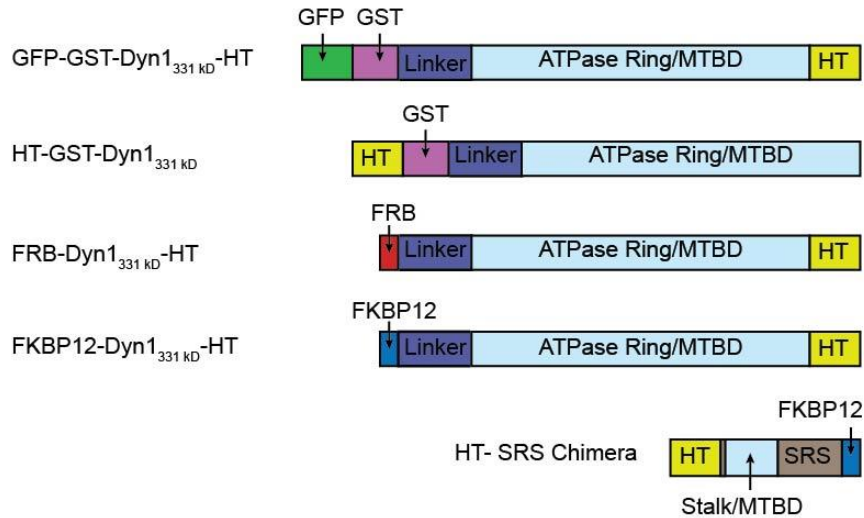
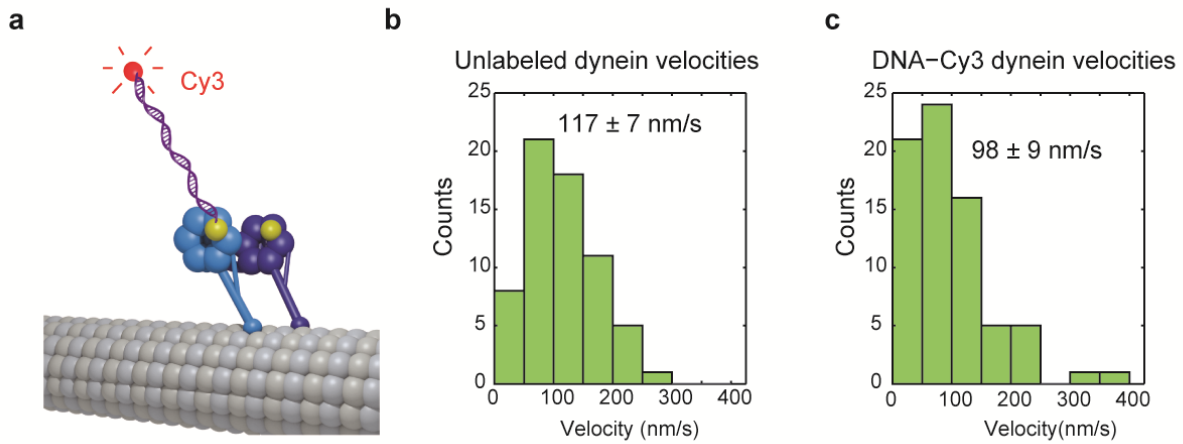


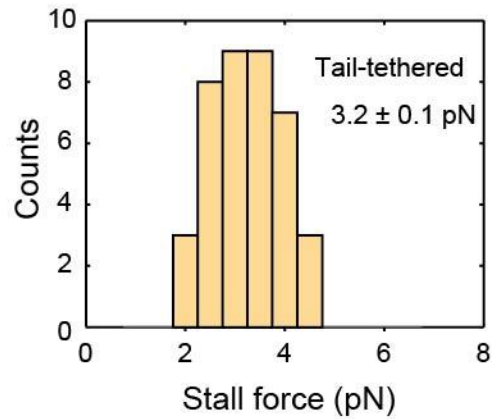
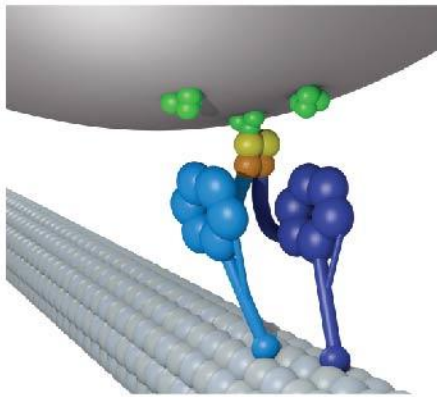
Supplementary Figures:



