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The kinesin-8 Kip3 depolymerizes microtubules with a collective force-dependent mechanism

M. Bugiel[1], M. Chugh[1], T. J. Jachowski[1], E. Schäffer[1,*], and A. Jannasch[1,*]

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ABSTRACT Microtubules are highly dynamic filaments with dramatic structural rearrangements and length changes during the cell cycle. An accurate control of the microtubule length is essential for many cellular processes in particular, during cell division. Motor proteins from the kinesin-8 family depolymerize microtubules by interacting with their ends in a collective and length-dependent manner. However, it is still unclear how kinesin-8 depolymerizes microtubules. Here, we tracked the microtubule end-binding activity of yeast kinesin-8, Kip3, under varying loads and nucleotide conditions using high-precision optical tweezers. We found that single Kip3 motors spent up to 200 s at the microtubule end and were not stationary there but took several 8-nm forward and backward steps that were suppressed by loads. Interestingly, increased loads, similar to increased motor concentrations, also exponentially decreased the motors' residence time at the microtubule end. On the microtubule lattice, Kip3 had a different binding behavior suggesting that the observations are distinct for the microtubule end. The force dependence of the end residence time enabled us to estimate what force must act on a single motor to achieve the microtubule depolymerization speed of a motor ensemble. This force is higher than the stall force of a single Kip3 motor, supporting a collective force-dependent depolymerization mechanism. Understanding the mechanics of kinesin-8's microtubule end activity will provide important insights into cell division with implications for cancer research.

Key words: Kinesin; microtubules; optical tweezers; force feedback

STATEMENT OF SIGNIFICANCE

Kinesin-8 motors are important for microtubule length regulation and overexpressed in different types of cancer. Yet, on the molecular level, it is unclear how these motors depolymerize microtubules. Using high-precision optical tweezers, we measured how single yeast kinesin-8 motors, Kip3, interacted with the microtubule end. Interestingly, we found that single Kip3 motors were still motile at the microtubule end. The force dependence of how long single motors were associated with the microtubule end enabled us to estimate what force motors must exert onto each other to achieve the collective microtubule depolymerization speed of many motors. Our data support a collective force-dependent depolymerization mechanism. A better understanding of Kip3's microtubule end activity has implications for cell division and associated diseases.

INTRODUCTION

The mitotic spindle and the microtubule cytoskeleton are highly dynamic structures. During cell division, assembly and disassembly of the mitotic spindle is a crucial step. The mitotic spindle consists of cytoskeletal microtubules (MTs). Therefore, a proper control of the microtubule length is important. Microtubules are built of protofilaments—chains of tubulin dimers. These microtubules have a polar structure with a more dynamic plus end and a less dynamic minus end. There are many microtubule-associated proteins (MAPs) that are involved in MT polymerization (1), stabilization (2, 3), and depolymerization (4–6) and, thus, MT length control. Furthermore, members of the kinesin-8 family are involved in control of the MT architecture (7–10), knock-out mutants in budding yeast exhibit unnaturally long microtubules (6), and an over-expression in humans leads to cancer (11, 12). While high concentrations of the budding yeast kinesin-8 *Kip3* lead to collective, length-dependent microtubule depolymerization (5, 6, 13), individual motors at low Kip3 concentrations have been proposed to stabilize MT plus ends (14). The length-dependent depolymerization is explained by an antenna model and results from the combination of the microtubule depolymerase activity with the very high processivity and average run length of 12 µm (5, 15). Compared to conventional kinesin-1, Kip3 is slow and stalls already at a force of ≈1.2–1.5 pN (16, 17). Kip3 also has an additional MT-binding site at its tail that enables it to cross-link MTs (15) and together with its weakly-bound slip state (16) contributes to its extraordinarily long run length. However, how kinesin-8 depolymerizes microtubules is still not clear and under debate.

In one model, Varga et al. suggested that Kip3 depolymerizes microtubules with a *bump-off* mechanism (13) in which one Kip3 is waiting at the MT end until another Kip3 reaches the microtubule end and bumps the waiting Kip3 off the microtubule. The model suggests that the bumped-off Kip3 takes one to two tubulin dimers with it. This model is motivated by the observation that the long microtubule end residence time of single Kip3 decreases exponentially with higher Kip3 concentrations, accompanied by increased MT depolymerization. The bump-off model requires Kip3 to reach the MT end. While this feature can be well explained by Kip3's long run length and its suggested ability to bypass obstacles on the microtubule (17–20), the activity of Kip3 at the MT end, i.e. the depolymerization mechanism itself, is poorly understood.

