Loading direction regulates the affinity of ADP for kinesin

Sotaro Uemura[1] and Shin'ichi Ishiwata[1,2]

Published online 17 March 2003; doi:10.1038/nsb911

Kinesin is an ATP-driven molecular motor that moves processively along a microtubule. Processivity has been explained as a mechanism that involves alternating single- and double-headed binding of kinesin to microtubules coupled to the ATPase cycle of the motor. The internal load imposed between the two bound heads has been proposed to be a key factor regulating the ATPase cycle in each head. Here we show that external load imposed along the direction of motility on a single kinesin molecule enhances the binding affinity of ADP for kinesin, whereas an external load imposed against the direction of motility decreases it. This coupling between loading direction and enzymatic activity is in accord with the idea that the internal load plays a key role in the unidirectional and cooperative movement of processive motors.

Kinesin, which was initially purified from squid neural tissue[1], bovine brain[2] and sea urchin eggs[3], is a motor protein widely distributed in eukaryotic cells[4]. Kinesin is a processive motor that 'walks' along a microtubule toward its plus end and is essential for the transport of vesicles and organelles[5–7]. Upon binding to a microtubule, kinesin takes >100 consecutive steps before dissociation. Each 8 nm step is coupled to one cycle of ATP hydrolysis, indicating a tight coupling between enzymatic reaction and mechanical event. The high processivity of kinesin has been explained by a model in which the two heads of kinesin alternate iteratively between single-headed and double-headed binding to a microtubule[8–12].

How can kinesin molecules convert chemical energy to mechanical work, and how do two heads communicate with each other to realize a coordinated processive movement along a microtubule[8–12]? This communication has been proposed to be mediated by the internal load that is imposed on kinesin when both of its heads are bound to the microtubule. It is assumed that, in this configuration, the leading head and the trailing head experience a load backward and forward, respectively, through the connection between the two heads[13–17]. However, there is as yet no direct experimental evidence supporting this hypothesis.

Recently, we measured the unbinding force of single kinesin–microtubule complexes in nucleotide-free, ADP-bound and AMP-PNP (adenosine 5′-[β,γ-imido] triphosphate, an ATP analog)–bound conditions to determine how kinesin binds to the microtubule in these states: with single-headed or double-headed binding and with weak or strong binding[10,11]. We were

