

A nucleotide code governs Lis1's ability to relieve dynein autoinhibition

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Dynein-1 is a microtubule motor that transports numerous cytoplasmic cargoes. Activation of motility requires it first overcome an autoinhibited state before its assembly with dynactin and a cargo adaptor. Studies suggest that Lis1 may relieve dynein's autoinhibited state, although evidence for this is lacking. We first determined the rules governing dynein–Lis1 binding, revealing that their binding affinity is regulated by the nucleotide-bound states of each of three nucleotide-binding pockets within dynein. We also found that distinct nucleotide ‘codes’ coordinate their binding stoichiometry by impacting binding affinity at two different sites within the dynein motor domain. Electron microscopy revealed that a 1 dynein:1 Lis1 complex directly promotes an uninhibited conformational state of dynein, whereas a 1:2 complex resembles the autoinhibited state. Cryo-electron microscopy revealed that the structural basis for Lis1 opening dynein relies on interactions with the linker domain. Our work reveals the biochemical basis by which Lis1 relieves dynein autoinhibition.

Cytoplasmic dynein-1 is important for numerous processes, including tissue morphogenesis and error-free mitosis¹. This minus-end-directed motor complex, which comprises light, light–intermediate, intermediate and heavy chains, transports numerous cargoes, including membrane-enclosed vesicles, RNAs and the nucleus. To engage in processive transport, dynein must first associate with dynactin and a cargo adaptor protein^{2,3}. As a consequence of linking dynein to dynactin and a cargo, these latter molecules activate dynein motility⁴.

As a check against the inappropriate delivery of cargoes, dynein and its regulators use numerous means of regulation. Among them are autoinhibitory mechanisms for the dynactin complex, as well as for several of the adaptor proteins^{4–10}. Dynein itself adopts an autoinhibited conformation referred to as the ‘phi’ particle (because of its resemblance to the Greek letter)^{11–13}. The phi conformation or analogous states have been observed for cytoplasmic dynein-1, dynein-2 (refs. 14,15) and the axonemal outer-arm dynein¹⁶. Although somewhat different, these states all involve intracomplex contacts between various dynein complex subunits and reduce the ability of dynein to bind

microtubules and/or assemble into motile dynein–dynactin–adaptor (DDA) complexes^{12,14,16}.

Although relief of autoinhibition is required for assembly of dynein-1 into motile DDA complexes¹², how this occurs is unclear. Recent studies have suggested that the phi particle can be ‘opened’ by the lissencephaly-related protein Lis1 (refs. 13,17–20), a critical conserved regulator of dynein. In addition to this proposed function, Lis1 also helps link dynein to dynactin during assembly of DDA complexes^{5,17,18} and promotes the association of dynein–dynactin with the plus ends of dynamic microtubules^{21–23}, which is thought to be important for their delivery to various cargoes^{20,24–27}. A role for Lis1 in opening phi dynein or somehow stabilizing the open state is based on compelling but indirect evidence.

A complicating factor may be an incomplete understanding of the rules governing dynein–Lis1 binding and the consequent inability to directly correlate the Lis1-bound state of dynein with the open versus phi state. Moreover, Lis1 appears to have variable dynein-binding modes: one in which a single Lis1 WD40 domain is bound to the dynein

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motor and another in which two WD40s from a Lis1 dimer are bound to a single motor domain^{28–31}. Although evidence suggests that the nucleotide-bound state of dynein coordinates Lis1 binding^{29,31–33}, much remains unknown. For example, it is well established that ATP plus vanadate (ATP-Vi; which mimics an ADP-Pi state³⁴) stimulates dynein–Lis1 binding³². In addition, we recently made the confounding finding that both AMPPNP (a nonhydrolyzable ATP analog) and apo (nucleotide depletion) conditions promote their binding³¹. These seemingly contradictory results are likely a result of dynein possessing multiple ATP-binding modules³⁵ (that is, ATPase associated with various cellular activities (AAA) domains), thus complicating the determination of the exact nucleotide state of the motor domain in previous studies. Therefore, to understand whether and how Lis1 affects dynein autoinhibition, it is important that we first clarify the rules governing their binding.

In this study, we use a combination of biochemical and electron microscopy (EM)-based approaches to understand the rules and the consequences of dynein–Lis1 binding. We find that the nucleotide-bound states of AAA1, AAA3 and AAA4 all coordinate to affect dynein–Lis1 binding affinity. In addition to affecting the strength of binding, we find that specific nucleotide ‘codes’ impact dynein–Lis1 binding stoichiometry and determine whether Lis1 binding promotes an ‘open’ state of dynein. We use cryo-EM to resolve the structural details that account for Lis1 opening phi dynein and cell biological tools to assess the consequences of disrupting this process. Our results support a model in which Lis1 can indeed open and/or stabilize an uninhibited conformation of dynein but only when the AAA domains are in specific nucleotide-bound states.

Results

Microtubules and nucleotides impact dynein–Pac1 binding

We recently found that locking dynein in a microtubule-bound conformation (MT-B; Fig. 1a) greatly reduces dynein–Pac1 (yeast homolog of Lis1) and dynein–Lis1 binding affinity compared to a microtubule-unbound (MT-U) state³¹. Although different nucleotide conditions change the relative binding of Pac1 and Lis1 to both the MT-U and MT-B mutants, the MT-U state exhibits higher affinity for Pac1 and Lis1 in most cases (Fig. 1). This led us to ask two questions. Firstly, at which AAA module is each nucleotide condition acting to affect dynein–Pac1 binding? Secondly, does nucleotide occupancy at any of the AAA domains affect the MT-B/U state to govern dynein–Pac1 binding affinity? To address these questions, we assessed the binding of full-length Pac1 (a constitutive dimer) to truncated monomeric MT-U and MT-B dynein motors (dynein_{MOTOR}) with mutations that render each AAA module either unable to bind nucleotide (by mutating the Walker A motif, ‘WA’) or unable to hydrolyze ATP (by mutating the Walker B motif, ‘WB’; Fig. 1a). We mutated the primary site for ATP hydrolysis (AAA1)³⁶, as well as the two other AAA domains that possess ATPase activity (AAA3 and AAA4)^{37–40}.

We mixed equimolar concentrations of dynein_{MOTOR} and Pac1 in different nucleotide conditions and quantitatively assessed the proportion of unbound and bound species by mass photometry. As we noted previously³¹, two distinct complexes become apparent: a 1 dynein_{MOTOR}:1 Pac1^{dimer} complex (expected mass = 513 kDa) and a second species that is indicative of a 2 dynein_{MOTOR}:1 Pac1^{dimer} complex (expected mass = 872 kDa) (Fig. 1b and Extended Data Fig. 1a)³¹. We refer to these latter species as 2:1 complexes.

In almost all cases, the MT-U mutants bound Pac1 to a greater extent than the corresponding MT-B mutant (Fig. 1c–j and Extended Data Figs. 1–3). Because of the poorer binding of most of the MT-B dyneins to Pac1, we focus our analysis on the MT-U data. Our results show that both nucleotide-bound and unbound (apo) AAA1 and AAA3 pockets stimulate Pac1 binding, albeit to different extents and in different ratios. Whereas the AAA1^{WB} and AAA3^{WB} mutants robustly assemble 1:1 complexes with Pac1 (Fig. 1d,e), the corresponding WA mutants assemble 2:1 complexes (Fig. 1h,i). Our results further indicate that both apo-AAA1 and apo-AAA3 (that is, AAA1^{WA} and AAA3^{WA}) must possess at least one other nucleotide-bound AAA pocket for maximal 2:1 Pac1 binding, as apo conditions reduced this complex for each (for example, apo for AAA1^{WA} and AAA3^{WA}; Fig. 1h,i). In the case of AAA3^{WA}, ATP or ADP were required for maximal 2:1 complex formation.

Whereas the MT-U AAA4^{WA} mutant binds Pac1 poorly in most conditions (except ATP-Vi and apo), the MT-U AAA4^{WB} mutant exhibits a similar pattern of 1:1 complex formation to the wild-type (WT) motor. This indicates that the AAA4 pocket must be bound to nucleotide for dynein to adopt a high-Pac1-affinity state. Furthermore, whereas the MT-U AAA4^{WA} and AAA4^{WB} mutants assemble low levels of 2:1 complexes in most cases, apo conditions stimulate this binding, suggesting an apo-AAA4 can promote assembly of this particular complex.

We next assessed the consequences of combining the AAA1 and AAA3 mutations. Although the single AAA1^{WA} mutant assembles the 2:1 complex to a high degree (Fig. 1k), pairing this mutation with either AAA3^{WA} or AAA3^{WB} leads to poor Pac1 binding (Fig. 1l). On the other hand, we observed even higher levels of Pac1 binding when we combined AAA1^{WB} with either AAA3^{WA} or AAA3^{WB}. Whereas the former assembles both 1:1 and 2:1 complexes with Pac1, the latter almost exclusively assembles 1:1 complexes. Although AAA3 is a critical regulator of binding stoichiometry, we note one instance in which AAA1 can also do so; when the AAA3^{WA} single mutant is treated with ATP-Vi, we observe low degrees of 2:1 complex assembly but high levels of 1:1 binding (Fig. 1e,i). Given that AAA1 is the likely site at which ADP-Vi binds^{31,34}, these data indicate that AAA1 and AAA3 both affect the affinity and stoichiometry of dynein–Pac1 binding (Fig. 1m,n).

In almost all cases (with apo conditions for many of the mutants being one exception), the MT-U state binds Pac1 to a greater extent than the corresponding MT-B state, which is consistent with the model that microtubule binding by dynein reduces dynein–Pac1/Lis1 binding

Fig. 1 | The role of the AAA WA and WB motifs in dynein–Pac1 binding.

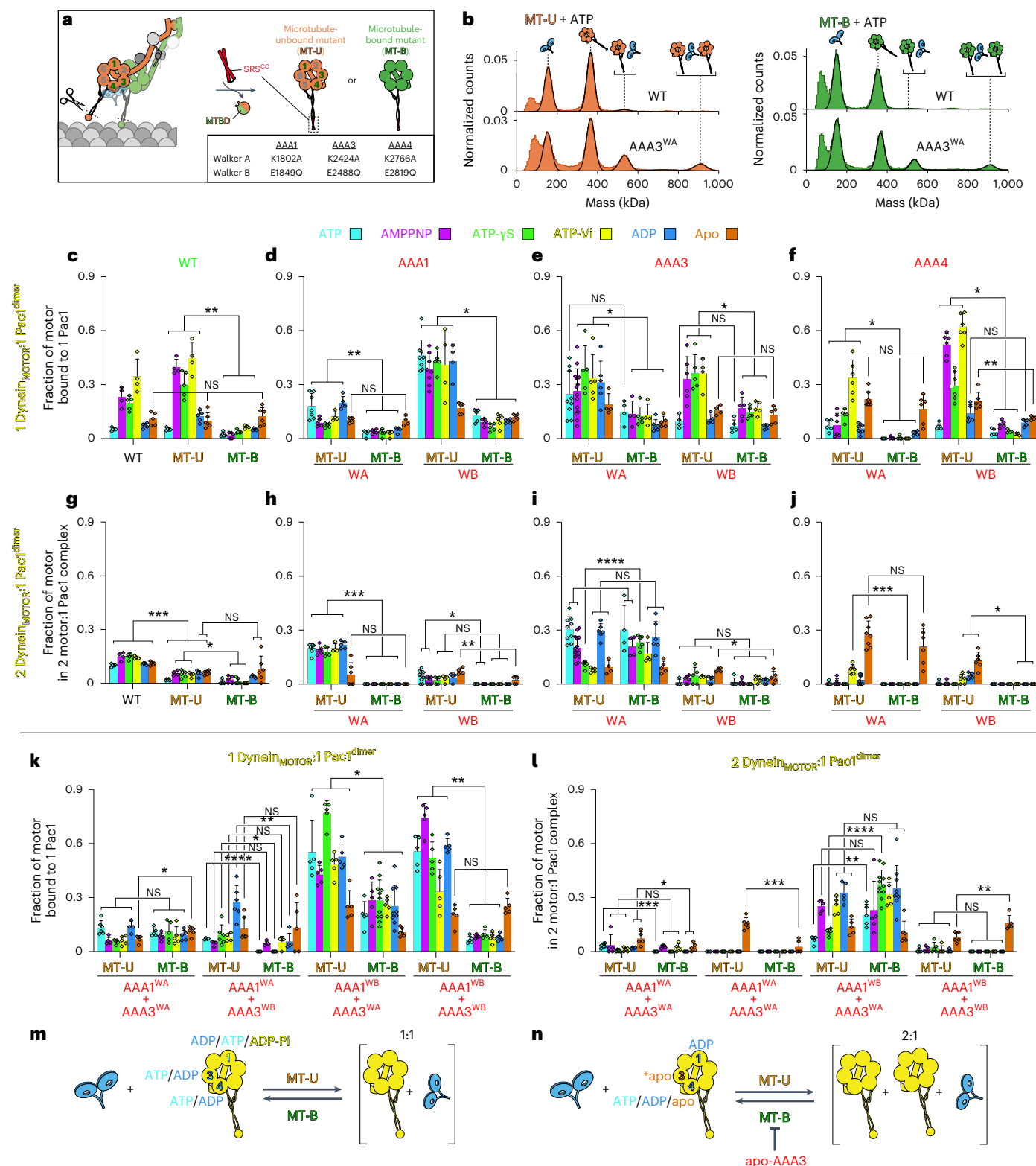
a, Cartoon depicting a microtubule-bound full-length dynein complex (with a translucent Pac1 to indicate region of binding) and monomeric dynein_{MOTOR} domains used for binding assays. The fragments were engineered to be locked in either a microtubule-unbound (MT-U) or -bound (MT-B) conformation by replacing the microtubule-binding domain (MTBD) with a rigid coiled-coil derived from seryl tRNA synthetase (SRS^{CC})^{31,58}. **b**, Representative mass histograms obtained from mass-photometry-based binding experiments with WT and AAA3^{WA} mutant (K2424A) dynein_{MOTOR}^{MT-U} and dynein_{MOTOR}^{MT-B}. **c–l**, Plots depict results (mean ± s.d. along with individual data points) from mass photometry binding assays. Plots indicate the fraction of motors within the 523-kDa peak, which corresponds to 1 Pac1^{dimer}:1 dynein_{MOTOR} monomer complexes (**c–f,k**), or those within the 897-kDa peak, which corresponds to 1 Pac1^{dimer}:2 dynein_{MOTOR} complexes (**g–k**). A cartoon schematic of complexes is shown above each respective peak in **b** (also see Extended Data Figs. 1–3). For **c,g**, from left to right, $n_{WT} = 4/4/5/4/5/5$, $n_{MT-U} = 4/4/5/4/5/6$ and $n_{MT-B} = 4/5/5/4/5/6$ independent replicates; for **d,h**, from left to right, $n_{MT-U}^{WA} = 7/6/5/4/5/7$,

$n_{MT-B}^{WA} = 5/5/5/4/5/4$, $n_{MT-U}^{WB} = 9/8/5/4/4/5$ and $n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for **e,i**, from left to right, $n_{MT-U}^{WA} = 9/15/5/5/6/4$, $n_{MT-B}^{WA} = 4/4/6/4/6/5$, $n_{MT-U}^{WB} = 4/6/5/4/5/4$ and $n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for **f,j**, from left to right, $n_{MT-U}^{WA} = 5/6/5/6/8/8$, $n_{MT-B}^{WA} = 5/5/5/5/5/6$, $n_{MT-U}^{WB} = 6/6/6/6/5/6$ and $n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for **k,l**, from left to right, $n_{MT-U}^{WA/WA} = 6/5/5/5/4/5$, $n_{MT-B}^{WA/WA} = 5/5/5/5/5/5$, $n_{MT-U}^{WA/WB} = 4/4/6/4/5/6$, $n_{MT-B}^{WA/WB} = 5/5/5/5/5/5$, $n_{MT-U}^{WB/WA} = 5/5/5/5/5/5$, $n_{MT-B}^{WB/WA} = 5/5/5/5/5/5$, $n_{MT-U}^{WB/WB} = 5/5/5/5/5/5$ and $n_{MT-B}^{WB/WB} = 5/5/5/5/5/5$ independent replicates). **m,n**, Results from binding assays indicate nucleotide conditions at each AAA domain that lead to maximal binding of 1:1 or 2:1 complexes. Although a main determinant of 1:1 versus 2:1 binding is the nucleotide state of AAA3 (as indicated by ‘apo’ near AAA3), AAA1 also has a key role. Our data also suggest that an apo-AAA3 precludes microtubule-binding-induced allostery from reducing the extent of 2:1 complex assembly. *P* values were calculated using an unpaired two-sided *t*-test with Welch’s correction. *****P* ≤ 0.0001, ****P* ≤ 0.0010, ***P* ≤ 0.01 and **P* ≤ 0.05; NS, not significant (*P* > 0.05). In cases in which multiple datasets are cross-compared, only the highest *P* value is indicated (to simplify data presentation).

affinity³¹. Thus, in most cases, the MT-U or MT-B state is dominant to the nucleotide-bound state. However, one notable exception is in the case of the AAA3^{WA} mutants. Although both single AAA3^{WA} and double AAA1^{WB};AAA3^{WA} mutants exhibit reduced assembly of 1:1 complexes in the context of the MT-B protein with respect to MT-U, this is not the case for the 2:1 complex. These data indicate that the 2:1 complex, which is induced in part by apo-AAA3, is refractory to MT-U/MT-B-induced conformational differences (Fig. 1n).

A Pac1 dimer bridges two monomeric motor domains

Negative-stain EM of the 2 dynein_{MOTOR}:1 Pac1^{dimer} complexes revealed the presence of closely linked pairs of motor domains (Fig. 2a). The motor domain pairs are sufficiently homogeneous that several of the resulting class averages reveal detailed images of the motor domains (Fig. 2b). Although the motors are held together with fairly consistent spacing and conformational conformity, they exhibit notable rotational freedom and exist in a roughly equal mixture of ‘aligned’



(with the coiled-coil ‘stalks’ pointing in the same direction) and ‘inverse’ configurations (with stalks pointing in opposite directions) (Fig. 2c). We manually fit a previously obtained three-dimensional (3D) model of a Pac1-bound monomeric motor domain²⁸ to our images, which permitted us to identify densities corresponding to the motor domains and the bound Pac1. We then manually docked various elements from previous high-resolution structures, including the dynein-2 motor domain³⁴, WD40 domains from Lis1 (ref. 31), and the N-terminal LisH domain⁴¹ (Fig. 2c).