In contrast, a more recent study by Arellano et al. proposed a different *switching* model (21). The authors measured that Kip3 has a higher affinity for curved tubulin protofilaments. In addition, once Kip3 is bound to curved tubulin dimers, the motor's ATPase activity is suppressed. Therefore, Kip3 accumulates at MT ends where single protofilament overhangs are curved and unstable and, thereby, promotes MT depolymerization through strong binding to curved protofilaments. This model is supported by the finding that monomeric, immotile Kip3 can promote MT depolymerization by binding directly to the microtubule end showing that motility is not required to reduce end residence times. However, the switching model does not rule out a cooperative bump-off model since high motor concentrations were used and motors, instead of walking to the last bound Kip3, may have bound directly next to the last motor out of solution. The observed switch of Kip3 into a non-motile state at the microtubule end raises the question how Kip3's binding state at the microtubule end differs from the state on the microtubule lattice under different nucleotide conditions. Moreover, the influence of loads on Kip3 at the microtubule end—that are expected to be present if large numbers of Kip3 push each other from the microtubule in a kind of traffic jam—is unknown.

Here, we used high-precision optical tweezers (22) in a two-dimensional force-feedback mode (23) to apply constant assisting and hindering loads along the microtubule axis on motile Kip3 while stepping towards and remaining at the microtubule end. We found that both the end residence time and the forward/backward motion at the microtubule end decreased exponentially with higher loads. Controls with Kip3 walking on the microtubule lattice and bound to microtubules in the presence of ADP and AMP-PNP, respectively, showed that the detachment mechanism from the microtubule end is distinct. Altogether, we propose that Kip3 depolymerizes microtubules with a collective, force-dependent mechanism requiring a detachment force of about 4–5 pN to effectively remove a tubulin dimer.

MATERIALS AND METHODS

Microtubule preparation

Porcine tubulin (3 µM) was polymerized in PEM buffer (80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$, pH = 6.9) with 1 mM $MgCl_2$ and 1 mM guanosine-5'-[(α,β)-methyleno] triphosphate (GMPCPP, Jena Bioscience, Jena, Germany) for 1.5 h at 37 °C. Afterwards, the GMPCPP-stabilized microtubules were diluted with PEM, spun down in a Beckman airfuge, and resuspended in PEM. If not noted otherwise, all chemicals were from Sigma. Microtubules were visualized with differential interference contrast employing a light emitting diode (LED-DIC) (24). For total internal reflection fluorescence (TIRF) assays, 10 % rhodamine-labeled tubulin was used to image microtubule growth.

Functionalized microsphere preparation

To specifically bind enhanced green fluorescent protein (eGFP)-tagged proteins, carboxylated polystyrene microspheres (mean diameter 0.59 µm, Bangs Lab., Fishers, USA) were coated covalently with a small fraction of anti-GFP nanobody (kindly provided by Ulrich Rothbauer, NMI, Reutlingen, Germany), surrounded by monofunctional PEG molecules (mPEG, 2 kDa, Rapp Polymere, Tübingen, Germany) in a ratio of $1 : 10^4$, based on protocols described in (25) and as depicted in Fig. 1A. The mPEG forms a polymeric brush that suppresses unspecific binding.

Sample preparation and assay

Experiments were performed in flow cells that were constructed using silanized, hydrophobic glass cover slips and parafilm as described before (19). Full-length budding yeast Kip3 (Kip3-eGFP-His_6) was expressed and purified according to (16, 26). The motility buffer for Kip3 stepping assays was PEM supplemented with 1 mM ATP, 0.1 mg/ml casein, 112.5 mM KCl, and an anti-fading mix (10 mM DTT, 20 mM glucose, 20 µg/ml glucose oxidase, 8 µg/ml catalase) (16, 19, 25). Experiments with motility buffer and different nucleotides apart from ATP used the same nucleotide concentration (1 mM) unless mentioned otherwise. Functionalized microspheres were mixed with motor proteins in motility buffer to a motor-to-microsphere ratio for which every third microsphere showed motility, implying single-molecule conditions with 95 % confidence (25, 27). The channels of flow cells were washed with PEM, filled and incubated successively with anti β-tubulin I (monoclonal antibody SAP.4G5 from Sigma in PEM), Pluronic F-127 (1 % in PEM), and microtubules in PEM. Finally, the kinesin-microsphere suspension was flowed in.