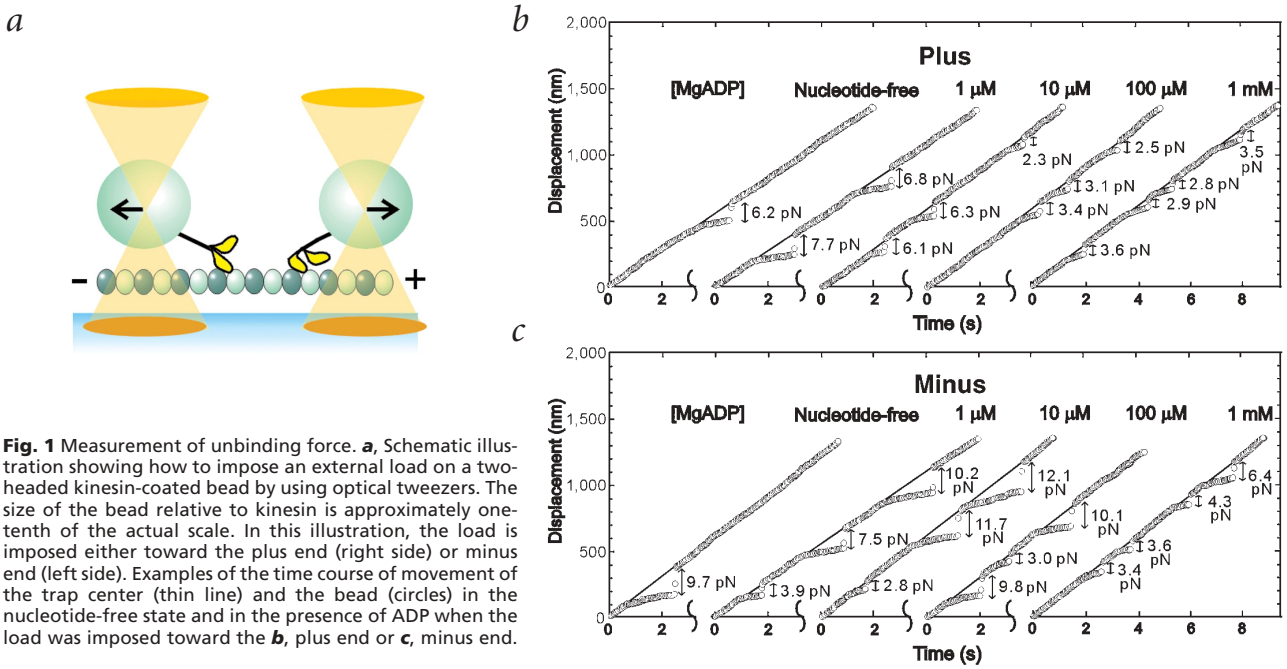

Fig. 1 Measurement of unbinding force. ***a***, Schematic illustration showing how to impose an external load on a two-headed kinesin-coated bead by using optical tweezers. The size of the bead relative to kinesin is approximately one-tenth of the actual scale. In this illustration, the load is imposed either toward the plus end (right side) or minus end (left side). Examples of the time course of movement of the trap center (thin line) and the bead (circles) in the nucleotide-free state and in the presence of ADP when the load was imposed toward the ***b***, plus end or ***c***, minus end.

[1]Department of Physics, School of Science and Engineering, and [2]Advanced Research Institute for Science and Engineering, Waseda University, 3-4-1 Okubo, Shinjuku-ku, Tokyo 169-8555, Japan.

Correspondence should be addressed to S.I. *e-mail: ishiwata@waseda.jp*

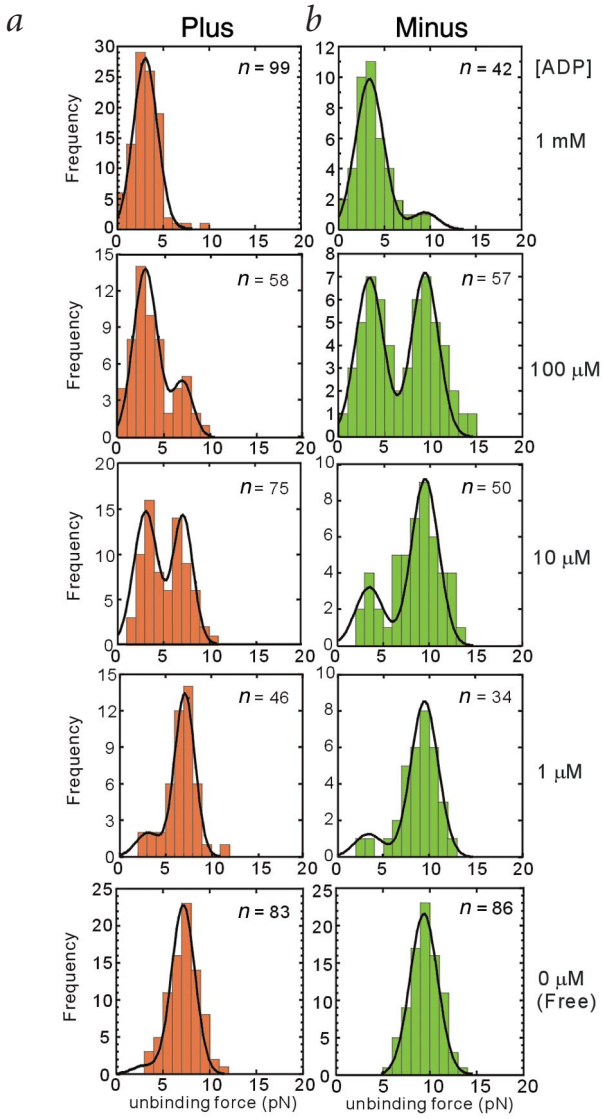

Fig. 2 Unbinding force distributions at various ADP concentrations. The ADP concentrations are shown on the right side of each distribution. ***a***, Plus-end loading is shown in orange, and ***b***, minus-end loading is shown in green. The solid curves are results of globally fitting the histograms to two Gaussian distributions for the plus-end and minus-end loading separately[24].

able to distinguish between single-headed and double-headed binding by analyzing the shape of the unbinding force distribution as unimodal and bimodal, respectively. Furthermore, we were able to distinguish between weak and strong binding on the basis of the peak value of the unbinding force distribution. We observed that double-headed, strong binding is predominant in the AMP-PNP–binding state, whereas single-headed, strong binding is predominant in the presence of AMP-PNP plus ADP[11]. In contrast, in the ADP binding state, single-headed binding is predominant and the binding is weak[10]. In the nucleotide-free state, the binding is strong but single-headed binding is predominant when unbinding is observed at a low loading rate[10–12]. In this study, we monitored the effects of loading direction on the affinity of ADP for kinesin.