Pac1 WD40 domains within a dimer bind to two distinct sites on a single dynein motor domain: one between AAA3 and AAA4 (site^{ring})^{28,42} and another at the base of the coiled-coil stalk (site^{stalk})²⁹. The close alignment of our two-dimensional (2D) averages to that from the 3D model²⁸ strongly suggests that Pac1 is likely bound to site^{ring}. Moreover, comparison of our 2D averages to a recent structure of dynein bound to two Lis1 WD40 domains²⁹ reveals a lack of density at site^{stalk} (Fig. 2d). To confirm this contact we generated three mutants: (1) dynein_{MOTOR} fragments with substitutions at site^{stalk} or (2) site^{ring} that perturb Pac1 binding^{29,42}, and (3) Pac1 with a substitution predicted to disrupt contacts at site^{stalk} but not site^{ring} (R247A) (Fig. 2e). For experiments 1 and 2, we used dynein_{MOTOR}^{WA} fragments with the native WT stalk and microtubule-binding domain (MTBD), whereas we used dynein_{MOTOR}^{MT-U;WA} for the latter. These experiments revealed that neither disruption of site^{stalk} nor the Pac1 R247A substitution affects assembly of the 2:1 complex (Fig. 2f,g). However, mutation of site^{ring} eliminates this species, indicating that site^{ring} but not site^{stalk} is indeed required for 2:1 binding.

Nucleotide binding alters Pac1 affinity at two dynein regions

Our data thus far suggest that the two different dynein–Pac1 complexes are a consequence of the two Pac1 binding sites on dynein separately modulating their affinity. In particular, we hypothesized that apo-AAA3 in combination with ADP-AAA1 simultaneously leads to higher binding affinity for Pac1 at site^{ring} and lower affinity at site^{stalk}. ATP-AAA3 with either ATP or ADP-Pi-bound AAA1, on the other hand, leads to weakened Pac1 affinity at site^{ring} and increased affinity at site^{stalk}.

To test whether site^{ring} changes affinity because of the AAA3 state, we assessed the ability of monomeric Pac1 WD40 domains (that is, Pac1^{AN}, which lacks the N-terminal dimerization domain) to bind to dynein_{MOTOR}. This fragment was found to specifically bind site^{ring} and can thus be used to interrogate binding affinity at this site^{28,31}. We observed very little binding between WT dynein_{MOTOR} and Pac1^{AN}, except in ATP-Vi and apo conditions (Extended Data Fig. 4a), indicating that this fragment binds dynein with low affinity (likely because of lack of avidity). However, we observed robust binding to the AAA3^{WA} mutant in all conditions except apo, indicative of apo-AAA3 promoting high Pac1-binding affinity at site^{ring}. If, instead of enzymatically depleting nucleotide, we simply reduced ATP concentrations by dilution in nucleotide-free buffer (to 10 μ M), we observed robust binding for the AAA3^{WA} mutant. The same was true for the WT dynein motor. This is

consistent with AAA1 (and AAA4) requiring nucleotide for high-affinity binding at site^{ring}. In contrast to the AAA3^{WA} mutant, we observed no binding between the AAA3^{WB} mutant and Pac1^{AN} in all conditions, consistent with ATP-AAA3 lowering affinity at site^{ring}.

We next tested whether Pac1 binding to site^{stalk} is affected by the nucleotide-bound state of AAA3. Consistent with the importance of site^{stalk} in dynein–Pac1 binding when AAA3 is bound to ATP, we observed reduced binding between Pac1^{R247A} and the AAA3^{WB} mutant (Extended Data Fig. 4b). We observed a similar reduction in binding between WT Pac1 and the AAA3^{WB} dynein mutated at site^{stalk} (Extended Data Fig. 4c). These data are also supported by previous cryo-EM data showing that a site^{stalk}-bound Pac1 WD40 domain only becomes apparent when AAA3 is bound to ATP (through an AAA3^{WB} mutation)²⁹. Taken together, our data support a model in which Pac1-binding affinity at both site^{ring} and site^{stalk} is modulated by the nucleotide-bound states of AAA1 and AAA3.

Nucleotide binding coordinates dynein–Pac1 stoichiometry

Given that a dynein complex consists of two motor domains with four Pac1 WD40 domain-binding sites (two site^{ring} and two site^{stalk}), we wondered how the different dynein–Pac1 binding modes described above translate to such a context. We purified a well-characterized glutathione S-transferase (GST)-dimerized dynein_{MOTOR} fragment with or without AAA3^{WA} or AAA3^{WB} mutations and assessed their binding to Pac1 (Extended Data Fig. 5). Whereas the WT and AAA3^{WB} mutant assembled into 1 GST–dynein_{MOTOR}:1 Pac1^{dimer} and 1:2 complexes, the AAA3^{WA} mutant formed predominantly 1 GST–dynein_{MOTOR}:1 Pac1^{dimer} complexes.

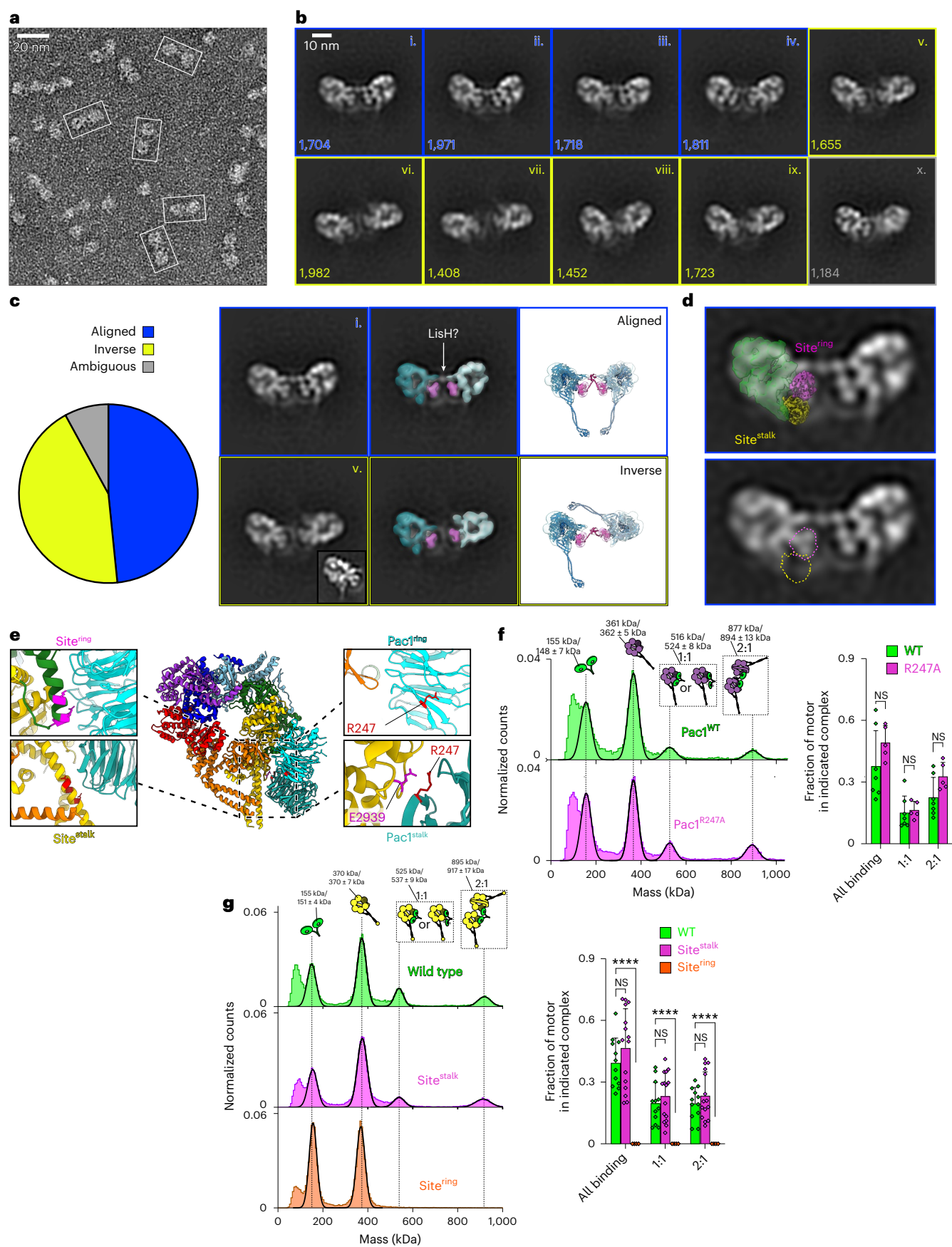
As expected from our data above, the AAA3^{WA} mutant bound Pac1 best in the presence of ATP, AMPPNP and ADP, likely because of the adoption of a high-Pac1-affinity state at site^{ring}. In all these cases, we observed an almost exclusive 1:1 stoichiometry of binding. This is consistent with each WD40 domain of a Pac1 dimer binding to site^{ring}, with no binding at site^{stalk}, likely because of this latter site adopting a low-affinity state. Of note, the AAA3^{WA} mutant can assemble with two Pac1 dimers in the presence of ATP-Vi, conditions that promote strong 1 dynein_{MOTOR}:1 Pac1^{dimer} binding and very little 2:1 binding (Fig. 1).

To determine whether these findings apply to the native dynein complex, we purified full-length, intact complexes (WT, AAA3^{WA} and AAA3^{WB}) from Sf9 cells. Mass photometry of the purified complexes revealed masses of $1,255 \pm 13$ kDa, $1,257 \pm 11$ kDa and $1,257 \pm 10$ kDa for the WT, AAA3^{WA} and AAA3^{WB} mutants, respectively, which very closely match the predicted mass of these complexes (1217 kDa). Pac1-binding experiments with each revealed an identical nucleotide-dependent pattern for the full-length dynein to that of GST–dynein_{MOTOR} (Fig. 3a–c). Specifically, AMPPNP and ATP-Vi stimulated assembly of both 1 dynein:1 Pac1^{dimer} and 1:2 species with the WT and AAA3^{WB} mutant, whereas the AAA3^{WA} mutant assembled into 1:1 complexes in the presence of ATP and ADP and 1:2 complexes in ATP-Vi conditions. Taken together, we identify a nucleotide-dependent code that coordinates the assembly of two distinct dynein–Pac1 complexes.

Fig. 2 | Pac1 bridges two dynein_{MOTOR} domains through site^{ring} contacts.

a,b, Representative electron micrographs (**a**) and 2D class averages (**b**) from negative-stain grids prepared with Pac1-bound dynein_{MOTOR}^{MT-U;K2424A} (in the presence of ADP). White boxes in **a** delineate closely apposed pairs of motor domains. Classes i–iv represent motor domains aligned in a parallel manner, classes v–ix are in an inverse orientation and class x is ambiguous. **c**, Quantitation of indicated motor orientations from 2D classification along with superimposition of 3D maps from EMD-6008 (ref. 28) (middle; one on each motor domain) on classes i and v from **b**. Colors depict the motor domain (shades of cyan) and Pac1 (magenta). Various elements from high-resolution structures were manually docked into the EM density (motor domain from PDB 4RH7 (ref. 34), linker from PDB 3VKC (ref. 59) and WD40 domain from PDB 8FDT (ref. 31)). Although unclear, the unassigned density may represent the N-terminal LisH dimerization domain of Pac1. Inset, focused 2D classification of a

monomeric motor domain. **d**, The 2D class average (class i) with superimposition of 3D map from EMD-8706 (ref. 29), which represents a dynein motor domain with Pac1 WD40 domains bound at both site^{ring} (magenta) and site^{stalk} (yellow). Note the lack of EM density for Pac1 at site^{stalk} in our 2D averages (dashed yellow outline). **e**, Close-up view of site^{ring} and site^{stalk} in yeast dynein_{MOTOR} (with an AAA3^{WB} mutation)²⁹. **f,g**, Representative mass photometry histograms (left) and plots (right; mean $\pm$ s.d.) showing binding between dynein_{MOTOR}^{MT-U;K2424A} and either WT Pac1 or Pac1^{R247A} (**f**; $n_{WT} = 7$ and $n_{R247A} = 5$ independent replicates) or between Pac1 and WT, site^{ring} (K2721A;D2725G;E2726S;E2727G) or site^{stalk} (E3012A;Q3014A;N3018A) mutants of dynein_{MOTOR}^{K2424A} (**g**; $n_{WT} = 12$, $n_{site-stalk} = 15$ and $n_{site-ring} = 4$ independent replicates) all in the presence of ADP. Expected and calculated molecular weight (mean $\pm$ s.d.) values for indicated species are shown above each peak. *P* values were calculated using an unpaired two-sided *t*-test with Welch's correction. *****P* $\leq$ 0.0001.



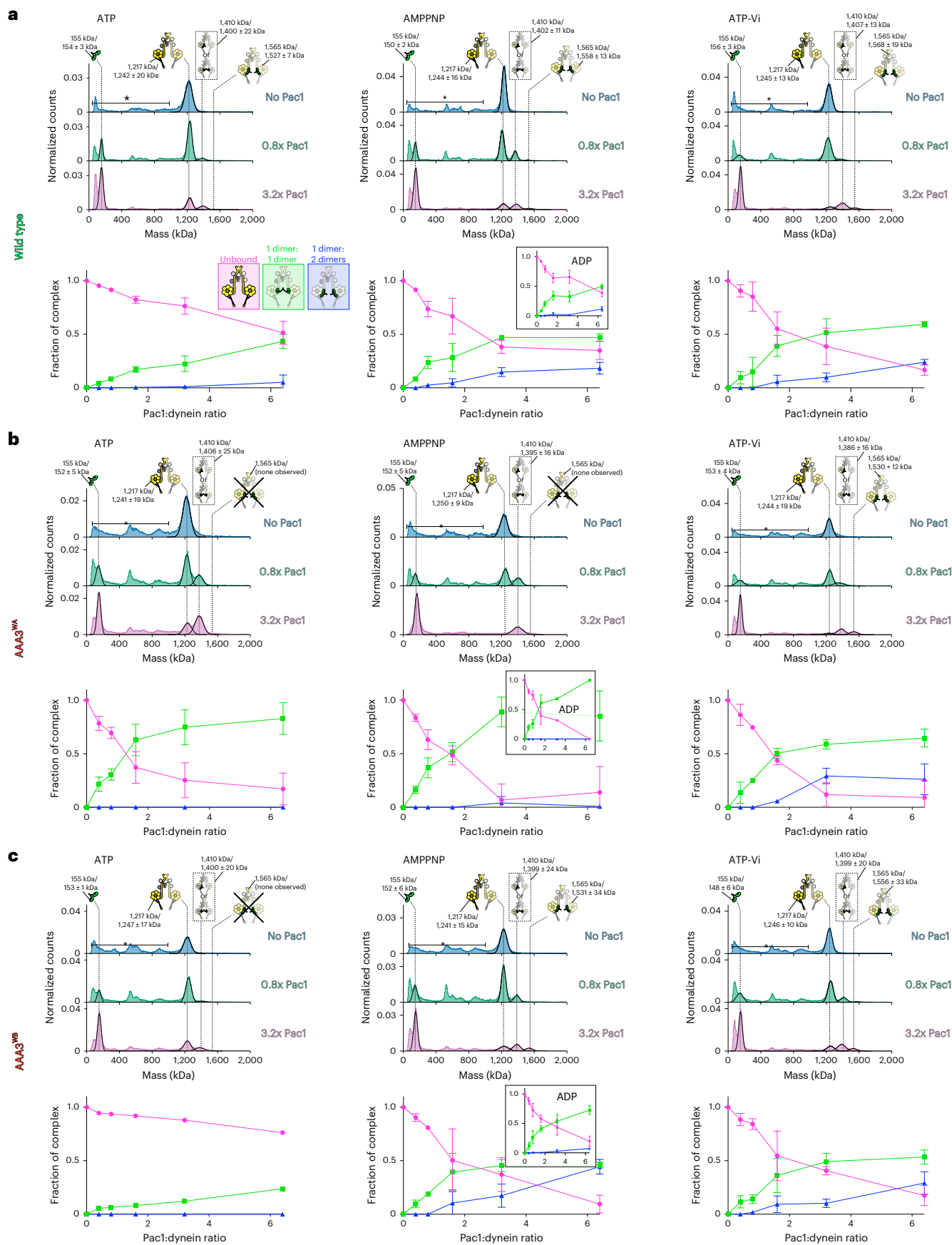


Fig. 3 | Nucleotide occupancy coordinates stoichiometry of binding between Pac1 and full-length dynein. **a–c**, Representative mass photometry histograms and associated plots (mean fraction of binding $\pm$ s.d.; in order of increasing Pac1 concentrations, $n_{WT/ATP} = 3/4/3/3/4$, $n_{WT/AMPPNP} = 3/5/6/3/3$, $n_{WT/ATP-Vi} = 3/4/4/4/3$ and $n_{WT/ADP} = 3/4/5/4/4$ independent replicates; $n_{AAA3-WA/ATP} = 4/3/3/5/3$, $n_{WA/AMPPNP} = 4/3/4/5/3$, $n_{WA/ATP-Vi} = 4/3/3/3/3$ and $n_{WA/ADP} = 3/3/3/3/3$ independent replicates; $n_{AAA3-WB/ATP} = 3/3/3/3/3$, $n_{WB/AMPPNP} = 3/3/4/4/3$, $n_{WB/ATP-Vi} = 4/6/3/3/6$ and $n_{WB/ADP} = 4/5/3/4/4$ independent replicates) depicting the extent and stoichiometry of binding between Pac1 and either WT full-length dynein complex (**a**), AAA3^{WA}

mutant (**b**) or AAA3^{WB} mutant (**c**) (all purified from SF9 cells) over a range of dynein–Pac1 ratios. Cartoon schematics above each peak indicate respective protein species but not necessarily the conformation (that is, phi or open). Binding experiments were performed in indicated nucleotide conditions (ATP, left; AMPPNP and ADP, middle; ATP–Vi, right). Expected and calculated molecular weight (mean $\pm$ s.d.) values for indicated species are shown with each respective cartoon schematic. Note that values indicated by asterisks in histograms were attributed to ‘noise’ or contaminants from protein purifications or buffer components (nucleotide mixtures).