Optical tweezers setup

Measurements were performed in a single-beam optical tweezers setup combined with LED-DIC to visualize single micro-

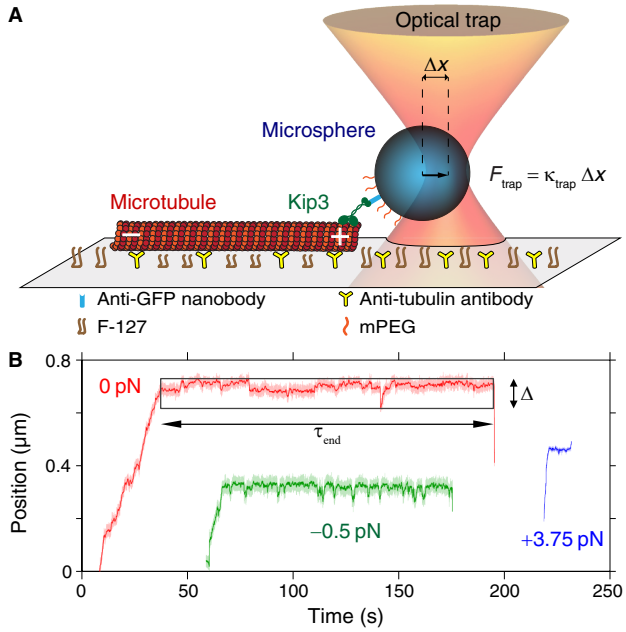

FIGURE 1 Probing the activity of Kip3 at the microtubule plus end using optical tweezers. (*A*) Schematic of the Kip3 stepping assay (side view, not to scale). Loads F were applied along the MT axis until Kip3 detached from the microtubule end. (*B*) Exemplary traces of Kip3 on the microtubule for three different loads offset for clarity (assisting and hindering loads are positive (blue) and negative (green), respectively). Motors walked to the microtubule plus end, stepped forward and backward after reaching it, and finally detached from the MT end. The end residence time τ_{end} was calculated as the time between the arrival at and detachment from the microtubule end. The end distance Δ was calculated as the difference between maximal and minimal position while the Kip3 was at the microtubule end.

tubules as described in (22, 24, 28). The setup is equipped with a millikelvin precision temperature control (22) set to 29.500 °C and a two-dimensional (2D) force clamp with a feedback rate of 1 kHz and a sampling rate of 4 kHz as described in detail in (23). Forces along the microtubule axis, are denoted by F. The optical trap was calibrated by a combined power spectral density–drag force method (29, 30). We used a lateral trap stiffness of 0.03–0.05 pN/nm with a laser power in the trapping focus of about 6–10 mW.

Tracking the activity of Kip3 at the microtubule plus end and on the microtubule lattice