Unbinding force at various ADP concentrations

We have examined the effect of imposing a load on the equilibrium between the weak binding state (realized predominantly in the presence of ADP) and the strong binding state (realized predominantly in the absence of nucleotide). To accomplish this, we measured the unbinding force at various ADP concentrations (1,000, 100, 10, 1 and 0 μM) for either the plus- or minus-end loading at a low loading rate (5.5 ± 0.14 pN s^{-1}, mean ± s.e.m., $n = 630$) using a previous reported method[10–12]. Here, a narrow distribution of the estimated loading rate (the rate of increase in the imposed load) exists because it was determined from the difference between the velocity of the bead moving along a microtubule (kept constant in the experiments) and the average extension of the protein–bead complex (differed for each measurement). The loading rate in the current study is slower than the rates of ADP binding and dissociation, as well as the rate of interconversion between single-headed and double-headed binding. Under this condition, the microtubule-bound kinesin is expected to sample predominantly the weak, single-headed binding state (ADP bound)[10] and strong, single-headed binding state (nucleotide free)[10–12] throughout the course of the experiments.

A kinesin-coated bead was first trapped by optical tweezers and placed in contact with a microtubule for 20–30 s to allow the kinesin–microtubule binding to reach equilibrium. Then, the unbinding force was measured by pulling the bead toward the plus end or the minus end of the microtubule at a constant velocity (Fig. 1*a*). Typical time course data (Fig. 1*b*,*c*) indicate that the unbinding events occurred repeatedly when the bead was kept moving along a microtubule. This was true not only at 1 mM ADP but also at intermediate ADP concentrations. In contrast, rebinding/unbinding events did not occur during the movement of the bead in the nucleotide-free state[10–12] (Fig. 1*b*,*c*).

We determined the unbinding force distributions obtained at various ADP concentrations (Fig. 2). As previously reported, in

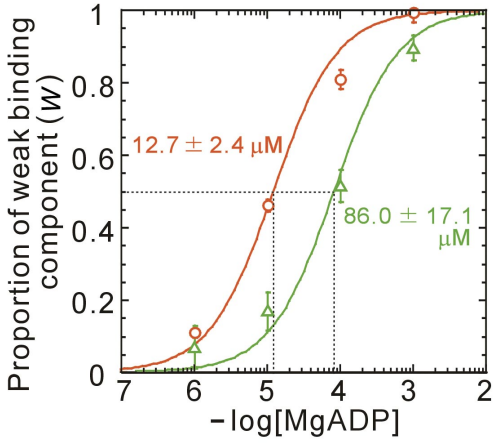

Fig. 3 Proportion of weak binding component, *W*, at various ADP concentrations. The proportion of weak binding component was estimated from the ratio of the area (peak × width) of the left peak of Gaussian distribution to the total area (sum of two Gaussian distributions) of the unbinding force distribution obtained by the global fit as shown in Fig. 2. Orange circles and green triangles show the data for plus-end and minus-end loading respectively. Data were fit to a hyperbola (orange and green lines), $W = [ADP] / (K + [ADP])$, in which the dissociation constant K was determined as 12.7 ± 2.4 μM for plus-end loading (K_+) and 86.0 ± 17.1 μM for minus-end loading (K_-). Error bars were obtained from the global fit.