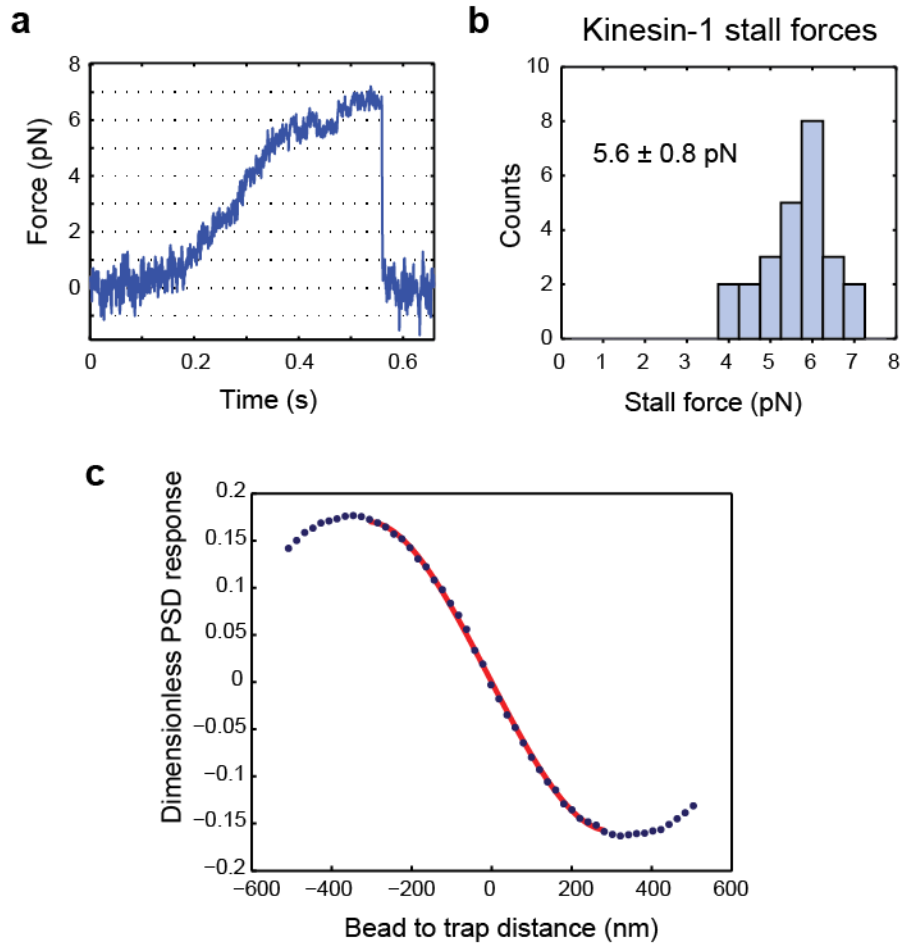
Supplementary Figure 1. Dynein and SRS constructs used in this study. The length of each segment is scaled proportionally to the length of the corresponding genetic sequence. All sequences are shown with their N-termini on the left and C-termini on the right. HT refers to HaloTag.