Kip3 experiments were performed in the way described previously (16, 17, 19). Briefly, Kip3-coated microspheres were trapped and positioned on top of microtubules to await Kip3-initiated motility. When the motor started to pull on the microsphere, the 2D force clamp was switched on with assisting or hindering loads applied on the kinesin along the MT axis, denoted as positive and negative, respectively, as illustrated in Fig. 1A. The 2D feedback ensured that no sideward loads were applied. Kip3's zero-load speed of ≈55 nm/s and stall force of about 1.5 pN were consistent with previous results (16, 17, 19). Microsphere positions were processed with a running median filter over 500 data points. We recorded a total of 510 traces of 93 different single-Kip3-powered microspheres. We defined the arrival of Kip3 at the microtubule plus end as the time point when apparent directed microsphere motion stopped. By visual inspection of the traces and LED-DIC images, it was confirmed that indeed the microtubule end was reached. The time between arrival and detachment from the microtubule end was defined as end residence time τ_{end} (Fig. 1B). During this period, we determined the end distance Δ by first subtracting the minimal from the maximal filtered position. Based on a normal distribution, the minimal and maximal positions differed on average about three standard deviations from the mean. Therefore, we subtracted six standard deviations of the normally distributed position noise from the latter difference resulting in Δ. Note that hindering loads of less than −1 pN were not used for microtubule end tracking experiments because such loads are close to the stall force and Kip3 rarely if at all reached the MT plus end under these conditions. To measure microtubule depolymerization speeds, we repeatedly performed Kip3 runs to the microtubule end using the same microsphere on the same microtubule with the same starting position and continuous data acquisition for up to 10 min. Each run lasted on average about 1 min. For each run, the maximal position was considered as the position of the MT plus end. The slope of lines fitted to these MT end positions versus their corresponding time points resulted in the depolymerization speed. According to the defined geometry, depolymerization speeds were denoted as negative. As a control on the MT lattice, we measured the run time and run length of Kip3 as a function of assisting loads (243 runs with 19 microspheres). Since the mean run length of Kip3 at zero load exceeded the range of our force-clamp, we were only able to measure run times and lengths for loads ≥2.5 pN. For these loads, run lengths were sufficiently small. Included in the MT lattice analysis were runs that had slip events (16); excluded in the lattice analysis were runs in which Kip3 reached the MT end or walked out of the force-clamp range. In another control, we measured the attachment time of Kip3 (123 events of 17 microspheres) as a function of load in the presence of the non-hydrolyzable ATP analog adenosine-5'-[(β, γ)-imido] triphosphate (AMP-PNP, Jena Bioscience). With AMP-PNP, kinesins bind tightly to the MT lattice (31). For this assay, the motility buffer contained 1 mM AMP-PNP instead of ATP. Because the attachment times in the presence of AMP-PNP were very long, they could only be measured for loads ≥3 pN. Even for these loads, a few events exceeded the maximal measurement time or were initiated at a lower load and then due to the lack of detachment the load was increased. Using this final load or maximal measurement time, these events were included in the analysis. Thus, measured times may be an underestimation of the true attachment times. Without kinesin motility in the presence of AMP-PNP, the polarity of the used microtubules was unknown. Therefore, loads were applied to Kip3 on the same microtubule repeatedly in both directions

along the MT axis until the Kip3-coated microsphere detached. For a load of 4.25 pN, we did not find a significant asymmetry between the attachment times as a function of load directions on a single microtubule (Mann-Whitney U-test, $p = 0.36$, number of attachment times for each direction $N = 15$). Thus, attachment times for both load directions were pooled and analyzed together.

TIRF stepping assays as control

As a control for Kip3 activity in the absence of load, we measured the end residence time, run time and run length of single GFP-tagged Kip3 in a TIRF microscope on rhodamine-labeled microtubules as described in (25). The mean end residence time was 80 ± 10 s (standard error if not noted otherwise, $N = 58$), the mean run time was 150 ± 30 s ($N = 26$), and the mean run length was 12 ± 1 µm ($N = 47$). The end residence time, run time, and run length were consistent with literature values (5, 13, 15). Because the GFP nanobody has a K_d of about 0.6 nM (32), we expect that in thermal equilibrium and under single-molecule conditions some of the Kip3 motors are not bound to the GFP-nanobody–functionalized microspheres, but are free in solution. These free motors are not seen in the optical trapping assay but may contribute to MT depolymerization. Therefore, we used the Kip3-microsphere motility suspension and measured the Kip3 flux to the microtubule plus end in a TIRF microscope. By counting the number of Kip3 run events to the microtubule plus end per time and microtubule, we measured a flux of about 0.018 Kip3/min/MT. Thus, only about once per hour a free Kip3 motor might contribute to MT depolymerization in the optical tweezers assay. Therefore, we can rule out a significant effect of free motors during trapping assays.

Spontaneous microtubule depolymerization

Spontaneous depolymerization of the microtubule plus end of immobilized, GMPCPP-stabilized microtubules was measured by image analysis of long-term videos recorded with LED-DIC microscopy over >1 h. Images were analyzed with Fiji.