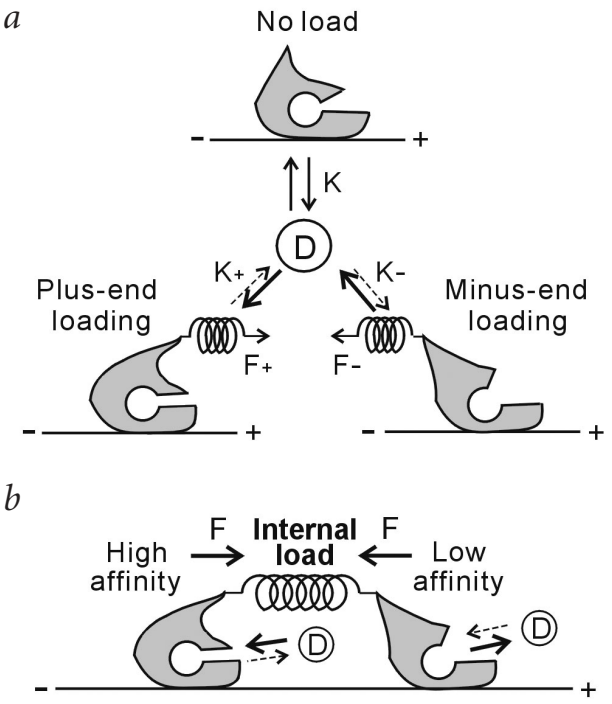

Fig. 4 Schematic illustrations showing equilibrium of ADP (D) binding to a complex of kinesin (nucleotide-binding pocket is shown) and microtubule (thin line, where both the plus and the minus ends are shown). ***a***, The effect of imposing a load toward the plus end (F_+) or the minus end (F_-) of a microtubule on the binding equilibrium. The dissociation constants of ADP in a kinesin-microtubule complex in the absence of load (K), under plus-end loading, K_+, and under minus-end loading, K_-, are also shown. ***b***, A possible role of an internal load within a two-headed kinesin molecule on the binding equilibrium. In (*b*), such a situation is illustrated that the internal load, which may be produced by extension of the link between the two heads upon binding to a microtubule, increases the binding affinity of ADP for the trailing head (left side) and decreases that for the leading head (right side). Here, we assume that the internal load imposed between the two heads is equivalent to the plus-end loading for the trailing head and the minus-end loading for the leading head.

1 mM ADP, binding of kinesin to microtubule was weak, with an average unbinding force of ~3 pN (ref. 10). Here we demonstrate that, at various ADP concentrations, only the proportion of the weak and strong binding components differed, maintaining constant values of the unbinding force. Specifically, the proportion of the weak-binding component increases as the ADP concentration increases, irrespective of the loading direction (Fig. 2). The ADP concentration at which the weak and strong binding components become equal appears to be lower for the plus-end loading (~10 μM ADP, Fig. 2*a*) than for minus-end loading (~100 μM ADP, Fig. 2*b*).

Affinity for ADP depends on loading direction

Next, we quantify the correlation between the relative proportion of the weak binding component and the ADP concentration. This correlation could be fit by a hyperbola, in which an apparent dissociation constant of ADP in the kinesin–microtubule complex was determined. There is a seven-fold difference in the affinity of kinesin for ADP: 12.7 ± 2.4 μM for plus-end loading and 86.0 ± 17.1 μM for minus-end loading (Fig. 3). This result indicates that the ADP binding is regulated by the direction of loading.

We summarized the results obtained above in a schematic illustration (Fig. 4*a*). The dissociation constant of ADP in the kinesin–microtubule complex in solution (K, in the absence of external load) has been reported to be ~50 μM[18,19], which is between the values of K_+ and K_- obtained here (Fig. 3). Thus, the dissociation constant of ADP seems to be decreased by plus-end loading and increased by minus-end loading. This result presents direct evidence for the regulation of the ATPase cycle by an imposed load and demonstrates that the effect of load is directionally dependent. The coupling between enzymatic activity and force is the essence of a mechano-enzyme. It is interesting to note that the intracellular concentration of free ADP in a rat brain was reported to be 54–128 μM[20], which falls within the range between K_+ and K_- obtained here, suggesting that the regulation of binding affinity of ADP by loading may occur *in vivo*.

Internal load and processivity

Thus far, several published reports suggest that the ATPase cycle of molecular motors depends on the external load[21–24]. Hancock and Howard[13] first suggested the positive role of internal load based on the comparison of the processivity and the ATPase activity of two-headed conventional kinesin homodimers with those of one-headed heterodimers. Thus, the mechanism of processivity of both kinesin[13–17] and myosin V[23,25,26] is usually explained by assuming an internal load imposed between the two heads. With double-headed binding, the leading and trailing heads of kinesin are pulled toward the minus and plus ends of a microtubule, respectively, such that the binding affinity of ADP becomes lower for the leading head and higher for the trailing head (Fig. 4*b*). The conformation of the nucleotide-binding site is predicted to be asymmetrically distorted by the internal load. This property is in agreement with the hand-over-hand model, which assumes that the dissociation of ADP occurs more easily at the leading head than at the trailing head. Taking into account that the binding lifetime of an attached head becomes ~150× longer upon dissociation of ADP and the internal load is assumed to be 4 pN (ref. 10), the average lifetime of the attached state of leading head is estimated to be an order of magnitude longer than that of the trailing head. Thus, the internal load is a possible key factor for the processivity of kinesin motility. Direct evidence of the existence of internal load is the next challenging target in the biophysical research of molecular motors.