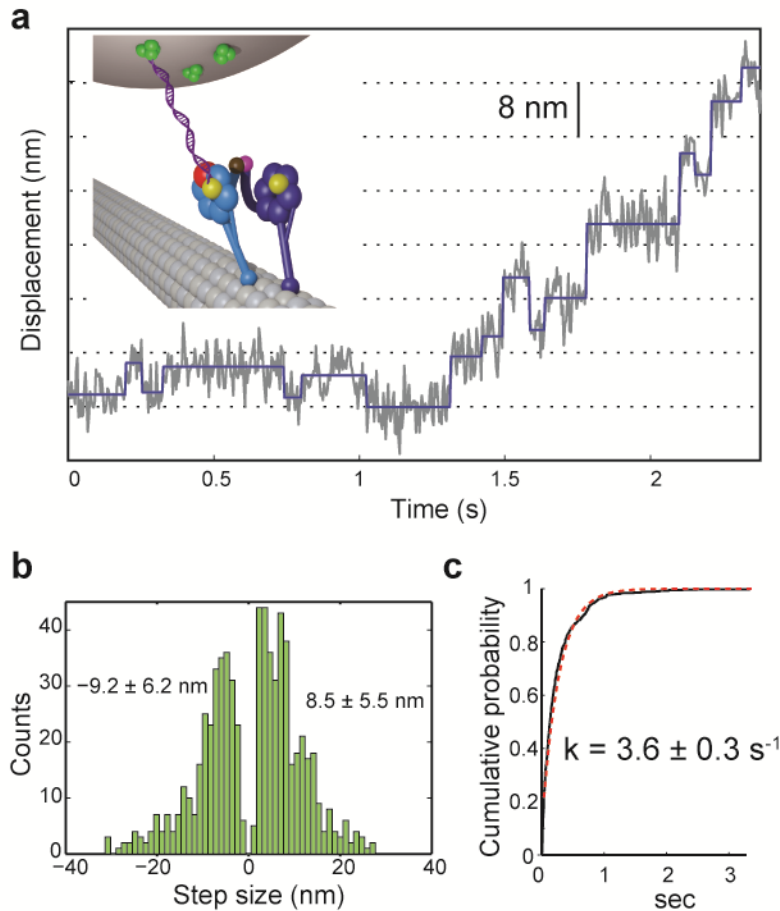
Supplementary Figure 2. Impact of DNA labeling on dynein velocity. (a) Geometry for labeling verification. The dsDNA molecule is identical to the one used in actual experiments, except the biotin at the distal end is replaced with a Cy3 fluorophore. (b) Velocity distribution obtained from GFP fluorescence kymographs of unlabeled WT/WT homodimers. (c) Velocity distribution of Cy3-DNA labeled WT/WT homodimers (mean \pm S.E.M.).



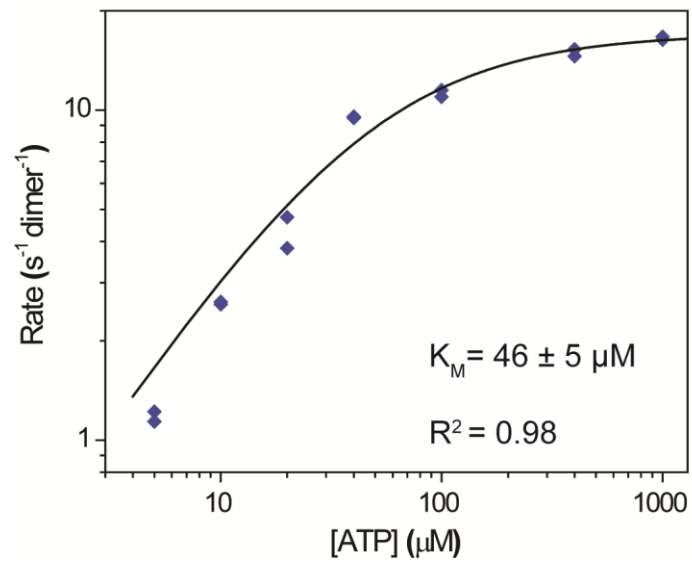
Supplementary Figure 3. Stall force of Dyn1_{331kD} with an N-terminal HaloTag. Dynein was attached to streptavidin-coated beads (streptavidin shown in green) via a biotinylated HaloTag ligand at the N-terminal HaloTag domain (yellow). A histogram of measured stall forces is shown on the right. The mean stall force measured in this geometry is similar to that of dynein motors that contain a C-terminal HaloTag domain, suggesting that C-terminal HaloTag does not significantly hinder dynein force production.