Pac1 ‘opening’ dynein is determined by binding stoichiometry

Given the proposed but untested model that Pac1 relieves dynein autoinhibition, we sought to understand how these distinct Pac1-binding modes might influence dynein’s adoption of the phi particle. We mixed full-length AAA3^{WA} and AAA3^{WB} dynein mutants with Pac1 in conditions that favored either 1:1 (AAA3^{WA} with ATP) or 1:2 (AAA3^{WB} with AMPPNP) binding and assessed dynein morphology by negative-stain EM (Extended Data Fig. 6). To enable unambiguous analysis of the resulting dynein–Pac1 complexes, we used sufficiently high Pac1–dynein ratios such that dynein binding was close to saturation (Fig. 4a,b).

In the absence of Pac1, approximately half of the AAA3^{WA} dyneins adopt an autoinhibited state, with the remainder existing in an open state (Fig. 4c). The 2D class averages of the phi dyneins reveal a morphology that is indistinguishable from WT dynein (Extended Data Fig. 7). Focused classification of the motor pairs in the open configuration revealed very low-resolution images of the motor domains that adopt a wide range of interhead spacing (Fig. 4c, right). The low resolution is a consequence of the high degree of flexibility for the open dyneins, as focused classification of individual motor domains from the same dataset revealed sufficient detail to identify various structural features (Fig. 4c, bottom right).

Addition of Pac1 to the AAA3^{WA} mutant resulted in a striking transition of dynein to an open state (Fig. 4d). Unlike the open state for Pac1-unbound dynein, those bound to Pac1 adopt a more consistent morphology with less rotational freedom and a smaller range of spacing between the motor domains, as evidenced by the higher-resolution 2D averages for the full-length complex, as well as our measurements of interhead spacing (Fig. 4e). Note that the interhead spacing with the Pac1-bound motor pairs is very similar to that of the 2 dynein_{MOTOR}:1 Pac1^{dimer} complexes (Figs. 2b and 4e). Focused classification of the motor pairs permitted the identification of densities that correspond to Pac1 WD40 domains bound to site^{ring} on each motor domain (Fig. 4d, right). This was even more apparent from focused classification of the individual motors, which revealed averages almost identical to those obtained from the monomeric Pac1-bound motor domains (Fig. 4d, bottom right). Thus, Pac1 indeed has the capacity to stabilize and/or promote an open dynein, at least when AAA3 is in an apo state and AAA1 is bound to either ADP or ATP.

A similar analysis of the AAA3^{WB} mutant revealed that it has a high propensity to adopt the autoinhibited conformation in the absence of Pac1 (Fig. 4f; note the lack of open classes). In addition to phi dynein, we observed a novel species in which two phi dyneins are bound through contacts between the coiled-coil stalks. The coiled-coil stalk domain and MTBD, which are normally too flexible to be visible in 2D averages, are apparent in this species, suggesting that the interdimer contact stabilizes this region (Fig. 4f, red arrow).

Unlike the AAA3^{WA} mutant, addition of Pac1 to the AAA3^{WB} mutant did not result in it adopting an open conformation (Fig. 4g). Instead of canonical phi dynein species, 2D averages revealed that the majority of these dyneins are phi-like, which appear to be distinct from phi. The low resolution of the averages of these species suggests a potential lack of symmetry in this phi-like state and may indicate that Pac1 binding partly opens phi dynein but only to a minor extent. Thus, whereas the 1 dynein:1 Pac1^{dimer} complex adopts an open state, the 1:2 complex does not.

Structural basis for 1 dynein:1 Pac1 complex assembly

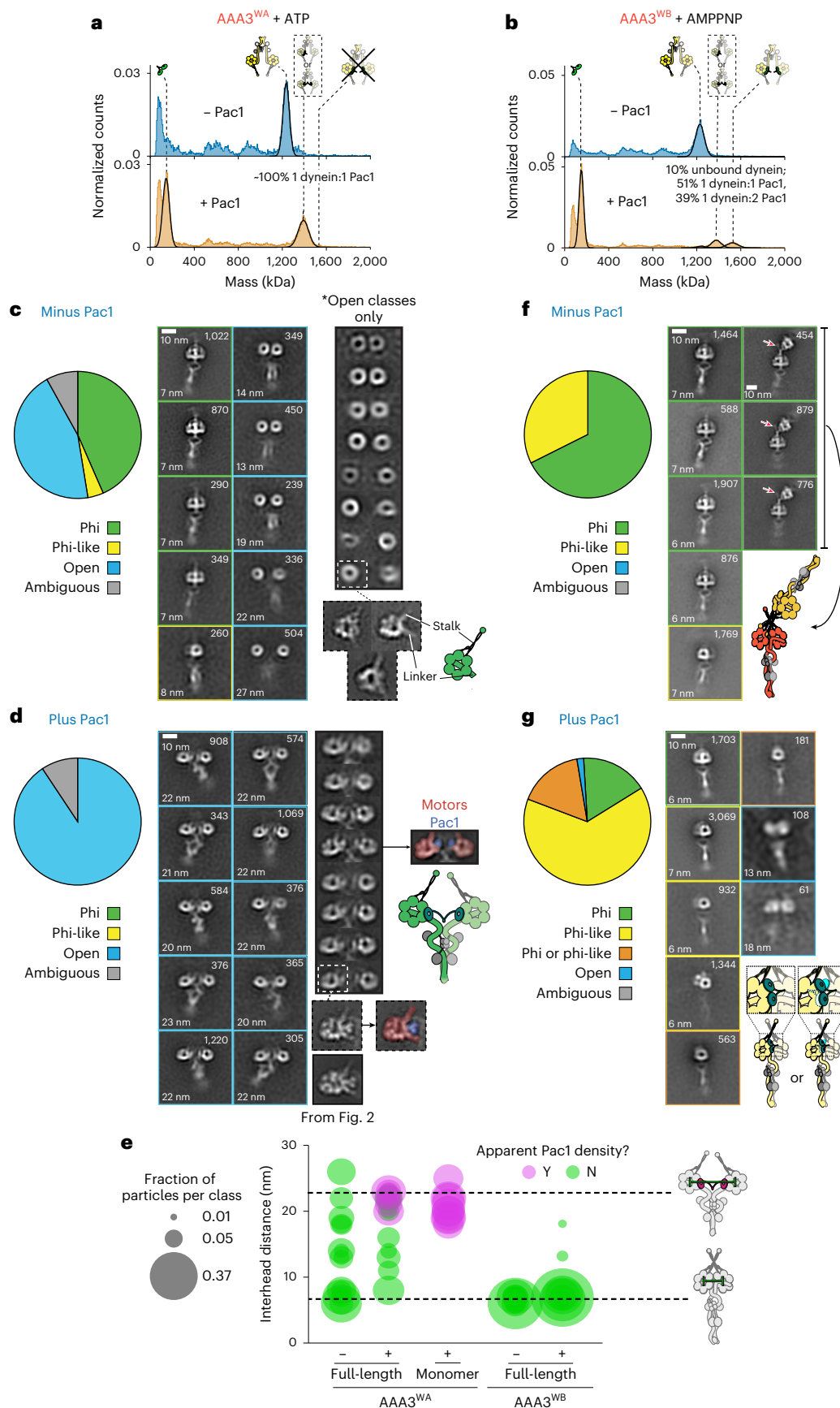
To gain insight into the mechanism by which one dynein complex binds one Pac1 dimer, thereby opening phi dynein, we sought to obtain a representative cryo-EM structure. We mixed full-length WT dynein with or without Pac1 in the presence of low concentrations of ATP (0.1 mM) (Fig. 5a, Extended Data Fig. 8 and Supplementary Table 1). For the purpose of this study, we focused exclusively on the motor domains of open dynein to understand the nature and consequence of dynein–Pac1 binding.

In the absence of Pac1, we obtained two classes: one with ADP–AAA1 and ADP–AAA3 and another with apo–AAA1 and ADP–AAA3 (Fig. 5b and Extended Data Fig. 9). Whereas those motors with apo–AAA1 exhibit a canonical postpowerstroke linker (‘post-1’; docked at AAA5), those with ADP–AAA1 have the linker in the recently identified post-2 state, in which the linker is docked at AAA4 (ref. 43). A post-2 docking mode, which was noted previously for yeast dynein, human dynein-1 and axonemal outer-arm dynein^{28,43,44}, was recently posited to have a critical role in dynein stepping⁴⁴.

In the presence of Pac1, we observed only a single class of motor-bound Pac1, in which AAA1 and AAA4 are bound to ADP and

Fig. 4 | Nucleotide occupancy and Pac1 binding mode dictate conformation of full-length dynein. **a,b**, Mass photometry histograms of samples containing full-length dynein (AAA3^{WA} or AAA3^{WB} mutant) with or without excess Pac1 that were used to directly prepare negative-stain EM grids, images of which are shown in **c,d,f,g**. Note that the cartoon schematics of dynein shown above each peak are not meant to convey the conformational state but rather to depict the binding ratios for each protein species. **c,d**, Representative 2D class averages showing full-length AAA3^{WA} dynein in the absence (**c**) or presence (**d**) of Pac1. Pie graphs show the relative fraction of indicated conformational state (determined from 2D averages). Numbers indicate the number of particles in each class (top) and the measured distance between the motor domains (bottom; from center to center). Focused classifications of motor dimers or monomers are shown (a Pac1-bound monomer from Fig. 2c is shown for comparison in **d**). The poor image quality for the dimers in **c** but not the monomers indicates a large range of conformational heterogeneity within the motor dimer pairs. Note the improved resolution for motor dimer averages in **d**, indicating the reduced heterogeneity for Pac1-bound

motor dimers compared to those unbound from Pac1 (Pac1 and dynein motor domain pseudocolored in blue and red, respectively). **e**, Bubble plot of interhead measurements taken from 2D class averages for indicated species. The bubble size reflects the proportion of particles per 2D class, whereas green and magenta indicate whether Pac1 density can be discerned from each average. Monomer data were measured from images shown in Fig. 2b. Dashed lines indicate the mean interhead distance for Pac1-bound AAA3^{WA} mutant (22 nm) and phi dynein (6.9 nm). **f,g**, Representative 2D class averages (and quantitation) showing full-length AAA3^{WB} dynein in the absence (**f**) or presence (**g**) of Pac1. We observed a novel species in which two phi dynein dimers are bound through apparent contacts between the motor domains (cartoon depiction in **e**) that appear to stabilize the stalk MTBD region (red arrows indicate improved resolution of this region; those classes missing measurements in **g** are because of an inability to clearly identify motor domain centers). Each dataset is representative of two independent replicates.



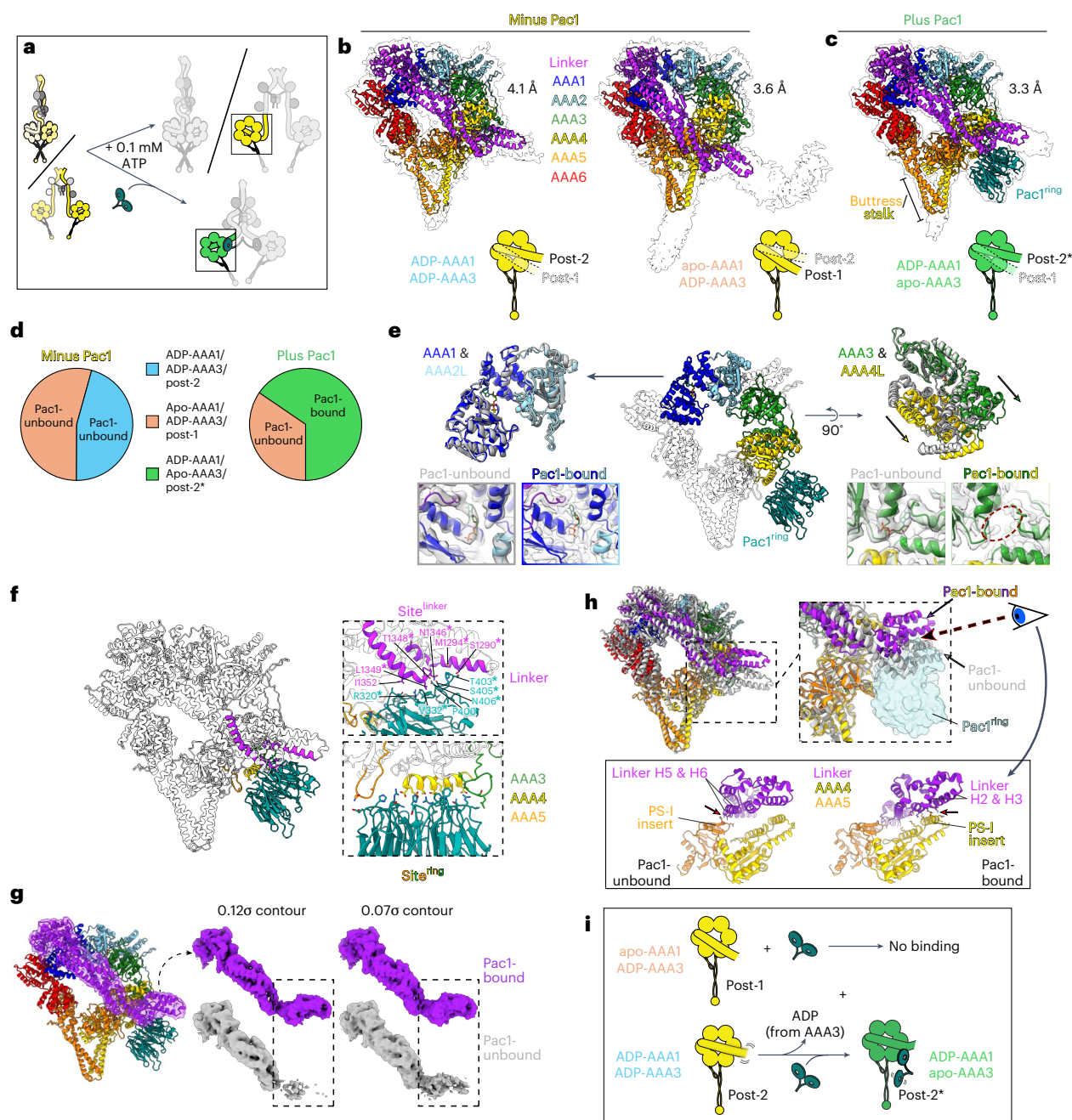


Fig. 5 | Cryo-EM of full-length dynein in the absence or presence of Pac1.

a, Schematic of experimental setup. Full-length WT dynein was incubated with or without Pac1 in the presence of 0.1 mM ATP before preparing grids for freezing. We performed a focused classification of individual motor domains of open dyneins. **b**, Molecular models of dynein motor domains without (b) or with (c) Pac1, along with corresponding density maps (indicated with outlines). The resolution of each structure is indicated. Subdomains are color-coded as indicated. Nucleotide occupancy at AAA1 and AAA3 and the linker position is indicated by the cartoon below each map (post-2* indicates a 'modified post-2' state). **d**, Pie graphs showing the relative fraction of dynein motors with indicated linker position and nucleotide occupancy at AAA1 and AAA3 for those motors without and with Pac1. Note that the post-2* state and the presence of apo-AAA3 are both unique to those bound to Pac1. **e**, Close-up views comparing AAA1 and AAA3 pockets in the absence (gray) or presence (blue and cyan for AAA1 and AAA2L; green and yellow for AAA3 and AAA4L) of Pac1. Note that, for minus Pac1,

we used the model with ADP-AAA1/ADP-AAA3 and post-2 linker. Right, arrows depict opening of the AAA3 pocket in the presence of Pac1. Insets, molecular models with EM density overlaid. The red dashed line circle delineates an empty AAA3 pocket. **f**, Close-up view of molecular model showing Pac1-dynein motor contacts at site^{ring} and new site^{linker}. Residues marked with asterisks are those mutated in site^{linker} mutant (Fig. 6). **g**, Molecular model and EM densities with two different contour settings illustrating that a Pac1 bound to dynein reduces flexibility of the N-terminal region of the linker (regions within dashed box). **h**, Comparison of Pac1-bound and unbound (the latter with ADP-AAA1/ADP-AAA3 and post-2 linker) dynein motors revealing a shift of the linker N terminus away from the stalk domain and MTBD. Close-up views show the linker in two slightly different post-2 docking modes: one in which the linker contacts residues in the PS-I of AAA5 (minus Pac1) and the other in which the linker contacts residues in the AAA4 PS-I (plus Pac1). **i**, Model indicating that high-affinity binding of Pac1 to site^{ring} and site^{linker} requires ADP binding to AAA1 and ADP release from AAA3.

AAA3 is in an apo state (Fig. 5c and Extended Data Figs. 8 and 9). Consistent with our biochemical data, these dyneins are bound to a single Pac1 WD40 domain through site^{ring}. Unlike previous structures of Pac1/Lis1-bound motors, ours possesses a post-2-like linker. Moreover, the AAA1 pocket is in an open state and the motor appears to be in a high-microtubule-binding-affinity state according to the conformation of the buttress and stalk (Fig. 5c). Note that this structure resembles a previously obtained low-resolution map (~20 Å) obtained for a monomeric dynein_{MOTOR} bound to Pac1 (ref. 28) and a previously determined cryo-EM structure solved for a Pac1-bound monomeric dynein_{MOTOR} with a truncated linker²⁹.

Although we observed motor domains with a post-2 linker in the absence and presence of Pac1, apo-AAA3 was exclusive to those bound to Pac1 (Fig. 5d), indicating that Pac1 binding stabilizes and/or promotes ADP release from AAA3. The Pac1-bound motor possesses a more open AAA3 pocket that is reflective of its empty state (Fig. 5e, right, and Extended Data Fig. 9). In addition to previously identified contacts at site^{ring}, we observed a novel interaction between Pac1 and the linker (site^{linker}; Fig. 5f). Although the linkers of both Pac1-unbound and Pac1-bound states are in post-2 positions, the N-terminal region of the Pac1-bound linker is positioned more distal from the stalk (Fig. 5g,h). This region is often truncated in previous structures. Pac1 binding shifts the linker from contacting AAA5 (through the presensor I (PS-I) insert) to instead contact the AAA4 PS-I insert (Fig. 5h, bottom). These data indicate that Pac1 binding stabilizes the linker in a modified post-2 conformation ('post-2*') and the AAA3 pocket in an apo state (Fig. 5i).

