs for 10:1, 2:1 and 1:1, respectively.

a shift from the fast to the slow population as the ATPγS concentration increased. Overlaid velocity histograms at different ATPγS intersected in a single point (Fig. 5b), results consistent with two-state inhibition of SRS-WT by ATPγS.

We calculated PD from residence times in 50-nm bins (Supplementary Note and Supplementary Fig. 7) and fitted the data to a Hill equation with n = 1 (Fig. 5c), consistent with two-state inhibition. The Kd was similar to that of WT (7.8 ± 2.4 µM versus 9.9 ± 1.6 µM, mean ± 95% CI), and pausing was saturated at 100 µM ATPγS. Unlike results for WT (Supplementary Fig. 5), the pause duration remained nearly constant (~15 s) at all ATP/ATPγS concentrations tested (Fig. 5d), thus suggesting that exit from a pause is limited by release of a single bound ATPγS, and consecutive pauses are separated by long runs of fast motility.

If AAA3 hydrolyzes an ATP at every step of the motor, the motor would be expected to pause after taking each step at saturating ATPγS9,31. If the AAA3 instead acts as a switch with a slower hydrolysis rate than that of AAA1, SRS-WT would be expected to take many fast steps between pauses because a persistently bound hydrolysis product at AAA3 would prevent reinhibition (Fig. 3a). The PDmax for SRS-WT was 0.0063 pause nm−1 (Fig. 5c), thus indicating that SRS-WT embarks on fast runs for many steps between long-lived pausing events.

The presence of extended fast runs between pauses was also apparent from traces of SRS-WT at saturating ATPγS (100 µM) and ATP (1 mM) (Fig. 6a). However, long runs of fast motility between pauses could also result from the high (10:1) ATP/ATPγS ratio used in these assays18,19,26. To exclude this possibility, we analyzed the pausing behavior of SRS-WT at saturating ATP/ATPγS intersected in a single point (Fig. 5b), results consistent with two-state inhibition of SRS-WT by ATPγS.

We found that AAA1 and AAA3 do not coordinate directly. Instead, AAA3 hydrolyzes ATP and results in reduced speed and strong MT attachment. After ATP hydrolysis at AAA3, the switch is on, and

between AAA1 and the MTBD. Using single-molecule enzyme-inhibition assays, we found that ATPγS specifically inhibits AAA3, thus granting a unique opportunity to investigate this site in further detail. We found that AAA1 and AAA3 do not coordinate directly. Instead, AAA3 hydrolyzes ATP and results in reduced speed and strong MT attachment.

Combining these results with previous work, we propose that AAA3 acts as a switch that regulates AAA1-directed MT release (Fig. 7). In the apo or ATP-bound state (mimicked by AAA3 ATP binding and hydrolysis mutants, respectively), AAA1 cannot allosterically direct MT release through the ring (Fig. 7)15,19,26. In this switch off state, dynein remains processive, albeit at a substantially lower speed26, presumably via tension generated on the MTBD through the power stroke of the linker17,26. Slow MT detachment in turn slows the hydrolysis cycle at AAA1, to result in reduced speed and strong MT attachment. After ATP hydrolysis at AAA3, the switch is on, and

DISCUSSION

Our results illuminate the role of the AAA3 site in the cytoplasmic dynein mechanochemical cycle. We found that ATPase mutations to AAA3 disrupt ATP-induced release of dynein from MT, results indicating that the AAA3 domain is essential for communication between AAA1 and the MTBD. Using single-molecule enzyme-inhibition assays, we found that ATPγS specifically inhibits AAA3, thus granting a unique opportunity to investigate this site in further detail. We found that AAA1 and AAA3 do not coordinate directly. Instead, AAA3 hydrolyzes ATP and results in reduced speed and strong MT attachment. After ATP hydrolysis at AAA3, the switch is on, and

Figure 6 The SRS-WT heterodimer takes long runs between pauses at equimolar concentrations of ATP and ATPγS. (a–c) Representative traces of SRS-WT motility at 10:1 (a), 2:1 (b) and 1:1 (c) ratios of ATP/ATPγS, respectively. Long runs of fast motility (>15 nm s−1) are shown in red. (d–f) Velocity (d), frequency of fast runs (e) and lengths of fast runs (f) of SRS-WT motility at different ratios of ATP to ATPγS. Error bars, s.e.m.