Methods

Proteins. Conventional two-headed kinesin homodimer and tubulin were prepared from bovine[27] and porcine brains[28], respectively. Both brain tissues were purchased from a local slaughterhouse. Polarity-marked microtubules labeled with tetramethyl-rhodamine succinimidyl ester (Molecular Probes) were prepared according to Hyman[29], except that *N*-ethylmaleimide–treated tubulin was not used so that polymerization at the minus end was not inhibited. We found that ~50% of the microtubules thus prepared were correctly marked (as shown in Fig. 2A of ref. 11), whereas almost all the others were block copolymers — that is, the plural nuclei strongly labeled with rhodamine existed in the same microtubules. These microtubules were not used for our experiments.

Unbinding force measurement. Kinesin-coated beads (1.0 μm in diameter, carboxylate-modified latex, Molecular Probes) were prepared according to the established procedure[27] with slight modifications. Kinesin molecules were mixed with the beads at a molar ratio of 2:1. The polarity-marked fluorescent microtubules in assay buffer (2 mM $MgCl_2$, 80 mM PIPES-KOH, pH 6.8, and 1 mM EGTA) were introduced into a flow cell and incubated for 2 min to allow binding to the glass surface. The solvent was exchanged three times with an assay buffer containing 0.7 mg ml^{-1} filtered casein to coat the glass surface with casein. The flow cell was then filled with an

assay buffer containing the kinesin-coated beads, filtered casein and an oxygen-scavenging enzyme system. The final solvent condition was ~0.1 pM kinesin-coated beads, 2 mM $MgCl_2$, 80 mM PIPES-KOH, pH 6.8, 1 mM EGTA, 0.7 mg ml^{-1} filtered casein, 10 μM taxol, 10 mM dithiothreitol, 4.5 mg ml^{-1} glucose, 0.22 mg ml^{-1} glucose oxidase, 0.036 mg ml^{-1} catalase and 1 U ml^{-1} apyrase (nucleotide-free state) or 1 mM ADP containing 1 U ml^{-1} hexokinase (ADP state). We were able to repeat the unbinding force measurements on the same microtubules several times on the same beads, presumably for the same kinesin molecules, by using optical tweezers for manipulation[10–12]. We confirmed that there was no significant difference in either the unbinding force distribution or binding properties regardless of the presence or absence of apyrase. Therefore, the denaturing of kinesin during measurement in the absence of nucleotides is unlikely. The average number of functional kinesin molecules on a bead was estimated to be one by statistical methods (considering the geometry of the kinesin on the bead, we estimate that only single kinesin molecules interacted with a microtubule in almost all the measurements)[5,27]. Global fits of unbinding force distribution to two Gaussian distributions were achieved by nonlinear optimization using SigmaPlot 8.0 (Windows)[24]. The microscopy system equipped with optical tweezers was as described[30]; the stiffness of the optical trap was estimated to be 0.05 pN nm^{-1} (ref. 30). All experiments were performed at 25 ± 1 °C.

Acknowledgments

We thank S.S. Rosenfeld and M. Chee for critical reading of the manuscript. We also thank D.D. Hackney and K. Kinosita Jr. for valuable discussion. This research was partly supported by Grants-in-Aid for Specially Promoted Research and for the Bio-venture Project from the Ministry of Education, Sports, Culture, Science and Technology of Japan. S.U. is a recipient of predoctoral fellowship of the Japan Society for the Promotion of Science.

Competing interests statement

The authors declare that they have no competing financial interests.

Received 6 November, 2002; accepted 10 February, 2003.