Pac1-linker contacts account for different binding modes

Although the Pac1-site^{ring} contacts in our structure largely overlap with those observed previously^{5,18,28–31,45}, we observed small but notable differences. A comparison to structures with two WD40s bound (one at site^{ring} and another at site^{stalk}) revealed that Pac1 is shifted downward toward site^{stalk} in our apo-AAA3 structure (by 7.6 Å; Fig. 6a,b). This downward shift, which may be because of the post-2* linker–Pac1 contact, appears to occlude binding of a WD40 domain at site^{stalk} (Fig. 6b; note steric clashes upon docking a WD40 at site^{stalk}). These observations suggest that the linker–Pac1 contact may account for (1) the increase in affinity of Pac1 for site^{ring} (through additional contacts at site^{linker}) and (2) the decrease in affinity of Pac1 for site^{stalk} (because of steric occlusion).

To test the importance of the linker–Pac1 contacts, we mutated this interface in both Pac1 and dynein (Supplementary Table 3 and Fig. 5f, asterisks). Although mutations at site^{linker} within dynein had no effect on its motility (Fig. 6c), they greatly reduced its binding to Pac1^{ΔN}, indicating these contacts are important for high-affinity binding at site^{ring} (Fig. 6d). If this contact is responsible for the downward translation of Pac1 toward the stalk, thereby occluding Pac1 binding

at site^{stalk}, then mutating this interface would be predicted to permit binding of Pac1 WD40s to both site^{ring} and site^{stalk}, thereby disfavoring assembly of 2 dynein_{MOTOR}:1 Pac1^{dimer} complexes. This was indeed true, as both Pac1 and dynein site^{linker} mutants could no longer assemble these complexes (Fig. 6e,f). Note that these mutants still robustly assembled 1:1 complexes, indicating that linker–Pac1 contacts have no role in high-affinity binding of the motor domain to Pac1 dimers.

If linker–Pac1 contacts are responsible for full-length dynein binding Pac1 in a 1:1 ratio, then mutating site^{linker} would result in the assembly of 1 dynein:1 Pac1^{dimer} complexes for the full-length AAA3^{WA} mutant instead of 1:1 complexes. This was indeed the case (Fig. 6g). To test the functional importance of site^{linker}, we engineered mutations into otherwise WT PAC1 or DYN1 loci in yeast cells (expressed at WT levels; Extended Data Fig. 10a) and measured dynein-mediated spindle movements^{46–48} (Fig. 6h,i and Extended Data Fig. 10b,c). Note that loss of PAC1 or DYN1 results in an elimination of these movements^{13,46}. Mutations at site^{linker} in either Pac1 or dynein greatly impaired in-cell dynein activity, as was apparent from the reduced extent and number of dynein-mediated spindle movements and from the diminished ability of dynein to translocate the mitotic spindle through the narrow bud neck.

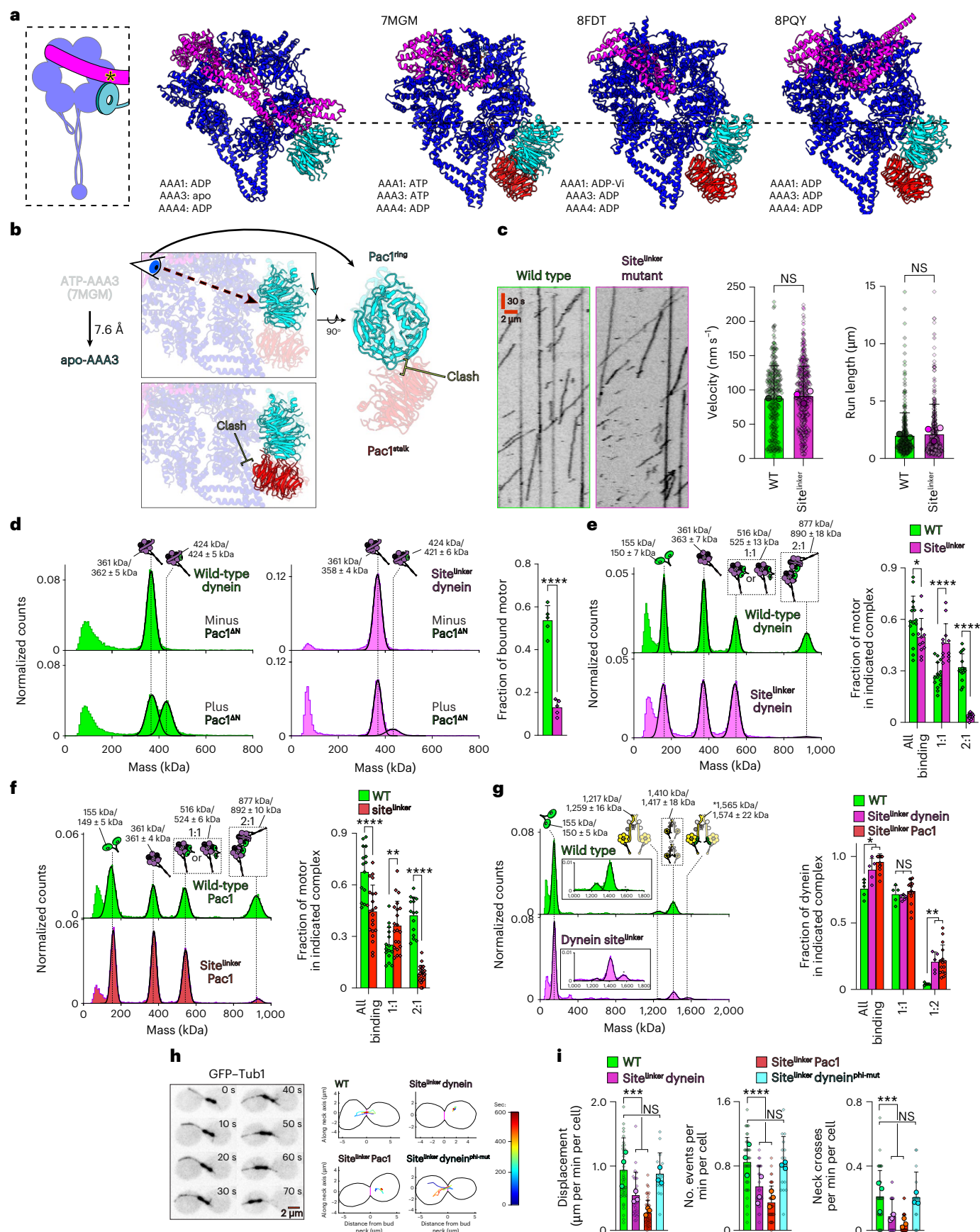
Our findings suggest that assembly of the 1 dynein:1 Pac1^{dimer} complex is required for Pac1 to open phi dynein, which is important for in-cell dynein function. If true, we reasoned that mutations that render dynein unable to adopt phi would be refractory to mutations at site^{linker}. Consistent with this idea, combination of mutations at both site^{linker} and at the phi interface (D2868K)¹³ resulted in dynein activity that was indistinguishable from that in WT cells (Fig. 6h,i and Extended Data Fig. 10b,c). Our findings indicate that site^{linker}–Pac1 contacts are specifically required for the assembly of 1 dynein:1 Pac1^{dimer} complexes, which are important for relieving dynein autoinhibition.

Discussion

Various conditions modulate the affinity of dynein for Pac1 and Lis1, including dynein's microtubule-bound and nucleotide-bound states^{31,32}. Here, we show that the latter is coordinated by AAA1, AAA3 and AAA4, which affect not only Pac1 binding affinity but also the stoichiometry of their binding, which in turn impacts whether Pac1 opens phi dynein. Given that phi-disrupting mutations promote DDA complex assembly¹², we hypothesize that the Pac1-stabilized open state promotes assembly of dynein with dynactin and a cargo adaptor. This is supported by a recent study suggesting a similar phenomenon with human Lis1 (ref. 49). This Pac1-stabilized conformation may also assist dynein binding to microtubules^{18,29,32,50}, as the Pac1-bound motor domains appear to be in a high-microtubule-binding-affinity state as predicted by the buttress stalk conformation. A recent study showed that microtubule

Fig. 6 | Pac1-site^{linker} contacts account for different dynein–Pac1 binding modes. **a**, Cartoon and various structural models comparing the position of the site^{ring}-bound Pac1 in the various structures with two WD40 domains bound (from left to right, this study, PDB 7MGM (ref. 5), PDB 8FDT (ref. 31) and PDB 8PQY (ref. 45)). The asterisk in the cartoon delineates the approximate region of site^{linker} contacts mutated. Nucleotide occupancy for AAA1, AAA3 and AAA4 is indicated. **b**, Comparison of site^{ring}-bound Pac1 in our structure and a yeast dynein with two WD40s bound (PDB 7MGM)⁴⁵. Note that the 7.6-Å downward shift of Pac1 is predicted to occlude a site^{stalk}-bound Pac1 because of clashes. **c**, Representative kymographs and quantitation of single-molecule motility assays performed with either WT or site^{linker} mutant variants of GST–dynein_{MOTOR}. Small diamonds, all data points; larger circles, means for independent replicates; bars, mean ± s.d. (for WT and site^{linker}, *n* = 309 and 473 motors from two and three independent replicates, respectively). **d,e**, Plot and representative mass photometry histograms showing WT or site^{linker} mutant monomeric dynein_{MOTOR}^{MT-U}-E1849Q;K2424A in the absence or presence of either monomeric (**d**; Pac1^{ΔN}, *n*_{WT} = 5 and *n*_{site-linker} = 5 independent replicates) or dimeric (**e**; *n*_{WT} = 15, and *n*_{site-linker} = 12 independent replicates) Pac1 (in the presence of ADP). **f**, Plot and representative mass photometry histograms showing

monomeric dynein_{MOTOR}^{MT-U}-E1849Q;K2424A with either WT or site^{linker} mutant Pac1 (in the presence of ADP; *n*_{WT} = 15 and *n*_{site-linker} = 21 independent replicates). **g**, Plot and representative mass photometry histograms depicting extent and stoichiometry of binding between Pac1 and either full-length dynein-K2424A or dynein-K2424A with site^{linker} mutations (in the presence of AMPPNP; *n*_{WT} = 5, *n*_{Dyn1-site-linker} = 5 and *n*_{Pac1-site-linker} = 15 independent replicates). For **d–g**, plots depict the mean ± s.d. along with independent replicates. Expected and calculated molecular weight (mean ± s.d.) values for indicated species are shown above each peak. **h**, Representative montage of hydroxyurea-arrested WT cells expressing GFP–Tub1 and tracks of spindle centroids for indicated strains (cell outline in black; bud neck in magenta). **i**, Plots depicting indicated dynein-mediated spindle translocation parameters. Small diamonds, all data points; larger circles, means for independent replicates; bars, mean ± s.d. (*n*_{WT} = 45, *n*_{Dyn1-site-linker} = 33, *n*_{Pac1-site-linker} = 51 and *n*_{Dyn1-site-linker/phi-mut} = 26 HU-arrested cells from three independent replicates each). *P* values were calculated using either a Mann–Whitney test (for run length values in **c**) or an unpaired two-sided *t*-test with Welch's correction (for all other datasets). *****P* ≤ 0.0001, ****P* ≤ 0.0010, ***P* ≤ 0.0100 and **P* ≤ 0.0500. In cases in which multiple datasets were cross-compared, only the highest *P* value is indicated (to simplify data presentation).



binding by dynein–dynactin complexes can also promote the assembly of active DDA complexes³¹, providing yet another potential mechanism by which Lis1 may be activating dynein. The importance of site^{linker} and the 1:1 complex it coordinates is highlighted by the fact that mutating this site on either dynein or Pac1 results in a notable reduction in dynein activity in cells, in spite of these mutants having no measurable reduction in assembly of the 1 dynein:2 Pac1^{dimer} complex.

Our cryo-EM data show that Pac1 stabilizes an apo-AAA3. Given the lack of dyneins with apo-AAA3 in the absence of Pac1, this is likely a transient state. We hypothesize that Pac1 is an opportunistic binder that favors those dyneins that release ADP from AAA3 (possibly because of the post-2 linker that may be triggered by apo-AAA3) and consequently binds and enriches for their presence. This suggests a mechanism by which Pac1 can open the phi state in cells; provided that Pac1 is present at sufficiently high local concentrations, immediately subsequent to ADP release from AAA3, Pac1 binds because of its high affinity for site^{ring} and site^{linker}.

In light of the proposed importance of the post-2 linker in dynein stepping behavior^{43,44}, the Pac1-stabilized post-2* state and/or the apo-AAA3 pocket might account for the observed impacts of Pac1 and Lis1 on dynein force generation^{32,50}. The importance of the linker–Pac1 contact also explains why the addition of ATP-Vi weakens affinity for Pac1 at site^{ring} (Extended Data Fig. 4a) and reduces assembly of one Pac1^{WD40} per dynein^{MOTOR} in favor of one Pac1^{dimer} per dynein^{MOTOR}; treatment with ATP-Vi enriches for a prepowerstroke linker^{34,52–54}, effectively eliminating site^{linker}, which would be expected to lead to two Pac1 WD40 domains binding per dynein^{MOTOR}, as we observed.

The linker position also likely accounts for our binding data with the monomeric dynein^{MOTOR} fragment (Fig. 1). Specifically, those conditions that enrich for the 2 dynein^{MOTOR}:1 Pac1^{dimer} species may do so because they promote a post-2 linker position. This is supported by our cryo-EM data (Fig. 5d), which show that ~50% of the dynein motor domains in the minus-Pac1 conditions exhibit a post-2 linker state. Addition of Pac1 results in the elimination of dyneins with a post-2 linker (that is, they are likely converted to a Pac1-bound motor with post-2*) but only a modest reduction in those with a post-1 linker (which remain unbound from Pac1). Recent work suggests that a AAA4^{WA} mutation locks the linker in a postpowerstroke state³⁸. However, our findings with this mutant revealed very little site^{linker}–Pac1 binding, as evidenced by its low extent of 2 dynein^{MOTOR}:1 Pac1^{dimer} assembly. This may be a consequence of apo-AAA4 promoting a post-1 rather than post-2 linker.

Although the functional importance of the 1 dynein:2 Pac1^{dimer} complex is unclear, these dyneins are clearly not in an open state but rather a phi-like state. This complex is likely similar to a recently identified Lis1-bound phi dynein, disruption of which leads to defects in dynein activity^{55,56}. As predicted by our binding assays, this complex possesses ADP-AAA1 and ADP-AAA3. Unlike phi dynein, phi-Lis1 lacks C₂ symmetry, which may explain our inability to obtain high-quality 2D averages.

We posit the following model to account for these distinct dynein–Pac1 complexes (Extended Data Fig. 10d). Phi dynein with ADP-AAA1 and ADP-AAA3 (ref. 44) is primed to bind Pac1 WD40s at both site^{ring} and site^{stalk}, leading to adoption of a phi-like conformation with either one or two Pac1 dimers bound (step 1). Given the high concentrations required to obtain complexes with two Pac1 dimers, it is more likely that dynein binds only one in the cell (as observed in recent structures^{55,56}). Dynein transiently adopts a partially open state, which results in ADP release from AAA3, the adoption of a post-2 linker state (step 2) and Pac1 dissociating from site^{stalk} and binding to site^{ring} on the second motor domain (steps 3 and 4). This Pac1-binding mode stabilizes dynein in an open conformation that is primed for assembly into motile DDA complexes (step 5). Upon microtubule binding and initiation of motility, Pac1 dissociates from the DDA complex³¹ (step 6). Given that the AAA3^{WA} mutant adopts the phi particle at a lower frequency, we cannot rule out the possibility that ADP release from AAA3 precedes and is causal of the transient opening of this state (in step 2).

A recent cryo-EM structure of a Pac1-bound monomeric dynein^{MOTOR}–AAA3^{WB} identified a potential intermediate state referred to as ‘chi’ dynein⁵⁷. This may reflect the transition state between the Pac1–phi complex and ADP release from AAA3 (step 2). Another recent cryo-EM structure of a Lis1-bound DDA complex on microtubules (with the Jip3 adaptor and two dynein dimers) showed a fully open and motile-competent configuration⁵. Whereas the MT-B dynein dimer has no Lis1, the MT-U dynein is bound to two Lis1 dimers, with ADP in both AAA1 and AAA3, conditions we found to promote binding of two Pac1 WD40 domains per motor. Given that this complex includes dynactin and a cargo adaptor, it likely reflects a later point in the activation mechanism. Future studies will be required to tease out how all of these distinct dynein–Pac1/Lis1 complexes relate to each other and their respective roles in the dynein activation process.

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/s41589-025-02096-8>.

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Methods

Media and strain construction

Yeast strains were derived from W303 or YEF473A (ref. 60) and are available upon request (Supplementary Table 2). We transformed yeast strains using the lithium acetate method⁶¹. Strains carrying mutations or tagged components were constructed by PCR product-mediated transformation⁶² or by transforming expression plasmids encoding for affinity-tagged proteins (Supplementary Tables 2 and 3). All mutagenesis was confirmed by whole-plasmid sequencing. To integrate dynein_{MOTOR} expression plasmids, the plasmids were first digested with either *Apa*I or *Sbf*I (both of which cut within the *URA3* gene; the latter was used for those plasmids with a rigid coiled-coil derived from seryl tRNA synthetase) before transformation into a yeast strain deleted for the native *DYN1* gene. Strains overexpressing WT or mutant *Pac1* were generated by transforming pRS306:GAL1p:8×His-ZZ-2×TEV-Pac1-FLAG-SNAPf (WT or mutant) linearized by digestion with *Apa*I. Integration of all plasmids were confirmed by diagnostic PCR. Yeast synthetic defined (SD) medium was obtained from Sunrise Science Products.