RESULTS

Kip3 was motile at the microtubule end taking 8-nm forward and backward steps

To determine the activity of Kip3 at the microtubule end, we tracked microspheres functionalized with single motors as a function of time while applying constant loads with the optical tweezers (Fig. 1A). To this end, we first placed motor-coated microspheres several micrometers away from the end of the microtubules and engaged a 2D force clamp (23). Conveniently, due to the high processivity of Kip3, motors automatically walked to and localized at the MT plus end with nanometer precision. Surprisingly, once at the microtubule end, microspheres did not stop but continued a slow forward and backward movement before finally detaching from the microtubule (Fig. 1B). Apart from this slow motion and also during the approach to the microtubule end, we observed motor slipping in the direction of loads consistent with previous results (16). During the end residence time, we determined the difference between maximal and minimal mean position that we call the end distance Δ (Fig. 1B). In the absence of loads and for hindering loads, this distance was on the order of 100 nm and showed a large variation that we attribute to slip events (Fig. 2A). With assisting loads, the end distance decreased and reached a constant value of 7.8 ± 0.8 nm ($N = 6$) suggesting discrete, single 8-nm, center-of-mass steps typical for kinesin motors. Indeed, with a magnified view of the end motion, discrete steps are visible (Fig. 2B). Based on a histogram analysis, the motion—including normal slow steps and fast slipping events—occurred in multiples of about 8 nm (Fig. 2B and Fig. S1). While there was forward and backward motion at the microtubule end, there was no net bias to the motion. The mean velocity at the microtubule end was not significantly different from zero (confirmed by a χ^2-test, 0.2 ± 0.1 nm/s, $N = 15$, Fig. S2). Therefore, microtubules were

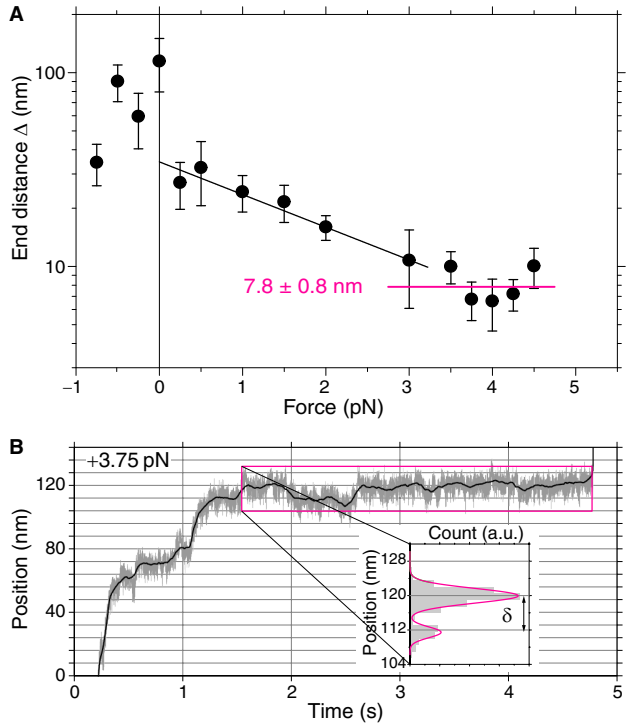

FIGURE 2 End distance of forward/backward motion of Kip3 at the MT plus end decreased with load. (*A*) End distance Δ (full black circles) as a function of load with exponential fit (black line). Error bars are standard errors. The horizontal magenta line refers to a weighted mean of end distances for loads >3 pN with weighted mean and standard error given. (*B*) Exemplary trace of Kip3 stepping to the microtubule plus end showing forward and backward steps during the end residence. Inset: Histogram of filtered position at the microtubule end with multiple Gaussian fits (magenta line). The step size δ is defined as difference between the peak positions of the Gaussians.