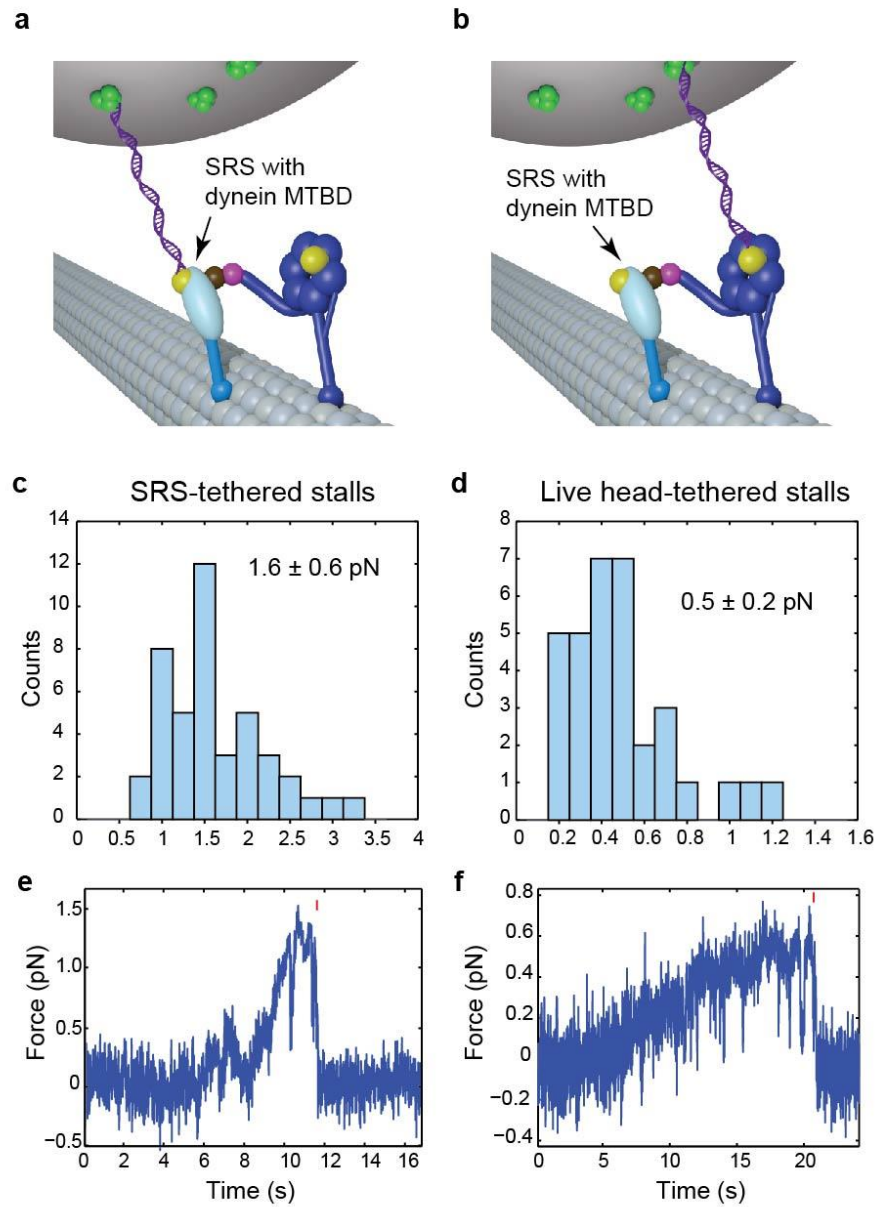
Supplementary Figure 4. The calibration of optical trap. (a) Sample kinesin-1 stall trace, stalling at 6.5 pN (trap stiffness is 0.06 pN/nm). (b) Kinesin-1 stall force distribution. The reported value of 5.6 ± 0.8 pN (mean \pm s.d.) agrees well with the previous finding that kinesin-1 stalls at 5-6 pN³⁶ (c) Sample response curve used to convert dimensionless detector signals into nanometers using a cubic polynomial fit. The data points in the curve are obtained by rapidly raster-scanning the trapping laser across the bead in one dimension. The red solid curve is a plot of the resulting cubic fit, which is used to convert raw PSD signals to nanometers.