Plasmid and BACmid construction

A plasmid encoding an affinity-tagged WT dynein_{MOTOR} fragment was generated as a starting point for the production of numerous mutants used throughout the manuscript (to enable simpler and more rapid engineering of mutants; Supplementary Table 3). This plasmid was made by amplifying the entire gene expression cassette from a yeast strain that encodes this same fragment (strain 2675; Supplementary Table 2) using PCR primers that span the 5' end of the *GAL1p* promoter to the 3' end of the HaloTag situated at the N terminus of dynein_{MOTOR}. This plasmid, which was assembled into pRS306 (with a *URA3* selection cassette) by traditional Gibson assembly, includes the following elements: GAL1p:ZZ-2×TEV-6×His-dynein_{MOTOR}-HaloTag (P1532). Supplementary Table 3 outlines mutations engineered into this plasmid by Gibson assembly.

For expression of biochemical quantities of high-purity full-length yeast dynein complex, we generated a multicistronic plasmid that contains expression cassettes for all four dynein complex genes (*DYN2*, *DYN3*, *PAC11* and *DYN1*, all codon-optimized for insect cells), with N-terminal affinity tags (6×His-ZZ) followed by 2×TEV cleavage sites and a SNAPf tag on *DYN1*. This plasmid (P825) was assembled using the biGbac technique⁶³. In brief, codon-optimized open reading frames for *DYN2*, *DYN3* and *PAC11* were each assembled into pLib, whereas a codon-optimized 6×His-ZZ-2×TEV-SNAPf-*DYN1* was assembled into pbiG1a (containing 5' polH promoters and 3' SV40 terminators). Gene expression cassettes for *DYN2*, *DYN3* and *PAC11* (that is, each with a polH promoter and SV40 terminator) were amplified from their respective pLib plasmid and then assembled into pbiG1b to generate pbiG1b:*DYN2/DYN3/PAC11*. The *DYN2/DYN3/PAC11* polygene cassette and 6×His-ZZ-2×TEV-SNAPf-*DYN1* were then combined together into pbiG2ab by cutting them out of their respective pbiG1 plasmids (by *Pme*I) and assembling them into *Pme*I-digested pbiG2ab, generating pbiG2ab:*DYN2/DYN3/PAC11/6×His-ZZ-2×TEV-SNAPf-DYN1* (P825; hereafter referred to as pbiG2ab:yeast dynein). Substitutions indicated throughout the paper (that is, K2424A, E2488Q or site^{linker} substitutions) were engineered directly into this plasmid.

For expression and purification of *Pac1* from Sf9 cells (with N-terminal 8×His-ZZ-2×TEV tags and C-terminal SNAPf tags), we assembled a codon-optimized gene fragment for *Pac1* into pFastBac (P1199). This plasmid was subsequently engineered with site^{linker} substitutions: T403A, S405A, N406A, R320A and V332A. All plasmids were validated by whole-plasmid sequencing.

To generate BACmids (used for transfection into Sf9 cells), pbiG2ab:dynein-dynein (WT or mutant) or pFastBac:8×His-ZZ-TEV-Pac1-SNAPf (WT or site^{linker} mutant) were transformed into DH10 EMBacY cells (Geneva Biotech) according to the manufacturer's

protocol. Proper transposition and BACmid generation were confirmed by blue-white colony screening. Resulting colonies were inoculated into Luria-Bertani medium supplemented with 7 µg ml⁻¹ gentamycin, 10 µg ml⁻¹ tetracycline and 50 µg ml⁻¹ kanamycin, grown overnight at 37 °C and purified as previously described^{12,20}. Bacteria were pelleted at 4,000g for 10 min and resuspended in 0.2 ml of ZymoPURE miniprep buffer P1, followed by 0.2 ml of P2 buffer. After 5 min of incubation, 0.4 ml of P3 buffer was added and incubated on ice for 5–10 min. After a 10-min centrifugation at 15,000g, the supernatant was added to 0.64 ml of room-temperature isopropanol and incubated for 1–2 h on ice. BACmid DNA was pelleted for 10 min at 15,000g, washed three times in 0.5 ml of 70% ethanol, air-dried for 1 min and then resuspended in DNA elution buffer. BACmids were stored at 4 °C and used within 2 weeks for subsequent virus production, as described below.

Supplementary Table 3 provides a list of plasmids generated and used throughout this study.

Protein purification

Purification of dynein_{MOTOR} fragments (GAL1p:ZZ-2×TEV-6×His-dynein_{MOTOR}-Halo, GAL1p:ZZ-2×TEV-6×His-GFP-GST-dynein_{MOTOR} or similar) was performed as previously described with minor modifications¹³. In brief, yeast cultures were grown in YPA medium supplemented with 2% galactose for 3–6 h, collected, washed with cold water and then resuspended in a small volume of water. The resuspended cell pellet was drop-frozen into liquid nitrogen and then lysed in a coffee grinder. After lysis, 0.25 volumes of 4× dynein lysis buffer (1× buffer: 30 mM HEPES pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate and 0.2 mM EGTA) supplemented with 1 mM dithiothreitol (DTT), 0.1 mM Mg-ATP and 0.5 mM Pefabloc SC or protease inhibitor tablets (Pierce) (concentrations for the 1× buffer) was added and the lysate was clarified by centrifugation at 310,000g for 1 h. The supernatant was then incubated with IgG Sepharose 6 fast flow resin (GE) for 1–3 h at 4 °C, which was subsequently washed three times in 5–10 ml of lysis buffer and twice in 5–10 ml of TEV buffer (50 mM Tris pH 8.0, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA and 10% glycerol) supplemented with 0.005% Triton X-100, 1 mM DTT, 0.1 mM Mg-ATP and 0.5 mM Pefabloc SC. To label protein for single-molecule assays (some GST-dynein_{MOTOR}-HaloTag fragments), approximately 2–5 µM JFX650 Halo dye was added and incubated for 10–20 min at room temperature, after which the resin was washed with 10 ml of TEV buffer three times. The resin was then incubated with TEV protease for 30 min at room temperature (for monomeric dynein_{MOTOR} fragments) or 1 h at 16 °C (for GST-dynein_{MOTOR}-HaloTag fragments). After digestion with TEV, the bead-buffer slurry was filtered through an Ultrafree-MC microcentrifuge filters (to collect liquid phase), aliquoted, flash-frozen in liquid nitrogen and stored at –80 °C. Protein quality was assessed by SDS-PAGE and mass photometry. Some dynein_{MOTOR} mutants were not sufficiently pure for mass-photometry-based binding assays and required a subsequent polishing step. To do so, the TEV-cleaved protein was diluted 10–20-fold in buffer A (20 mM Tris pH 8.0, 1 mM magnesium acetate, 10% glycerol, 0.1 mM ATP and 1 mM DTT) and then injected on to a monoQ 10/100 GL pre-equilibrated in buffer A using an AKTA Pure system. After injection, the column was washed with 2–5 column volumes of buffer A and then bound proteins were eluted using 20 column volumes of a linear gradient from buffer A to buffer B (20 mM Tris pH 8.0, 1 mM magnesium acetate, 1 M NaCl, 10% glycerol, 0.1 mM ATP and 1 mM DTT). The peak fractions (typically 25–35% buffer B) with the highest purity (determined by mass photometry) were pooled, concentrated, aliquoted, flash-frozen and then stored at –80 °C. Note that one mutant in particular (dynein_{MOTOR}^{MT-U}-K2766A) required a gel filtration step instead of ion exchange to obtain protein of sufficient purity for our mass-photometry-based binding assays. To this end, the concentrated peak fractions from the monoQ were applied to a Superose 6 10/300 using GF150 (25 mM HEPES pH 7.4, 150 mM KCl, 1 mM MgCl₂, 1 mM DTT and 0.1 mM Mg-ATP) and the highest-purity

fractions (determined by mass photometry) were then pooled and concentrated before aliquoting, freezing and storage at -80°C .

GST-dimerized dynein motors (expressed from yeast cells expressing ZZ-2 $\times$ TEV-6 $\times$ His-GFP-GST-dynein_{MOTOR}-Halo) used for mass photometry experiments underwent tandem affinity purification from yeast cell cultures as previously described⁶⁴. Briefly, following cell lysis, 0.25 volumes of 4 $\times$ Ni-NTA dynein lysis buffer (1 $\times$ buffer: 30 mM HEPES pH 7.2, 150 mM potassium acetate, 2 mM magnesium acetate and 10% glycerol) supplemented with 1 mM β -mercaptoethanol, 0.1 mM Mg-ATP and 0.5 mM Pefabloc SC (concentrations for the 1 $\times$ buffer) was added and the lysate was clarified as described above. The supernatant was then bound to Ni-NTA agarose for 1 h at 4°C , which was subsequently washed three times in 5 ml of Ni-NTA lysis buffer. The protein was eluted in Ni-NTA lysis buffer supplemented with 250 mM imidazole by incubation on ice for 20 min. The eluate was then diluted with an equal volume of 1 $\times$ dynein lysis buffer, which was then incubated with IgG Sepharose 6 fast flow resin for 1 h at 4°C . The beads were washed three times with dynein lysis buffer and then twice with TEV buffer and the protein was eluted as described above (TEV protease treatment for 30 min at room temperature). Eluted protein was applied to a size-exclusion resin (Superose 6; Cytiva) preequilibrated in TEV buffer using an AKTA Pure system. Peak fractions (determined by absorbance at 260 nm and SDS-PAGE) were pooled, concentrated, aliquoted, flash-frozen and then stored at -80°C .

The intact full-length yeast dynein complex was expressed in and purified from insect cells (Sf9 cells from Thermo Fisher, 11496015) using a similar protocol to that used for the human dynein complex⁶⁵. Briefly, a six-well dish was seeded with 2 ml of Sf9 cells at 5×10^5 cells per ml, which were maintained in SF 900 II SFM Medium (Life Technologies). Cells were transfected with 2–6 μg of BACmid DNA using FuGENE HD transfection reagent and incubated at 27°C in a sealed box with a damp towel inside. The efficiency of transfection and virus production was monitored using yellow fluorescent protein (YFP) fluorescence. Then, 4–7 days following transfection (when 95–100% of cells expressed YFP reporter), the virus (P1) was collected by transferring the medium to a sterile tube. The virus was amplified by adding 1 ml of P1 virus to 50 ml of Sf9 cells at 5×10^5 cells per ml, which were incubated at 27°C in a flask shaking at 140 rpm. The resulting P2 virus was collected by centrifuging cell suspension and transferring virus-containing medium to a sterile tube, which was maintained at 4°C . For protein production, 20 ml of P2 was used to infect 2 L of Sf9 cells at 1.5×10^6 cells per ml. Then, 66–70 h after infection, the cells were harvested (2,000g, 20 min), washed with lysis buffer (50 mM HEPES pH 7.4, 100 mM NaCl and 10% glycerol, pH 7.2), pelleted again (1,810g, 20 min) and resuspended in an equal volume of lysis buffer supplemented with 1 mM DTT, 0.1 mM Mg-ATP and 1 mM PMSF. The resulting cell suspension was drop-frozen in liquid nitrogen and stored at -80°C . For protein purification, ~30 ml of fresh dynein lysis buffer supplemented with cOmplete protease inhibitor cocktail (Roche), 1 mM DTT and 0.1 mM Mg-ATP was added to the frozen cell pellet, which was then rapidly thawed in a 37°C water bath before incubation on ice. Cells were lysed in a Dounce-type tissue grinder (Wheaton) using ~60 strokes. Subsequent to clarification at 310,000g for 1 h at 4°C , the supernatant was applied to 3–4 ml of IgG Sepharose fast flow resin (Cytiva) preequilibrated in lysis buffer and incubated at 4°C for 3–5 h. Beads were then washed with 30–50 ml of lysis buffer and then 20–30 ml of TEV buffer (50 mM Tris pH 7.4, 150 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 10% glycerol, 1 mM DTT and 0.1 mM Mg-ATP). The beads were then incubated with 300 μg of TEV protease overnight at 4°C . The next morning, the recovered supernatant was applied to either a Superose 6 gel filtration column (Cytiva) or a TSKgel G4000 column. Peak fractions were pooled, concentrated, aliquoted, flash-frozen and then stored at -80°C . Sample quality was assessed using mass photometry, SDS-PAGE and/or negative-stain EM before cryo-EM grid preparation.

Pac1 was purified from either budding yeast (as previously described^{13,64}) or Sf9 cells. Note that we observed no difference in dynein binding between Pac1 purified from these two cell types, although our yields from Sf9 cells were substantially higher. Briefly, yeast or Sf9 cell lysate was prepared as described above. Proteins were prepared as described above for the dynein_{MOTOR} fragments with two differences. Instead of incubating with TEV protease for 30 min at room temperature, TEV digest was performed at 4°C overnight. Instead of polishing the protein by anion-exchange chromatography, the TEV eluates were applied to a size-exclusion chromatography resin (Superose 6, Cytiva) that was equilibrated in TEV buffer supplemented with 1 mM DTT using an AKTA Pure system. Peak fractions (determined by absorbance at 260 nm and SDS-PAGE) were pooled, concentrated, aliquoted, flash-frozen and then stored at -80°C .

Negative-stain EM

EM grids were prepared by applying freshly purified dynein complexes to glow-discharged carbon-coated 200-mesh copper grids. After a ~1-min incubation, 2% uranyl acetate was added. Micrographs were collected on a FEI Tecnai F20 200-kV transmission EM instrument equipped with a Gatan US4000 charge-coupled device (CCD) camera (model 984), at a nominal magnification of $\times 90,000$ with a digital pixel size of 1.449 Å. All image analysis was performed in RELION 4.0 on the University of Colorado Boulder high-performance computer cluster, Alpine. Particles were manually picked from ~20–30 micrographs (~300–400 particles), which were used to generate low-resolution 2D class averages. These 2D averages were then used to autopick particles used to generate our final 2D averages.

To visually assess the Pac1-bound AAA3^{WA} dynein_{MOTOR}^{MT-U} complex (Fig. 2a–d), we first enriched for this species by incubating an equimolar mixture of these proteins with ADP. After a 20-min incubation on ice, the mixture was applied to a size-exclusion chromatography resin (Superose 6, Cytiva) that was equilibrated in TEV buffer supplemented with 1 mM DTT and 1 mM ADP using an AKTA Pure system. Peak fractions enriched with 2:1 dynein_{MOTOR}^{MT-U:WA}-Pac1 complexes (determined by mass photometry) were pooled, concentrated and applied to an EM grid as described above.

Mass photometry

All proteins were diluted to either 50 nM or a fraction greater or lesser thereof (as indicated by x value in Fig. 3 and Extended Data Fig. 5) in assay buffer (50 mM Tris pH 8.0, 150 mM potassium acetate, 2 mM magnesium acetate and 1 mM DTT) with or without added nucleotide (1 mM of each). Then, 1–2 μl of each protein was mixed 1:1 (to 25 nM of each), incubated for 1–2 min and then diluted 1:5 on the stage (2 μl of mixed protein + 8 μl of same buffer with or without nucleotide) to a final concentration of 5 nM immediately before image acquisition. For apo conditions, residual ATP from the protein preparation was depleted using apyrase by mixing 4.5 μl of respective dynein_{MOTOR} protein with a 0.5 μl of apyrase (New England Biolabs) and incubating for 30 min at room temperature. Then, 1-min videos were acquired using Refeyn MP and all images were processed and analyzed using Discover MP. Calibration was performed with β -amylase and thyroglobulin. Axes were normalized using Discover MP such that the sum of all events for a given experiment was equal to 1.

Cryo-EM sample preparation and data collection

Dynein concentration was diluted to 2 mg ml⁻¹ (from 3 mg ml⁻¹) in GF150 buffer with or without Pac1 and incubated on ice for 1 h before cryo-EM sample vitrification (Extended Data Fig. 8b). Then, 3 μl of sample was applied to Quantifoil holey carbon grids (R2/1, 300-mesh gold or R2/1, 400-mesh) and incubated in the chamber of the Vitrobot Mark IV unit (FEI) for 5 s at 4°C and 100% humidity. Subsequently, the grids were blotted for 3–5 s and plunged into liquid ethane. The grids were then screened and data were collected on a 200-keV Glacios EM instrument

(Thermo Fisher Scientific) with a K3 direct detection camera (Gatan) at a magnification of $\times 45,000$, physical pixel size of $0.868 \text{ \AA}$ and total dose of $40 \text{ e}^- \text{ per } \text{\AA}^2$. In total, 2,179 (for dynein alone) and 7,036 (for dynein–Pac1 complex) videos at a defocus range of $-1.2 \text{ }\mu\text{m}$ to $-2.7 \text{ }\mu\text{m}$ were acquired and data collection was automated using SerialEM^{66,67}.

Cryo-EM image processing

Preprocessing steps, including motion correction, contrast transfer function (CTF) estimation and particle picking, were conducted either in cryoSPARC Live⁶⁸ or with an in-house script using MotionCor2 (ref. 69), GCTF⁷⁰ and Gautomatch. Scripts for real-time data transfer and on-the-fly preprocessing are available from GitHub (<https://github.com/JackZhang-Lab>). Reconstruction steps were conducted in cryoSPARC⁶⁸ (Extended Data Fig. 8c,d).

For both datasets, the initial particles were picked using blob picker or template matching and extracted at a box size of 128 and pixel size of $3.47 \text{ \AA}$ (bin 4). The particles were subjected to several rounds of 2D classification to obtain high-quality class averages of dynein motor domain. Ab initio reconstruction followed by heterogeneous refinement of these particles (six classes) revealed a medium resolution ($5\text{--}8 \text{ \AA}$) of the motor domains. Manual inspection was performed for different classes and similar classes were merged. Two different classes were identified and the particles were reextracted at a box size of 284 and pixel size of $1.157 \text{ \AA}$ for high-resolution reconstruction. Each class was subjected to homogeneous refinement and two rounds of global CTF, local CTF and local refinement. The resolutions of all maps were estimated by Fourier shell correlation calculations^{71,72} embedded in cryoSPARC. Local resolution analysis⁷³ was performed in cryoSPARC.

Model building and refinement

The previous published yeast dynein motor domain structures (PDB 4AKI (ref. 74), PDB 4W8F (ref. 52) and PDB 7MGH (ref. 45)) were used as initial models. A manual fitting step was conducted in ChimeraX⁷⁵ to fit individual AAA domains to the cryo-EM maps. Namdinator⁷⁶ was then used to automatically refine the model into the cryo-EM map.