Figure 7 A model for the role of AAA3 in dynein’s mechanochemical cycle. The ATP hydrolysis cycle at AAA1 drives the stepping motility of dynein. Left, when AAA3 is in the apo state, communication between AAA1 and the MTBD is blocked, thus resulting in slow, force-dependent MT release and consequent slow progression through the AAA1 hydrolysis cycle (τcycle = 1.2 s)15,18. Right, when ATP binds to AAA3 and is hydrolyzed, the allosteric circuit connecting AAA1 and the MTBD is completed. In this state, ATP binding at AAA1 leads to fast release from the MT and subsequent progression through the AAA1 hydrolysis cycle (τcycle = 0.13 s). Cycle times are calculated from the bulk ATPase rates per head. This AAA3-controlled switch may have an essential role in the reproposing of dynein for intracellular transport (i.e., fast MT release) and anchoring (i.e., slow MT release) functions. P_i, inorganic phosphate.
the allosteric circuit connects AAA1 to MTBD, thus allowing rapid, ATP-stimulated release from the MT (Fig. 7). This switch on post-hydrolysis state (either ADP-P or ADP) persists for an average of ~20 steps of the motor, allowing the motors to move at full speed. Conformational changes required for release from the MT is driven by ATP hydrolysis in AAA1 (refs. 18,23). Eventual release of products at AAA3 turns the switch off until the next nucleotide cycle.

A possible clue for the molecular basis of the AAA3 switch comes from structural studies of dynein and other AAA motors, in which allosteric communication across the ring is driven by coupled rigid-body motions of the subunits.15,16,35–38 In dynein, these motions initiate with ATP binding to AAA1 (refs. 16,36). The ATP-binding cleft at the AAA1 site has an open conformation in the apo state, which is consistent with its low affinity for ATP (Fig. 4f). ATP binding induces AAA1–AAA2 gap closure15,16,36, leading to rigid-body rearrangement of AAA2–AAA4 (refs. 15,35,36,38). These rearrangements in the AAA+ ring may create an allosteric circuit that connects AAA1 to the base of the stalk to couple ATP hydrolysis with control of MT binding affinity15,23,34. Our results indicate that AAA3 has a major regulatory role in this circuit. In the apo or ATP-bound states of AAA3, the communication between AAA1 and the base of the stalk is disrupted. In a post-hydrolysis state of AAA3, AAA1 can efficiently direct AAA3 rearrangement, which in turn couples to MT release through AAA4 and AAA5 (refs. 1,15,16).

Our model has implications for the role of AAA3 in dynein’s cellular functions. We showed that dynein switches between fast and slow motility according to the nucleotide state of AAA3. We speculate that regulation of AAA3 within cells can repurpose cytoplasmic dynein for different tasks, e.g., from rapidly transporting intracellular cargos along the length of an MT to anchoring astral MTs to the cell cortex during mitosis.6,39 Because the cellular concentration of ATP is saturating for dynein, the AAA3 switch is expected to be on for the majority of the time. The AAA3 switch may be allosterically regulated by adaptor proteins that bind to the dynein motor domain. One clue comes from studies of the dynein regulatory protein Lis1, which binds to the AAA+ ring near AAA3 (refs. 11,40). The Lis1–dynein complex also shows persistent MT attachment and bears a strong resemblance in its velocity to AAA3 mutants.11,40 Interestingly, a similar phenotype can also be recapitulated by mutating the sensor arginine of AAA4 that reaches into the AAA3 ATP-binding pocket.40 Further work on conformational changes within the AAA+ ring as a function of nucleotide state of AAA3 and on how dynein-associated proteins affect these conformational states is required to elucidate the regulation of the dynein motor domain.11,40–42.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.A.D. and A.Y. designed experiments, M.A.D. and C.A.C. performed single-molecule inhibition assays, M.A.D. and V.B. collected and performed single-molecule motility assays, M.A.D. performed bulk ATPase assays, F.B.C. performed optical-trapping assays and monomer release assays, M.A.D., C.A.C. and F.B.C. analyzed the data, and M.A.D. and A.Y. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cloning and molecular biology. We used an S. cerevisiae cytoplasmic dynein gene (DYFN1) truncated at the N terminus (encoding amino acids 1219–4093) and artificially dimerized through an N-terminal GST tag (referred to as GST-Dyn1131kD) as a template for mutagenesis17. Dynein constructs were tagged with a HaloTag (Promega) at either the N or C terminus for fluorescent labeling17,18. The K1694A mutation was inserted into GFP-GST-Dyn1131kD by homologous recombination. To construct the SRS-WT heterodimer, a monomeric dynein bearing an N-terminal FRB domain (FRB-Dyn1131kD-Halo) was dimerized with a chimeric construct, which consists of SRS fused with a dynein stalk-MTBD and a C-terminal FKBP12 tag. The stalk registry (85:82) of the SRS construct ensures tight binding of the MTBD to MTs23,34. Heterodimers were constructed by mixture of 1 µl of 500 nM FRB-Dyn1131kD with 1 µl of 5 µM FKBP12-SRS85:82 and 2 µl of 600 nM rapamycin13,34.