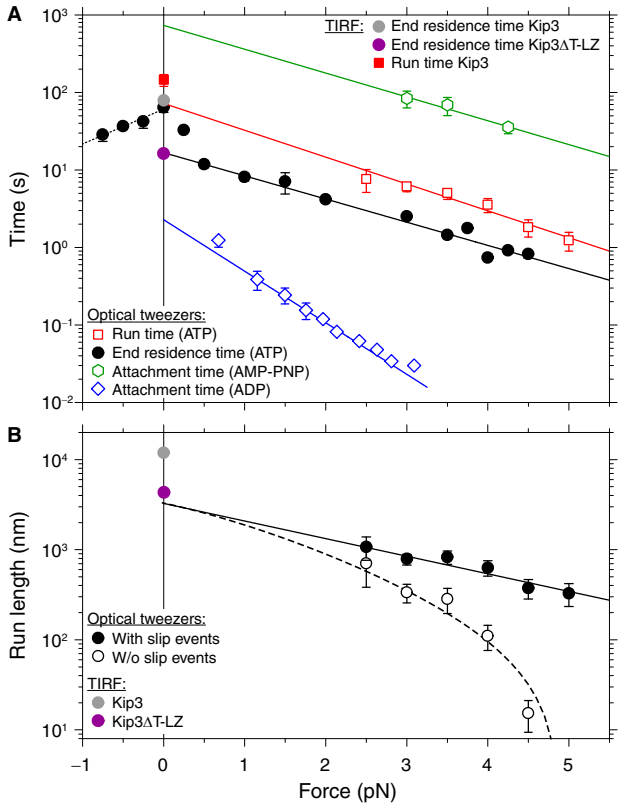

FIGURE 3 Microtubule end residence time of Kip3. (*A*) End residence time in ATP (*black circles*) with exponential fits for hindering loads (*black dotted line*) and assisting loads >0.5 pN (*black line*), run time on the MT lattice in ATP (*open red squares*) with exponential fit (*red line*), attachment time in AMP-PNP (*open green hexagons*) with exponential fit (*green line*), and attachment time in ADP (*open blue diamonds*) with exponential fit (*blue line*). For the last two measurements, data for assisting and hindering loads have been pooled and plotted together. In the absence of load, mean end residence time (*grey circle*) and run time (*red square*) from TIRF measurements together with the literature value for the end residence time of truncated Kip3ΔT-LZ (*purple circle*, (14)) are plotted. (*B*) Run length (*black circles*) of Kip3 as a function of load with exponential fit (*black line*) together with the zero-force run length measured in TIRF assays (*grey circle*) and run lengths from the literature (14) for truncated Kip3ΔT-LZ (*purple circle*). From the original run length data that included slipping events, the average cumulative slip distance from (16) was subtracted and plotted as *open black circles* together with a model (*black dashed line*, see text). All error bars are SEMs.

on average neither growing nor shrinking during the residence time. Together, before detachment, Kip3 was still motile at the MT end taking several 8-nm forward and backward steps with no net motion.

The end residence time of Kip3 exponentially decreased with assisting loads

Since the end residence time of free, single Kip3 motors decreased exponentially with increasing motor concentration (13), we asked the question whether this crowding effect is due to forces that motors exert onto each other as suggested by the bump-off model and whether we can achieve the same effect by applying an external load on a single Kip3 at the microtubule end. Thus, we measured the end residence time as function of load. For all loads, the end residence time was exponentially distributed (see Fig. S3 for examples). The mean end residence time of 64 ± 9 s for Kip3 without load was consistent with the TIRF microscopy control and literature values (13–15). Load decreased end residence times (Fig. 3A, black circles). Intriguingly, only for assisting loads larger than about 0.5 pN, end residence times decreased exponentially. Based on fits using the Bell model $\tau_{\text{end}}(F) = \tau^0_{\text{end}} \exp\left[-|F|\, x^{\ddagger}/(k_B T)\right]$, we determined the distance to the transition state $x^{\ddagger}$ for assisting and hindering loads to be $x^{\ddagger}_{+} = 2.9 \pm 0.1$ nm and $x^{\ddagger}_{-} = 4.3 \pm 0.6$ nm, respectively, where k_B is the Boltzmann constant and T is the absolute temperature. The transition distance is about half the size of a tubulin dimer. Thus, with a distance of ≈4 nm and the thermal energy $k_B T$ at room temperature being about 4 pNnm, a force of 1 pN reduced end residence times about e-fold. An overview of all measured transition distances $x^{\ddagger}$ is given in Table S1. To test whether sideward loads also reduced the end residence time, we analyzed data from previous side-stepping assays (19). Without any load applied along the MT axis, but with a sideward load of 0.5 pN, we measured an end residence time of 26 ± 4 s (SEM, $N = 14$, 10 microspheres). This time is significantly smaller than the unloaded case (confirmed by a Mann-Whitney U-test, $p = 0.027$), comparable to the one of hindering loads that have the same magnitude, but larger compared to the equivalent assisting loads. Thus, force acting in the direction of microtubule growth had the largest effect. Together, the load dependence of the end residence time suggests that removal of tubulin dimers is facilitated by force.