Supplementary Figure 5. Measuring steps of the mutant head-tethered construct. (a) Stepping trace obtained from mutant head-tethered WT/AAA1_{K/A} construct under 1.5 pN hindering load. The stepping trace (gray) is decimated to 250Hz. The output of the step-fitting algorithm is shown in blue. (b). Step size distribution of the mutant head-tethered construct under 1.5 pN hindering load. Values shown are mean \pm s.d. (c). Stepping rate of the mutant head-tethered construct under 1.5 pN hindering load, obtained by fitting cumulative probability distribution (solid black line) to a single exponential (dashed red line). The reported stepping rate is mean \pm 95% confidence interval boundaries.

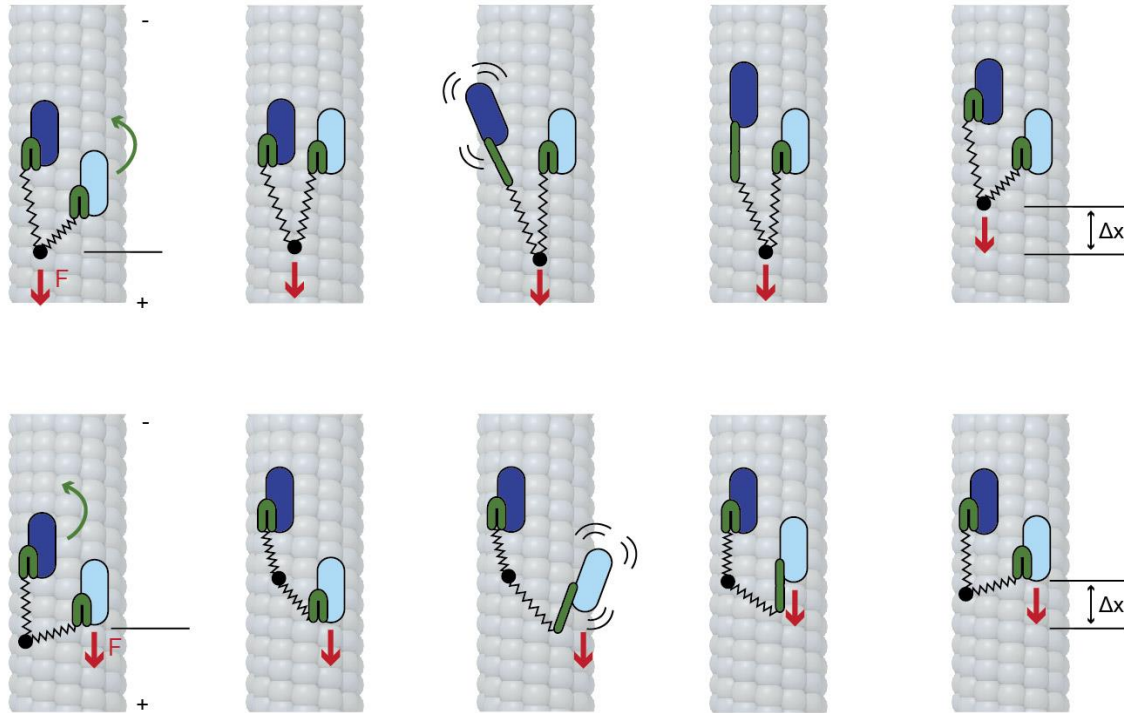


Supplementary Figure 6. ATPase rates of WT dynein at saturating MT concentrations. K_M of WT dynein is $46 \pm 5 \mu\text{M}$.