All models underwent iterative refinement using PHENIX real-space refinement (version 1.21rc1_5190)⁷⁷ and manual rebuilding in Coot^{78,79}. The quality of the refined models was assessed using MolProbity⁸⁰ integrated into PHENIX, with statistics reported in Supplementary Table 1.

Plots and molecular graphics

Plots were generated using GraphPad Prism 10.4. Molecular graphics were prepared using UCSF ChimeraX (version 1.9)⁷⁵.

Single-molecule motility assays

Single-molecule motility assays were performed as previously described with minor modifications^{13,44}. Briefly, flow chambers constructed using slides and plasma were cleaned and silanized coverslips attached with double-sided adhesive tape were coated with anti-tubulin antibody ($8 \text{ }\mu\text{g ml}^{-1}$, YL1/2; Accurate Chemical and Scientific Corporation) and then blocked with 1% Pluronic F-127 (Fisher Scientific). Taxol-stabilized microtubules assembled from unlabeled porcine tubulin (Cytoskeleton) were introduced into the chamber. After incubation for 2–5 min, the chamber was washed with lysis buffer (30 mM HEPES pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA and 10% glycerol) supplemented with $20 \text{ }\mu\text{M}$ taxol and 1 mM DTT. Dynein, diluted in an oxygen-scavenging motility buffer (lysis buffer supplemented with 50 nM protocatechuate 3,4-dioxygenase, 2.5 mM protocatechuic acid, 1 mM Trolox, 1 mM cyclooctatetraene, 1 mM 4-nitrobenzoyl alcohol, 1 mM DTT, $20 \text{ }\mu\text{M}$ taxol and 1 mM Mg-ATP) was then added. Total internal reflectance fluorescence (TIRF) microscopy images were immediately collected using an iLAS2 RING TIRF system on a Nikon Ti-2E inverted microscope equipped with a $\times 100$ (numerical aperture (NA): 1.49) TIRF objective,

a Ti2-SS-E motorized stage, piezo z control and an iXon LIFE 897 cooled electron-multiplying (EM)-CCD camera (Andor). A 640-nm laser housed in an LUN-F 3 (Nikon) was used along with a multipass quad filter cube set (C-TIRF for 405, 488, 561 and 638 nm; Chroma) and emission filter mounted in a filter wheel (700/75 nm; Chroma). To image nonfluorescent microtubules, we used interference reflection microscopy⁸¹. 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.

Live-cell spindle dynamics assay

For the spindle dynamics assay (Fig. 6h,i), mid-log-phase cells were arrested with 200 mM hydroxyurea (HU) for 2.5 h in SD complete supplemented with 2% glucose and then applied to slide-mounted agarose ‘pads’ comprising 1.7% agarose dissolved in SD complete supplemented with 2% glucose and 200 mM HU for confocal fluorescence microscopy. Full z stacks (21 planes with $0.2\text{-}\mu\text{m}$ spacing) of GFP-labeled microtubules (GFP–Tub1) were acquired every 10 s for 10 min. Images were collected on a Nikon Ti-E microscope equipped with a $\times 100$ (NA: 1.49) TIRF objective, a Ti-S-E motorized stage, piezo z control (Physik Instrumente), an iXon DU888 cooled EM-CCD camera (Andor) and a spinning disc confocal scanner unit (CSUX1, Yokogawa). A 488-nm laser housed in an LU-NV laser unit equipped with AOTF control (Nikon) was used to excite GFP, which was used with an emission filter mounted in a filter wheel (ET525/50M; Chroma). The microscope was controlled with NIS Elements (Nikon).

For those parameters plotted in Fig. 6i and Extended Data Fig. 10B, dynein-mediated spindle movements were manually scored from both xy and xz projections using custom-written MATLAB routines (‘CellCropGUI_4.m’ for cropping individual cell images and ‘spindle_tracker_v3.m’ for manually tracking dynein-mediated spindle movements are both available from Zenodo). Dynein-mediated spindle movements were identified as those events in which the spindle underwent a directed excursion toward the cell cortex that was coincident with a microtubule–cortex encounter. The visual representations of spindle dynamics over time shown in Fig. 6h and Extended Data Fig. 10c were generated with a custom MATLAB routine (‘SpindleTracker3D_v8.m’, for automated tracking of spindle centroid over time, is also available from Zenodo) that permits the user to delineate the cell outline and bud neck and tracks the spindle centroid over time with respect to these boundaries. To eliminate any dynein-independent contributions to spindle movements, these assays were performed in cells lacking Kar9, a protein that is required for an actin/myosin-mediated spindle orientation pathway^{82–84}. Data shown are from three independent replicates from at least two biological replicates.

Immunoblotting

To determine the relative expression levels of WT and mutant Dyn1 protein, cell pellets from log-phase growing cells were first resuspended in lysis buffer (30 mM HEPES pH 7.2, 50 mM potassium acetate, 2 mM magnesium acetate and 0.2 mM EGTA supplemented with 1 mM DTT, 0.1 mM Mg-ATP and cOmplete protease inhibitor). Glass beads (0.5 mm) were added to cell slurry, which were broken open by vortexing four times for 15 s each, with 1 min of incubation on ice in between. After clarification ($20,000g$, 10 min at 4°C), an equal amount of protein from each sample (determined by Bradford assay) was incubated with $15 \text{ }\mu\text{l}$ of GFP-Trap beads (ProteinTech) for 1 h at 4°C . After incubation, beads were washed twice in lysis buffer and then boiled in sample buffer before loading on a 5% SDS polyacrylamide gel. Separated protein was transferred to PVDF (in 25 mM Tris, 192 mM glycine supplemented with 0.05% SDS and 10% methanol), which was blocked (TBS supplemented with 5% milk) then probed with a monoclonal anti-GFP antibody (1:200; Abm) followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (at 1:10,000; Jackson ImmunoResearch Laboratories).

Expression levels of WT and mutant Pac1 (both tagged with 13Myc) were determined by loading an equal amount of clarified cell extracts (prepared as described above for Dyn1) directly onto a 4–20% SDS polyacrylamide gel (Bio-Rad). After transferring to PVDF, membranes were blocked (as above) and then probed with a rabbit anti-c-Myc antibody (1:500; GenScript) followed by an HRP-conjugated goat anti-rabbit antibody (at 1:10,000; Jackson ImmunoResearch Laboratories). Immunoblots were processed using SuperSignal West Pico PLUS chemiluminescent substrate (Thermo Fisher) and imaged on a Bio-Rad ChemiDoc.

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 Protein Data Bank and EM Data Bank under the following accession codes: PDB [9MFV](#) and EMD-[48239](#) for dynein-alone dataset class 1, PDB [9MFW](#) and EMD-[48240](#) for dynein-alone dataset class 2, PDB [9MFX](#) and EMD-[48241](#) for dynein–Pac1 dataset class 1 (motors unbound from Pac1) and PDB [9MFY](#) and EMD-[48242](#) for dynein–Pac1 dataset class 2 (motors bound to Pac1). All plasmids, yeast strains, datasets and raw video files that were generated during and/or analyzed during this study are available from the corresponding authors upon request. Source data are provided with this paper.

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Author contributions

S.M.M. designed the study. I.C.G., B.R.I., W.D.T., A.H.I., S.C.G., S.V.B. and S.M.M. purified the proteins for binding assays and negative-stain EM grids. I.C.G., B.R.I., W.D.T., S.C.G. and S.M.M. performed the in vitro binding assays. J.S.B. and S.M.M. performed and analyzed the live-cell

microscopy for spindle-positioning assays. P.C. and J.Y. acquired and analyzed the cryo-EM data and built the structural models with support from K.Z. P.C. and S.M.M. generated the figures. S.M.M. wrote the manuscript. All authors edited and revised the manuscript. S.M.M. and K.Z. acquired funding.

Competing interests

The authors declare no competing interests.

Additional information

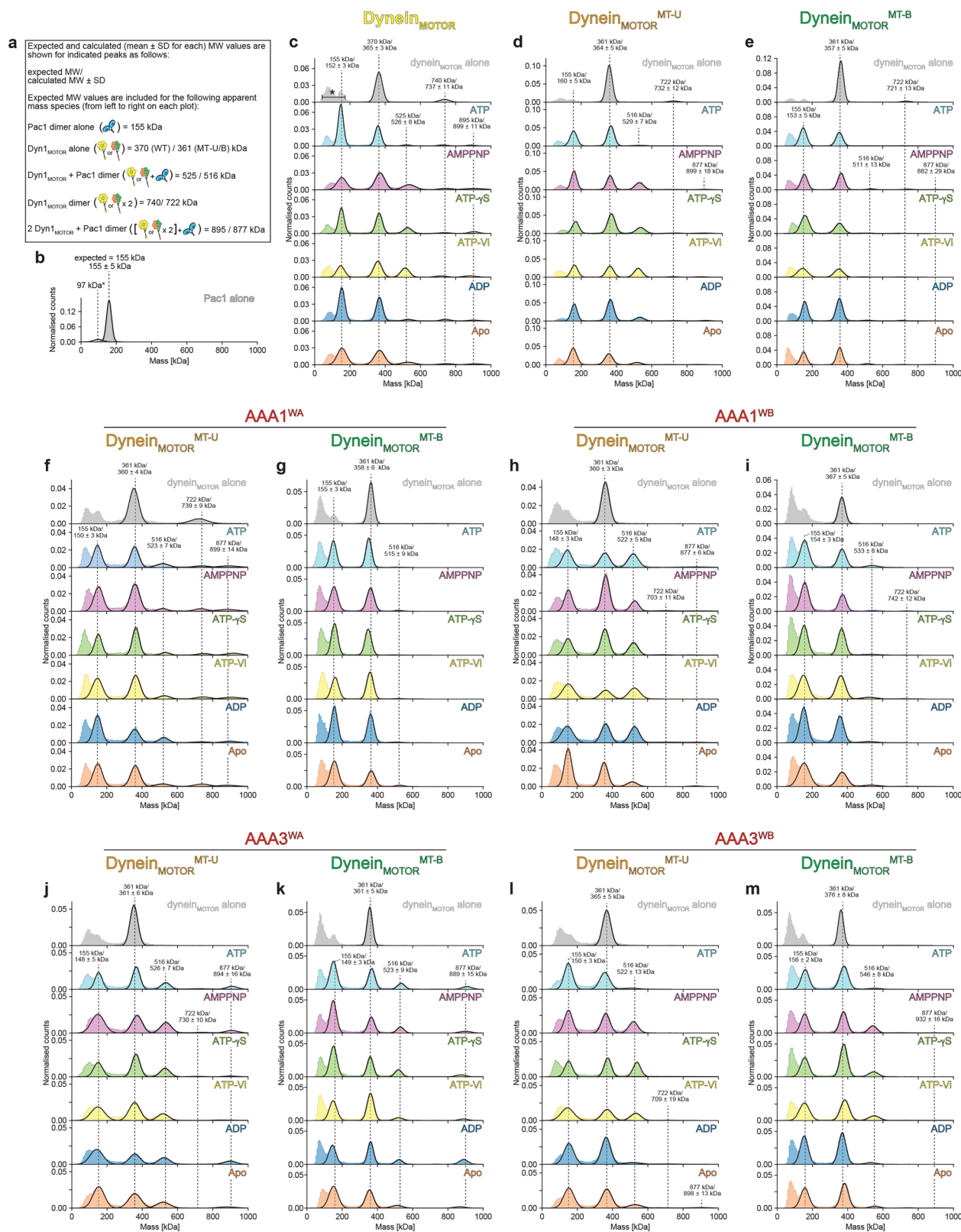
Extended data is available for this paper at <https://doi.org/10.1038/s41589-025-02096-8>.

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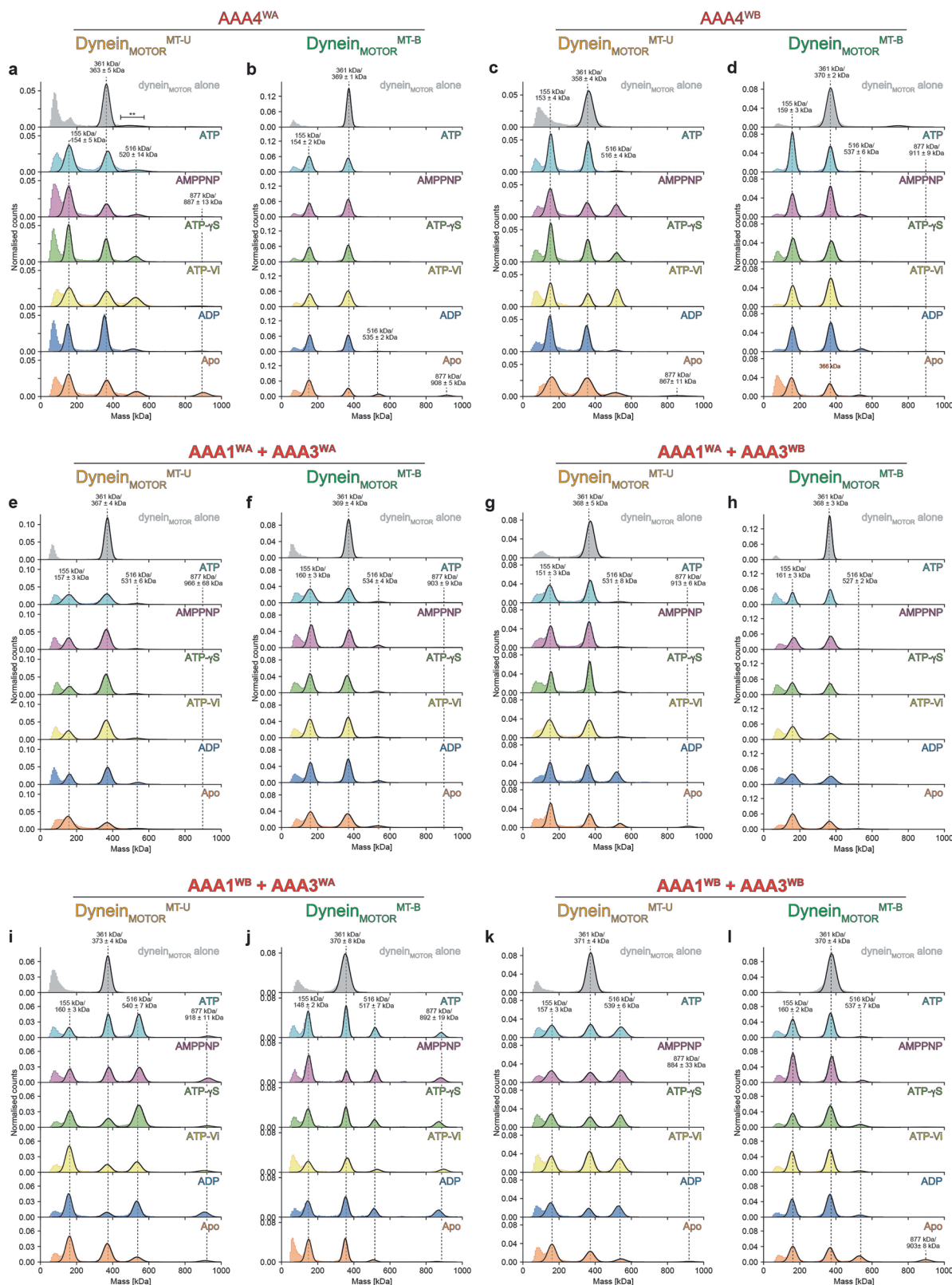
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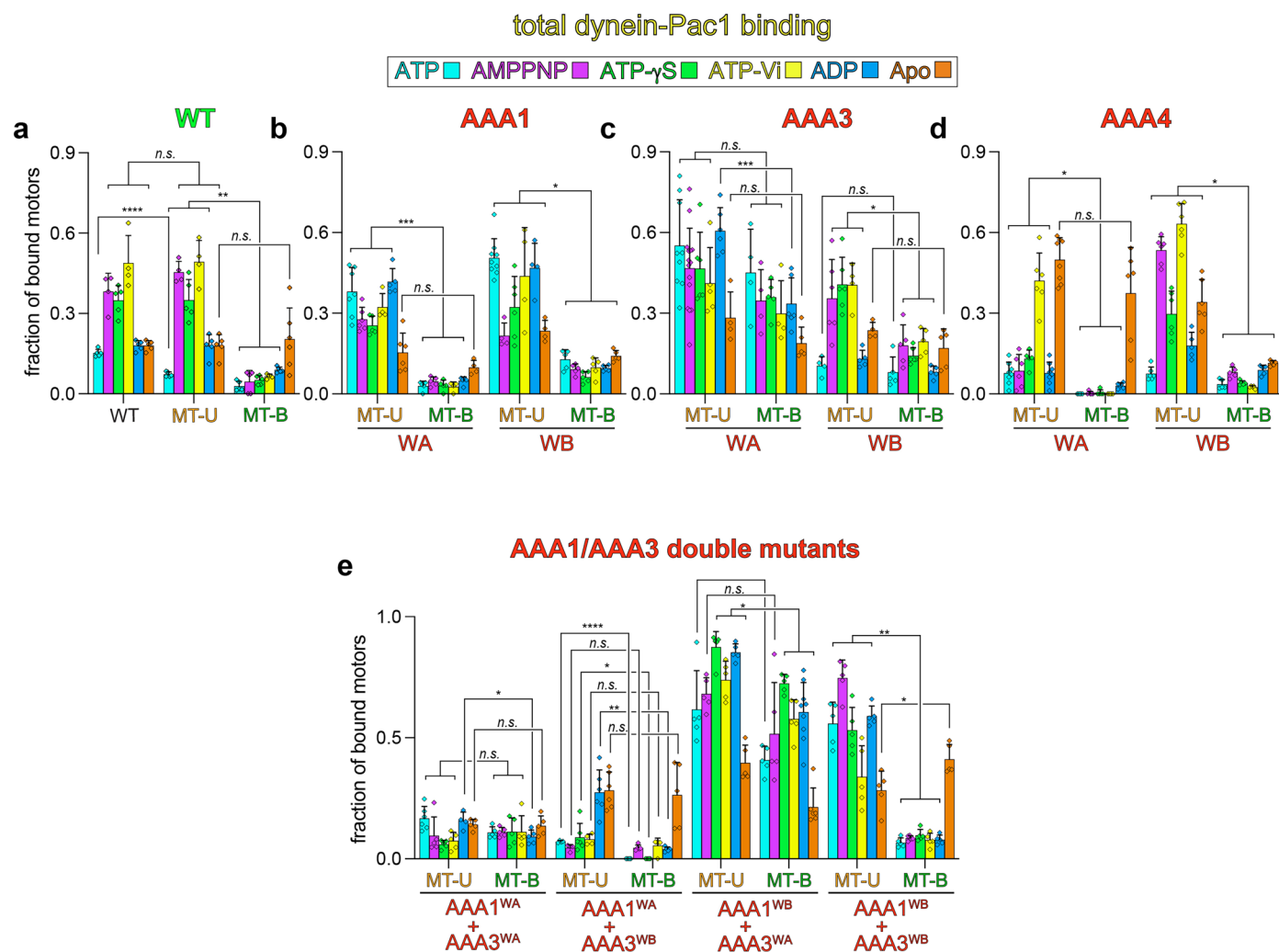


Extended Data Fig. 1 | Representative mass photometry histograms used to assess dynein-Pac1 binding. (a) Schematic depicting expected molecular weight values for protein species identified throughout Fig. 1 and elsewhere. (b – m) Representative mass histograms obtained from indicated proteins alone or in combination (as indicated). Expected/calculated molecular weight (mean $\pm$ standard deviation) values for indicated species are shown above

each peak. Note that values indicated by “*” in panel c (which are present in almost all binding experiments to varying extents) are attributed to “noise”, or contaminants either from protein purifications, or buffer components (nucleotide mixtures). As shown in panel b, these species are not representative of monomeric Pac1 (expected MW, ~78 kDa).