Protein expression, purification and labeling. Dynein proteins were expressed in yeast and affinity purified as previously described8. Active motors were purified by the MT bind-and-release assay8,9,45 and stored in dynein loading buffer (DLB, 30 mM HEPEs, pH 7.2, 2 mM MgCl2, 1 mM EGTA and 10% glycerol). Expression and purification of SRS-MTBd was carried out from Escherichia coli, as previously described8. For velocity measurements, the motors were labeled with 10 µM TMR HaloTag ligand for 1 h on ice during protein preparation, prior to washing of the immunoglobulin G beads. For high-resolution tracking measurements of WT dynein, amine-modified QDs emitting at 655 nm (QD655, Life Technologies) was labeled with 1 µl –5 nM monomeric TMR-labeled dynein in DLBC without ATP and washed twice with DLBC to remove excess motor. The sample was then washed with imaging buffer, which did not contain ATP and TMR fluorescence was monitored under TIRF illumination. MT-bound dynein motors were monitored for 100 frames at 280-ms temporal resolution before the assay solution was exchanged with imaging buffer plus nucleotide. The percentage of released dynein monomers was quantified by taking the average TMR fluorescence intensity along 5 MTs per movie (three movies per nucleotide condition) for 10 s before and after flow and by subtracting the background intensity from TMR fluorescence. The change in intensity before and after flow was normalized by the intensity before flow. The reduction of fluorescence intensity was not due to permanent photobleaching of TMR, which is minimal on the timescale of our experimental conditions, as we confirmed by repeating the flow experiment in the absence of nucleotide.

ATPase assays. MTs were prepared with standard techniques. Briefly, 3.5 mg ml−1 purified bovine tubulin in BRB80 buffer (80 mM Pipes, pH 6.8, 1 mM MgCl2 and 1 mM EGTA) was polymerized by addition of 1 mM GTP, and this was followed by stepwise addition of 10% volume each of 4 µM, 40 µM and 400 µM, respectively. 0.15 m M, we corrected for stage drift by tracking immobilized deep-red fiducial beads on the slide concurrently with data acquisition and subtracting their velocities from the measured velocities of dynein molecules. To measure the pausing behavior, QD-labeled dynein motors were imaged in an imaging buffer consisting of DLBC at 100–1,000 µM ATP and 0–1,000 µM ATPγS, supplemented with 10 mM β-mercaptoethanol to prevent blinking48, 2 mM phosphoenolpyruvate and 0.1 mg ml−1 pyruvate kinase (Sigma-Aldrich) to regenerate ATP.

ATPase assays. MTs were prepared with start standard conditions. Briefly, 3.5 mg ml−1 purified bovine tubulin in BRB80 buffer (80 mM Pipes, pH 6.8, 1 mM MgCl2 and 1 mM EGTA) was polymerized by addition of 1 mM GTP, and this was followed by stepwise addition of 10% volume each of 4 µM, 40 µM and 400 µM, respectively. Those were normalized by the intensity before flow. The reduction of fluorescence intensity was not due to permanent photobleaching of TMR, which is minimal on the timescale of our experimental conditions, as we confirmed by repeating the flow experiment in the absence of nucleotide.

Optical-trapping assays. Optical-trapping assays were performed with a custom-built optical trap, as described previously29. To apply forces through the C-terminal domain, dynein monomers with a C-terminal HaloTag were labeled with a short (74 bp) double-stranded DNA strand containing biotin at its distal end. Since the DNA–biotin complex was immobilized with streptavidin-coated polystyrene beads (0.86-µm diameter, Invitrogen). The motor-bead mixture was then diluted to 10% in DLB supplemented with 5 mg ml−1 casein, the PCA and PCD oxygen scavenging system and 2 mM ATP. For the apo condition, ATP was not added to the motor bead mixture, to result in residual ATP concentrations of ~40 nM. Those were monitored using a DynaQ assay kit (Quant, BioTek Instruments) set to record A360 at 60-s intervals against a buffer-only blank. The final bead concentration was 0.1% weight by volume. Cy5-labeled adenosines were adsorbed to the surface of a flow cell before addition of the bead and dynein mixture. The beads were trapped by a −50 nM, 1,064-nm laser beam (Compass 1064, Coherent) to achieve a spring constant of ~0.05 pN nm−1. Trapped beads were positioned over a Cy5-labeled axoneme and dynein motors, which were then monitored by single-molecule motility assays. Those that were not coated with antibody did not label the motor, and antibody-coated QDs did not label motors that were not tagged with GEP (not shown).

Microscopy and imaging. Single-molecule motility assays were performed on a custom-built objective-type total internal reflection fluorescence (TIRF) microscope equipped with an inverted microscope body (Nikon Ti-Eclipse) with perfect focusing system and a 1.45-NA 60x objective (Nikon, TIRF Plan Apochromat). The sample was illuminated with 488-nm and 532-nm laser beams (Coherent) to image QDs and TMR, respectively. Movies of TMR-labeled dynein were recorded with a 0.5–2.0 s exposure time under ~2-mW, 532-nm excitation. QD655-labeled dyneins were imaged with a 50–100-ms exposure time. Fluorescence signal was detected with an ORCA-Flash4.0 sCMOS camera (Hamamatsu).
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