Supplementary Figure 7. Confirmation of AAA1_{K/A} results using an SRS fusion ‘peg leg’.

(a) Geometry for mutant head-tethered experiments. The DNA tether is attached to the SRS ‘peg leg’, which is heterodimerized with a WT head, which is heterodimerized with an SRS ‘peg leg’ through rapamycin + FRB/FKBP. (b) Geometry for live head-tethered SRS experiments. The DNA tether is attached to a WT head. (c) Stall force histogram of the SRS-tethered WT/SRS construct (mean ± s.d.) (d). Stall force histogram of the live-head tethered WT/SRS construct (mean ± s.d.) (e,f) Stall force traces of SRS-tethered and live-head tethered constructs, respectively.



Supplementary Figure 8. Putative model for step measurements of dynein motors under load. (Top row) When load is applied from the tail, the leading head bears a larger fraction of the load and is inhibited from taking an additional step. When the heads are not located side-by-side, the load is mainly resisted by the leading head's linker. A step taken by the trailing head does not significantly change the position of the tail attachment point (black dot) under tension because of the high compliance of the tail in at low and intermediate tensions. Only the steps when either of the heads advances the leading position are detectable in this geometry (detectable step represented with Δx ; see **Methods** for a discussion of spatiotemporal constraints on step detection). (Bottom row) In a head-tethered trapping geometry, the tethered head likely remains in the trailing position and is assisted by the leading head via linker tension. Only the tethered head's steps are resolvable in this experiment. As a consequence, the trap generally detects steps of only one of the heads in both head- and tail- tethered assays, leading to the observed similarities between measured stepping rates and sizes.

Supplementary Note 1

Here, we present the derivation of the force-velocity (F-V) relation for a one-state motor model with force dependence in both the forward and backward stepping rates.

We start with the basic equation relating the forward and backward stepping rates of a one-state motor¹:

$$\frac{k_+(f)}{k_-(f)} = e^{-(\Delta G + Lf)/kT}, \quad (1)$$

where f is the external load on the motor, $k_+(f)$ is the force-dependent forward stepping rate, $k_-(f)$ is the force-dependent backward stepping rate, ΔG is the free energy from ATP hydrolysis biasing the motor to step forward, L is the step size, k is the Boltzmann constant, and T is the temperature.

We now assign variable force dependence to $k_+(f)$ and $k_-(f)$, introducing the dimensionless parameters a and b :

$$k_+(f) = k_+ e^{-aLf/kT} \quad (2)$$

$$k_-(f) = k_- e^{bLf/kT} \quad (3)$$

Where k_+ and k_- are the constant forward and backward stepping rates in unloaded conditions.

Substituting equations (2) and (3) into (1), we obtain

$$\frac{k_+}{k_-} e^{-(a+b)Lf/kT} = e^{-(\Delta G + Lf)/kT}. \quad (4)$$

Because both the exponents and preexponents must be equal for all f for the equality above to hold, it follows that

$$\frac{k_+}{k_-} = e^{-\Delta G/kT} \quad (5) \text{ and}$$

$$a + b = 1. \quad (6)$$

Now, to obtain an expression for the velocity of the motor as a function of force, we note that the velocity is just the difference between the forward and backward stepping rates multiplied by the step size:

$$V(f) = L(k_+(f) - k_-(f)). \quad (7)$$

Substituting (2), (3), (5), and (6) and rearranging, we get the following expression for the F-V relation:

$$V(f) = Lk_+ e^{-aLf/kT} (1 - e^{(\Delta G + Lf)/kT}). \quad (8)$$

Supplementary References

1. Thomas, N., Imafuku, Y. & Tawada, K. Molecular motors: thermodynamics and the random walk. *Proc. R. Soc. B* **268**, 2113–22 (2001).