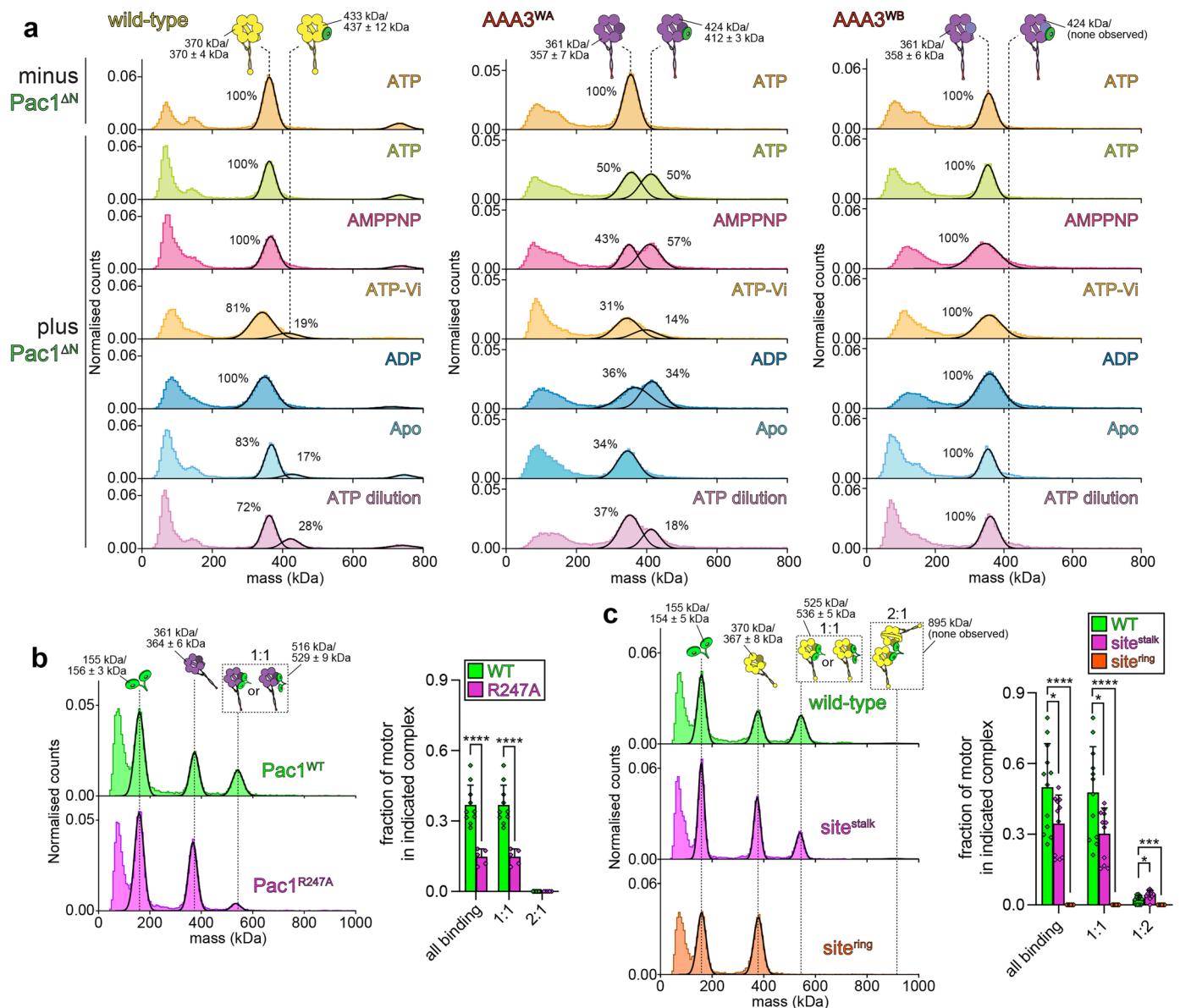
Extended Data Fig. 2 | More representative mass photometry histograms used to assess dynein-Pac1 binding. (a – l) Representative mass histograms obtained from indicated proteins alone or in combination (as indicated). See Extended Data Fig. 1 for more information.



Extended Data Fig. 3 | Assessment of total dynein_{MOTOR}-Pac1 binding.

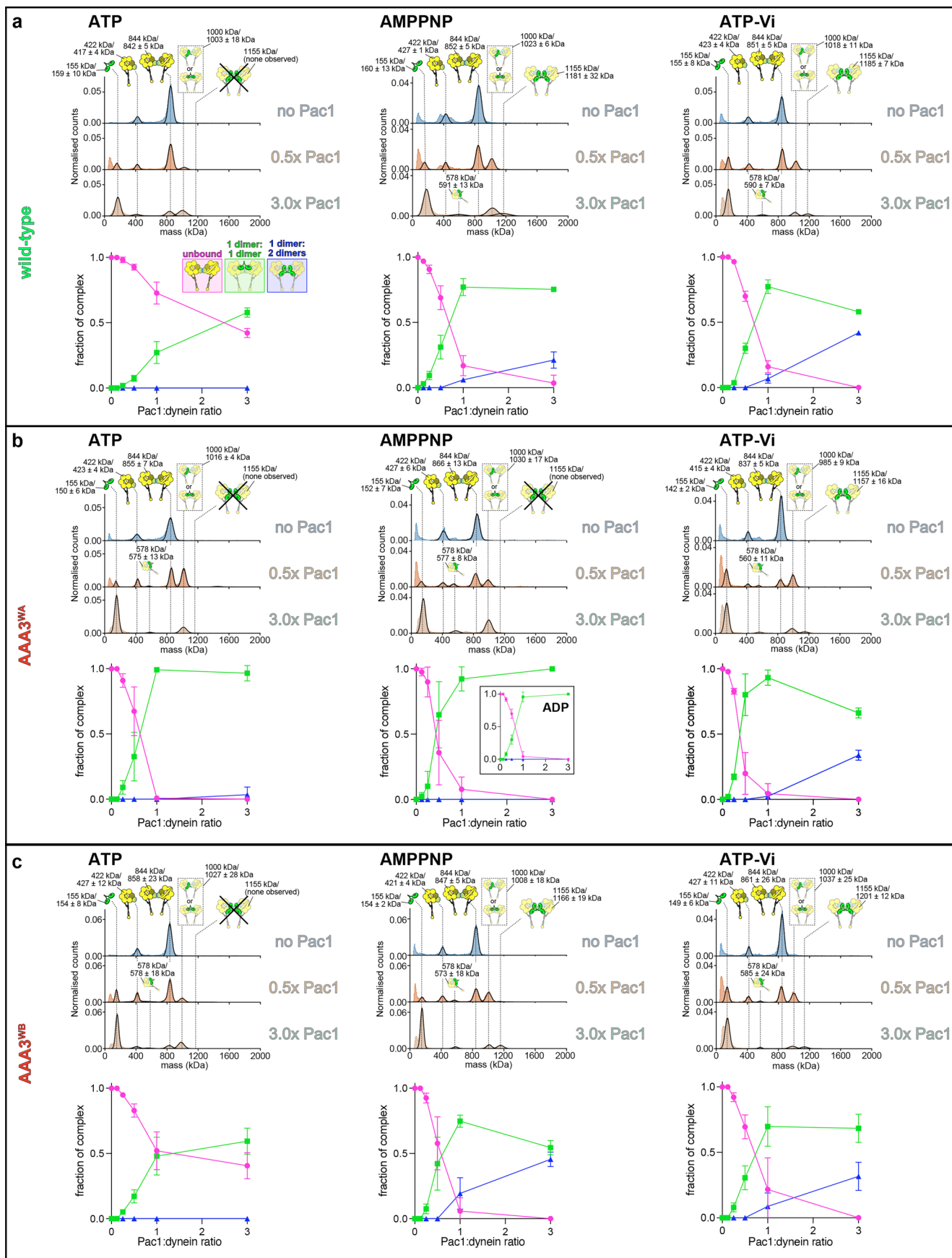
(a–e) Plots of total bound fraction for wild-type (a) or indicated WA or WB mutant in AAA1 (b) AAA3 (c) or AAA4 (d) mutant dynein_{MOTOR} fragments. Those with indicated combination of AAA WA or WB mutants are shown in panel e. Plots depict results from mass photometry binding assays (mean ± standard deviation along with individual data points; for panel a, from left to right, $n_{WT} = 4/4/5/4/5/5$, $n_{MT-U} = 4/4/5/4/5/6$, $n_{MT-B} = 4/5/5/4/5/6$ independent replicates; for panel b, from left to right, $n_{MT-U}^{WA} = 7/6/5/4/5/7$, $n_{MT-B}^{WA} = 5/5/5/4/5/4$, $n_{MT-U}^{WB} = 9/8/5/4/4/5$, $n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for panel c, from left to right, $n_{MT-U}^{WA} = 9/15/5/5/6/4$, $n_{MT-B}^{WA} = 4/4/6/4/6/5$, $n_{MT-U}^{WB} = 4/6/5/4/5/4$, $n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for panel d, from left to right, $n_{MT-U}^{WA} = 5/6/5/6/8/8$, $n_{MT-B}^{WA} = 5/5/5/5/5/6$, $n_{MT-U}^{WB} = 6/6/6/6/5/6$,

$n_{MT-B}^{WB} = 5/5/5/5/5/5$ independent replicates; for panel e, from left to right, $n_{MT-B}^{WA/WA} = 6/5/5/5/4/5$, $n_{MT-B}^{WA/WB} = 5/5/5/5/5/5$, $n_{MT-U}^{WA/WB} = 4/4/6/4/5/6$, $n_{MT-B}^{WB/WB} = 5/5/5/5/5/5$, $n_{MT-U}^{WB/WB} = 5/5/5/5/5/5$, $n_{MT-B}^{WB/WB} = 5/5/5/5/5/5$ independent replicates). Values represent total fraction of bound motors, including those within the 523 kDa peak and those within the 897 kDa peak. *P*-values were calculated using an unpaired two-sided *t* test with Welch's correction (****, $P \leq 0.0001$; ***, $P \leq 0.0010$; **, $P \leq 0.01$; *, $P \leq 0.05$; n.s., not significant, $P > 0.05$). In cases in which multiple data sets are cross compared, only the highest *P* value is indicated (to simplify data presentation). See associated Source Data file for all raw data and all individual *P* values.



Extended Data Fig. 4 | AAA1 and AAA3 coordinate Pac1-binding affinity at both site^{ring} and site^{stalk}. (a) Representative mass photometry histograms showing binding between monomeric Pac1 (Pac1^{ΔN}; Δ1-143) and indicated monomeric dynein_{MOTOR} (WT, left; MT-U/K2424 A, middle; MT-U/E2488Q, right). Each are representative of at least 3 independent replicates. Note that the relatively low mass of unbound Pac1^{ΔN} makes it undetectable by mass photometry in our conditions. (b and c). Representative mass photometry histograms (left) and plots (mean ± SD, along with all data points; right) showing

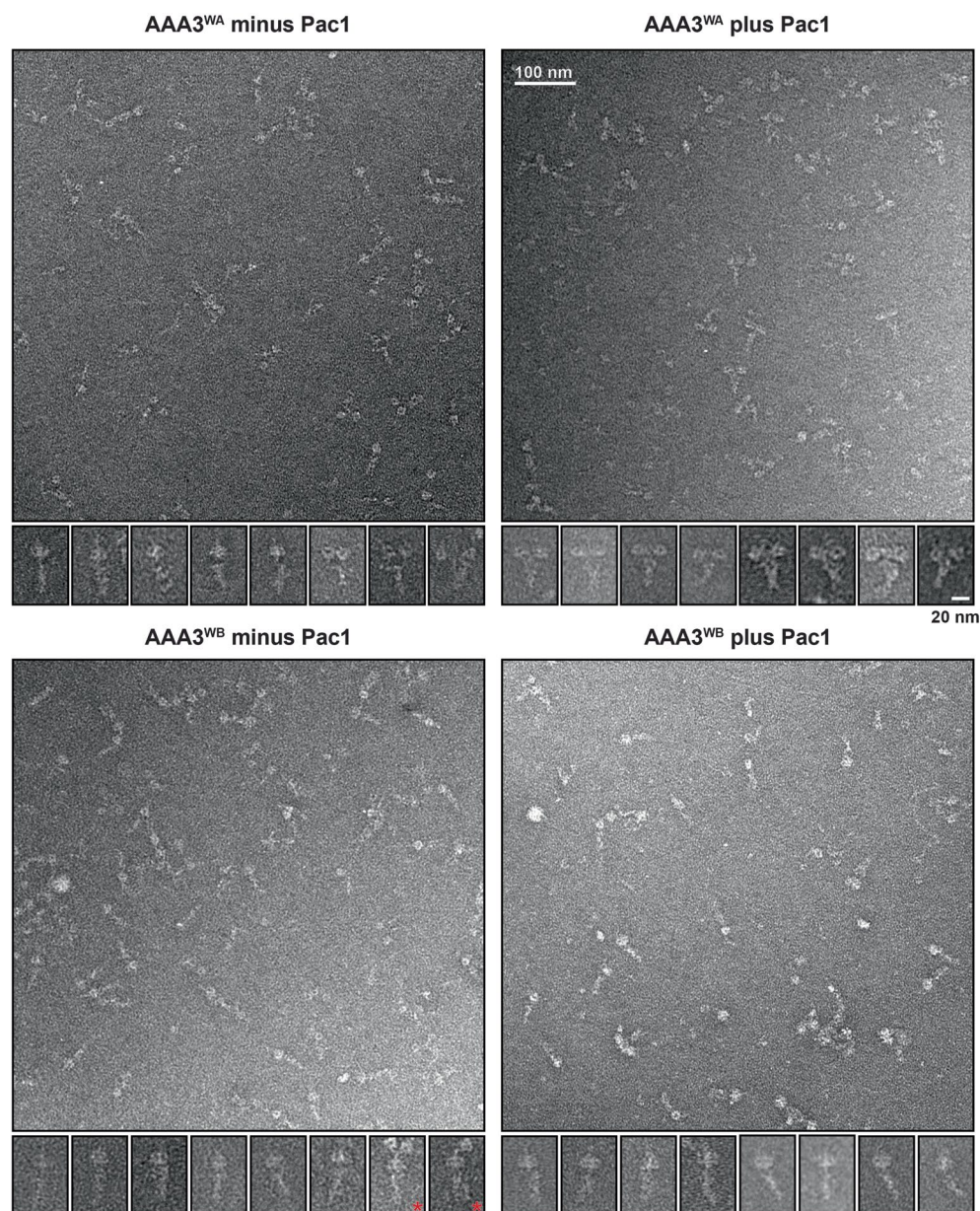
binding between full-length Pac1 (WT or R247A) and either dynein_{MOTOR}^{MT-U/E2488Q} (b; $n_{WT} = 13$, and $n_{R247A} = 9$ independent replicates) or dynein_{MOTOR}^{E2488Q} (c; $n_{WT} = 12$, and $n_{site-stalk} = 11$, and $n_{site-ring} = 6$ independent replicates) in the presence of AMPPNP. Expected/calculated molecular weight (mean ± standard deviation) values for indicated species are shown above each peak. *P*-values were calculated using an unpaired two-sided *t* test with Welch's correction (****, $P \leq 0.0001$; ***, $P \leq 0.0010$; *, $P \leq 0.05$). See associated Source Data file for all raw data and all individual *P* values.



Extended Data Fig. 5 | See next page for caption.