1. Vale, R.D., Reese, T.S. & Sheetz, M.P. Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* **42**, 39–50 (1985).
2. Brady, S.T. A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* **317**, 73–75 (1985).
3. Scholey, J.M., Porter, M.E., Grissom, P.M. & McIntosh, J.R. Identification of kinesin in sea urchin eggs and evidence for its localization in the mitotic spindle. *Nature* **318**, 483–486 (1985).
4. Hirokawa, N. Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* **279**, 519–526 (1998).
5. Svoboda, K. & Block, S.M. Force and velocity measured for single kinesin molecules. *Cell* **77**, 773–784 (1994).
6. Hackney, D.D. Evidence for alternating head catalysis by kinesin during microtubule-stimulated ATP hydrolysis. *Proc. Natl. Acad. Sci. USA* **91**, 6865–6869 (1994).
7. Rice, S. *et al*. A structural change in the kinesin motor protein that drives motility. *Nature* **402**, 778–784 (1999).
8. Vale, R.D. & Milligan, R.A. The way things move: looking under the hood of molecular motor proteins. *Science* **288**, 88–95 (2000).
9. Mandelkow, E. & Johnson, K.A. The structural and mechanochemical cycle of kinesin. *Trends Biochem. Sci.* **23**, 429–433 (1998).
10. Uemura, S. *et al*. Kinesin-microtubule binding depends on both nucleotide state and loading direction. *Proc. Natl. Acad. Sci. USA* **99**, 5977–5981 (2002).
11. Kawaguchi, K. & Ishiwata, S. Nucleotide-dependent single- to double-headed binding of kinesin. *Science* **291**, 667–669 (2001).
12. Kawaguchi, K., Uemura, S. & Ishiwata, S. Equilibrium and transition between single- and double-headed binding of kinesin as revealed by single-molecule mechanics. *Biophys. J.* **84**, 1103–1113 (2003).
13. Hancock, W.O. & Howard, J. Kinesin's processivity results from mechanical and chemical coordination between the ATP hydrolysis cycles of the two motor domains. *Proc. Natl. Acad. Sci. USA* **96**, 13147–13152 (1999).
14. Derenyi, I. & Vicsek, T. The kinesin walk: a dynamic model with elastically coupled head. *Proc. Natl. Acad. Sci. USA* **93**, 6775–6779 (1996).
15. Cross, R.A. *et al*. The conformational cycle of kinesin. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **355**, 459–464 (2000).
16. Crevel, I., Carter, N., Schliwa, M. & Cross, R. Coupled chemical and mechanical reaction steps in a processive *Neurospora* kinesin. *EMBO J.* **18**, 5863–5872 (1999).
17. Xing, J. *et al*. Kinesin has three nucleotide-dependent conformations. Implications for strain-dependent release. *J. Biol. Chem.* **275**, 35413–35423 (2000).
18. Ma, Y.Z. & Taylor, E.W. Kinetic mechanism of a monomeric kinesin construct. *J. Biol. Chem.* **272**, 717–723 (1997).
19. Ma, Y.Z. & Taylor, E.W. Interacting head mechanism of microtubule-kinesin ATPase. *J. Biol. Chem.* **272**, 724–730 (1997).
20. Askenasy, N. & Koretsky, A.P. Transgenic livers expressing mitochondrial and cytosolic CK: mitochondrial CK modulates free ADP levels. *Am. J. Physiol. Cell Physiol.* **282**, C338–346 (2002).
21. Visscher, K., Schnitzer, M.J & Block, S.M. Single kinesin molecules studied with a molecular force clamp. *Nature* **400**, 184–189 (1999).
22. Nishiyama, M., Higuchi, H. & Yanagida, T. Chemomechanical coupling of the forward and backward steps of single kinesin molecules. *Nat. Cell Biol.* **4**, 790–797 (2002).
23. Rief, M. *et al*. Myosin-V stepping kinetics: a molecular model for processivity. *Proc. Natl. Acad. Sci. USA* **97**, 9482–9486 (2000).
24. Schnitzer, M.J., Visscher, K. & Block, S.M. Force production by single kinesin motors. *Nat. Cell Biol.* **2**, 718–723 (2000).
25. Veigel, C., Wang, F., Bartoo, M.C., Sellers, J.R. & Molloy, J.E. The gated gait of the processive molecular motor, myosin V. *Nat. Cell Biol.* **4**, 59–65 (2002).
26. Spudich, J.A. & Rock, R.S. A crossbridge too far. *Nat. Cell Biol.* **4**, E8–E10 (2002).
27. Kojima, H., Muto, E., Higuchi, H. & Yanagida, T. Mechanics of single kinesin molecules measured by optical trapping nanometry. *Biophys. J.* **73**, 2012–2022 (1997).
28. Weingarten, M.D., Lockwood, A.H., Hwo, S.Y. & Kirschner, M.W. A protein factor essential for microtubule assembly. *Proc. Natl. Acad. Sci. USA* **72**, 1858–1862 (1975).
29. Hyman, A.A. Preparation of marked microtubules for the assay of the polarity of microtubule-based motors by fluorescence. *J. Cell Sci.* **s14**, 125–127 (1991).
30. Nishizaka, T., Miyata, H., Yoshikawa, H., Ishiwata, S. & Kinosita, K. Jr. Unbinding force of a single motor molecule of muscle measured using optical tweezers. *Nature* **377**, 251–254 (1995).