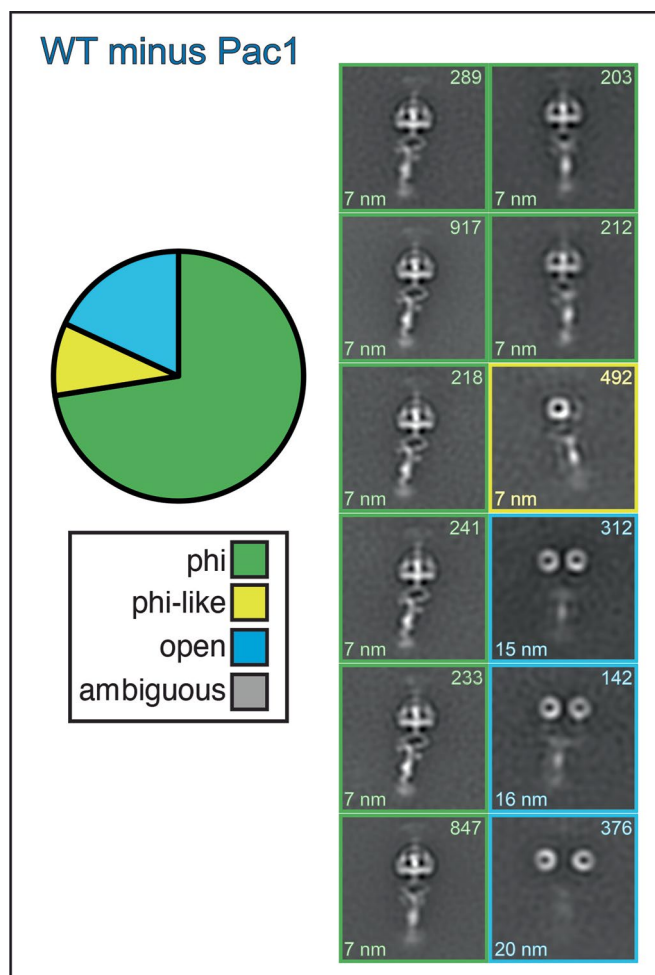
Extended Data Fig. 5 | Nucleotide occupancy coordinates stoichiometry of binding between Pac1 and GST-dimerized dynein_{MOTOR}. (a – c) Representative mass photometry histograms and associated plots (mean fraction of binding $\pm$ SD; $n = 3$ independent replicates for each) depicting extent and stoichiometry of binding between Pac1 and either wild-type (a), AAA3^{WA} (b) or AAA3^{WB} GST-dynein_{MOTOR} mutants at a range of dynein:Pac1 ratios. Cartoon schematics

above each peak indicate respective protein species. Binding experiments were performed in indicated nucleotide conditions (ATP, left; AMPPNP and ADP, middle; ATP + vanadate, right). Note that we observed no 1844 kDa species, which would be indicative of Pac1 linking two distinct GST-dynein_{MOTOR} fragments, including in conditions that enrich for 2 dynein_{MOTOR}:1 Pac1^{dimer} species (that is, AAA3^{WA} mutant with ATP or ADP).



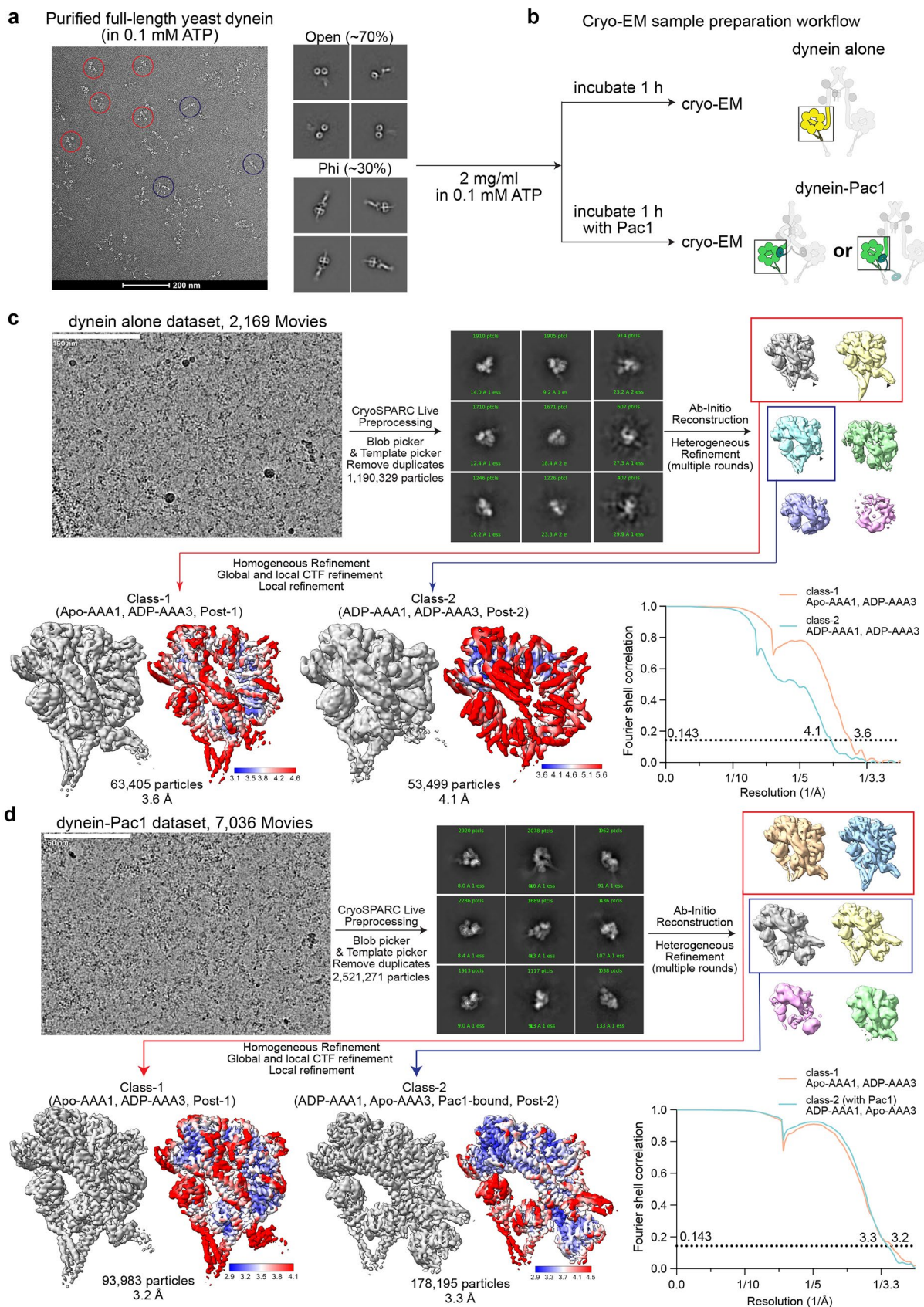
Extended Data Fig. 6 | Representative negative stain EM images of full-length dynein mutants. Representative unprocessed negative stain EM images of indicated full-length dynein mutants in the absence (left) or presence of excess Pac1 (see Fig. 4a, b). Panels of cropped images show representative

individual dynein complexes from each condition ("*" indicates 2x-phi dynein species observed in AAA3^{WB} minus Pac1). Similar results were obtained from 2 independent replicates.

**Extended Data Fig. 7 | Negative stain EM of wild-type full-length dynein.**

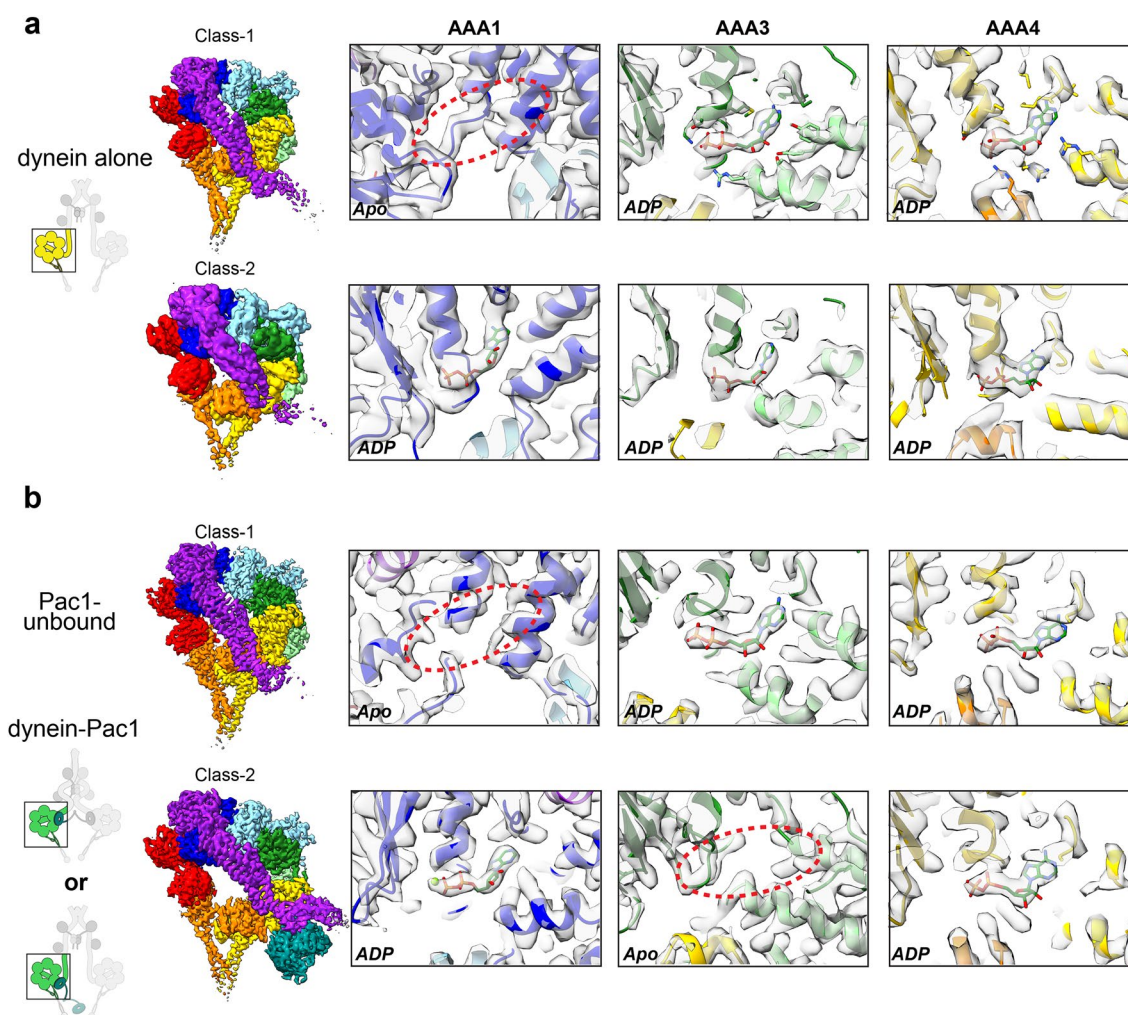
Representative 2D class averages showing indicated full-length dynein (purified from Sf9 cells) in the absence of Pac1. Pie graph shows relative fraction of

indicated conformational state (determined from 2D averages). Numbers indicate the number of particles in each class (top) and the measured distance between the motor domains (from center-to-center; bottom).

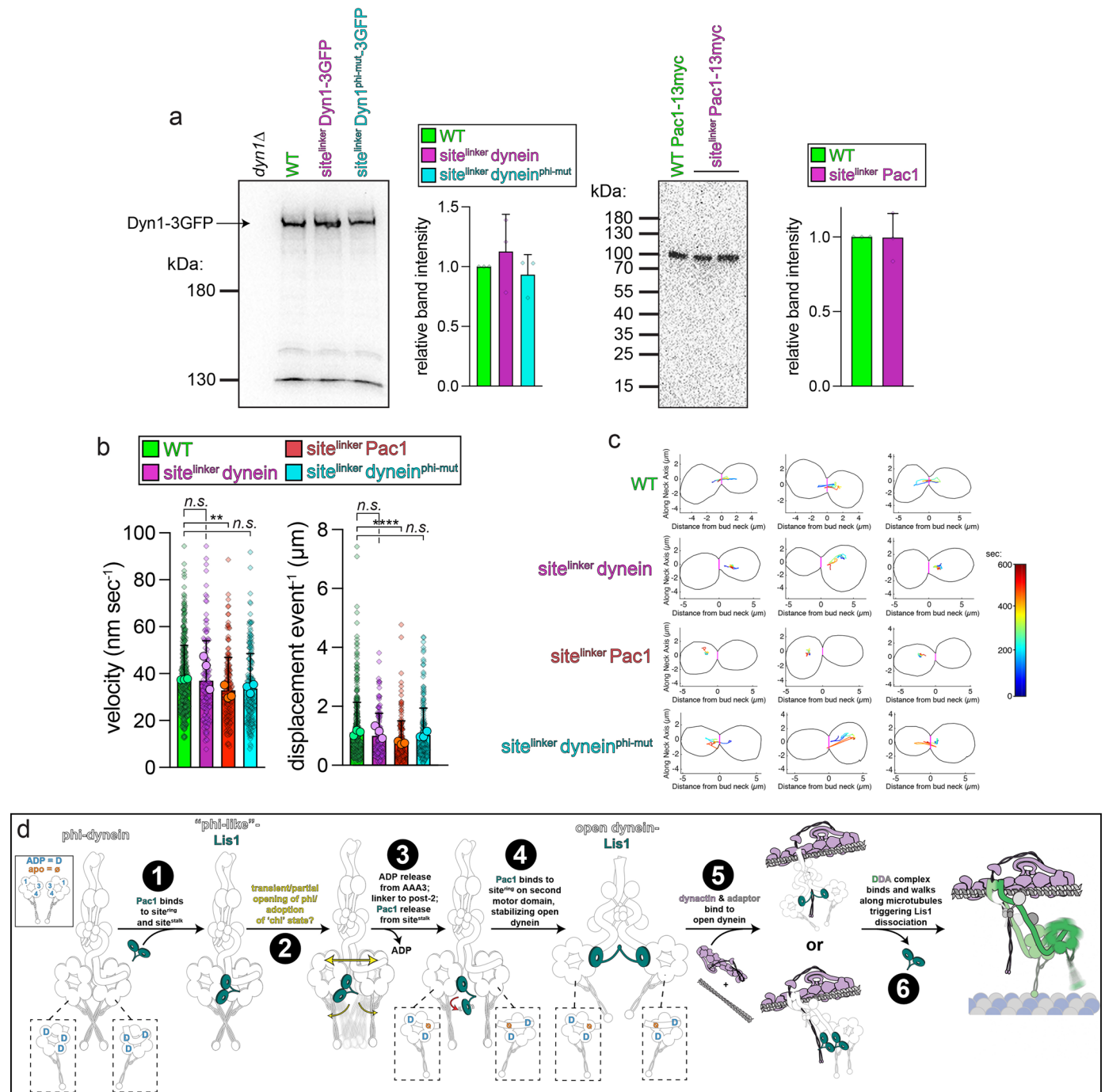


Extended Data Fig. 8 | Cryo-EM data processing workflow. (a) A representative negative staining image of purified yeast full-length dynein with corresponding 2D class averages. Red and blue circles indicate the open and phi dynein, respectively. (b) Cryo-EM sample preparation workflow of dynein alone or in complex of Pac in 0.1 mM ATP concentration. (c and d) Cryo-EM image processing

of the dynein alone dataset (c) or the dynein-pac1 dataset (d). Representative 2D class averages of motor domains are shown. Cryo-EM maps, local-resolution analysis and FSC plots are shown. Similar results were obtained from 2 independent replicates.



Extended Data Fig. 9 | Nucleotide states of AAA1, AAA3 and AAA4 from each cryo-EM map. (a and b) Two classes from the dynein alone dataset (a) or from the dynein-pac1 dataset (b). The Cryo-EM maps are shown in transparent surface mode fitted with PDB models. The red dashed circles highlight the missing nucleotide in the pocket (apo state).



Extended Data Fig. 10 | Additional parameters for dynein-mediated spindle movements in HU-arrested cells, and model for Lis1 opening of phi dynein.

(a) Representative immunoblots of Dyn1-3GFP (wild-type and indicated mutants) and Pac1-13myc along with quantitation (mean ± SD; n = 3; see Methods). Whereas cells lacking dynein were used as a control for the Dyn1-3GFP blots (*dyn1Δ*), those without a 13myc tag on Pac1 were used for the Pac1 blot (see "control" in Extended Data Fig. 10 source data). (b) Velocity and displacement values for dynein-mediated translocation of the mitotic spindle in hydroxyurea-arrested cells (see Fig. 6i for n values). *P*-values were calculated using a two-sided Brown-Forsythe and Welch ANOVA (for velocity values), or a two-sided Kruskal-Wallis test (for run length values) (****, *P* ≤ 0.0001; **, *P* ≤ 0.0100; *n.s.*, not significant, *P* > 0.05). See associated raw data file for individual values and all statistical data. (c) Additional representative tracks of spindle centroids for indicated strains (cell outline in black, bud neck in magenta). (d) We posit the following model to account for our data. Dynein primarily exists in the

autoinhibited phi state with ADP bound to AAA1, 3 and 4 (refs. 12,44). As a consequence of this nucleotide code, Lis1 can bind with one WD40 binding at site^{ring}, and the other at site^{stalk}. Our negative stain EM data indicate this binding mode does not result in dynein opening, consistent with two recent cryo-EM studies^{55,56}. To release ADP from AAA3 and adopt a post-2 linker state, dynein partially/transiently opens, which consequently leads to Pac1 unbinding from site^{stalk}, and binding to the 2nd motor domain. This new binding mode stabilizes an open conformation of dynein, which more readily binds to dynactin and an adaptor. Although assembly of the dynein-dynactin-adaptor complex likely promotes adoption of a parallel arrangement of the motor domains¹² (bottom), we cannot rule out dynein remaining in the Lis1-stabilized open conformation we observe by negative stain EM (top). Finally, upon binding to microtubules and initiating a motility event, Lis1 dissociates due to a weakened affinity at both site^{ring} and site^{stalk}³¹.

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Models and cryo-EM maps have been deposited in the PDB and EMDB as follows: PDB-9MFV/EMDB-48239 for dynein alone dataset class-1, PDB-9MFW/EMDB-48240 for dynein alone dataset class-2, PDB-9MFX/EMDB-48241 for dynein-Pac1 dataset class-1 (motors unbound from Pac1), and PDB-9MFY/EMDB-48242 for dynein-Pac1 dataset class-2 (motors bound to Pac1). All of the plasmids, yeast strains, datasets and raw video files that were generated during and/or analyzed during this study are available from the corresponding authors upon request. All data used to generate plots throughout the manuscript are included as Source Data files.

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Data exclusions	For EM averages (for both negative stain and cryo), particles with low quality (low contrast, ice contamination) were discarded.
Replication	Live cell and single molecule microscopy (i.e., mass photometry, motility) assays, negative stain and cryo-EM sample preparation and 3D reconstruction were reproduced from at least 2 biological and/or independent datasets collected from samples prepared with different protein purifications.
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Antibodies

Antibodies used Mouse anti-GFP (ABM, cat#G096), rabbit anti-myc (Genscript , cat #A00172), HRP-conjugated goat anti-mouse and anti-rabbit (Jackson ImmunoResearch).

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