Kip3 detachment from the MT end was different from MT lattice

To test whether force-induced detachment is faster at the microtubule end compared to the lattice, we investigated how the attachment time of Kip3 at the microtubule end differed from the one on the lattice. Therefore, we measured the run time in the presence of ATP as a function of assisting load on the microtubule lattice (open red squares in Fig. 3A). We found that also the mean run times on the MT lattice decreased exponentially with increasing assisting load. In this case, the distance to the transition state was $x^{\ddagger}_{+} = 3.3 \pm 0.4$ nm comparable to the one of the end residence time. However, at the same load, the run times themselves were significantly longer compared to the end residence times suggesting that the motor detachment mechanism from the lattice differed from the one at the microtubule end. Interestingly, the run times on the lattice extrapolated roughly to the zero-force end residence time and run time on the lattice. According to the switching model (21), the ATPase activity at the microtubule end is suppressed. Thus, we expect that on a curved tubulin dimer at the MT end, at least one of the kinesin heads remains

in a strongly bound ATP state (33). To mimic this strongly bound state, we measured the attachment time of Kip3 on the MT lattice in the presence of AMP-PNP—a non-hydrolyzable nucleotide analog of ATP—as a function of load (open green hexagons in Fig. 3A). The exponential force dependence resulted in a distance to the transition state of $x^{\ddagger} = 3.0 \pm 0.4$ nm again similar to the other cases above. More importantly, the AMP-PNP attachment times were more than an order of magnitude longer compared to the run times and end residence times in ATP. Finally, to show that Kip3 does not slip from the MT end in the weakly-bound ADP state, we compared the end residence times with the attachment times of Kip3 on the MT lattice in the presence of ADP (open blue diamonds in Fig. 3A). For this purpose, we reanalyzed data from Bormuth *et al.* (34), where Kip3 was pulled over microtubules in the presence of 2 mM ADP. Also, these attachment times decreased exponentially with assisting load. For ADP, the distance to the transition state of $x^{\ddagger} = 6.4 \pm 0.5$ nm was significantly larger compared to the other cases. Strikingly, the attachment times were at least an order of magnitude shorter than the end residence time. However, even if Kip3 slipped in a weakly-bound state to the microtubule end, motors remained there without instantaneous detachment (Fig. S4). Together, the data in different nucleotide states suggest that the accelerated Kip3 end residence time is not due to sliding off of the microtubule end, but due to force that is acting through the strongly bound Kip3 motor to break off tubulin dimers from the microtubule end.

Repeated microtubule end tracking on the same microtubule is consistent with motor-induced tubulin removal

To test whether we could measure depolymerization due to single-motor microtubule end activity, we repeatedly tracked the same Kip3-coated microsphere on the same microtubule (Fig. S5A). We observed that the maximal position along the microtubule—i.e. the position of the MT end—decreased over time. Based on a linear fit to the maximal positions over time, we determined the depolymerization speed. Over many events, the mean depolymerization speed was -0.30 ± 0.02 nm/s ($N = 56$, Fig. S5B) consistent with the literature value of ≈ -0.25 nm/s (13). However, the value differed from our internal control measured under identical conditions in the absence of Kip3. By tracking microtubule ends using LED-DIC (24), the spontaneous depolymerization speed was -0.20 ± 0.01 nm/s ($N = 137$, Fig. S5C) not affected by the trapping laser at the used power. While Kip3 increased the depolymerization speed, we cannot directly deduce how many tubulins were removed per dissociation event because we do not know the number of protofilaments the motor interacted with in different runs. The typical time between dissociation events was about 1 min (Fig. S5A) resulting in a measured motor-induced length decrease per event of approximately $0.1\ \text{nm/s} \times 60\ \text{s} = 6$ nm. If in each run the motor would have interacted with the same protofilament, this distance would correspond roughly to the removal of one tubulin dimer. However, if the motor interacted with a few protofilaments—lets assume the top three—than for each event on average $3 \times 6\ \text{nm} = 18$ nm corresponding to about two tubulin dimers would have to be removed. The more protofilaments are considered, the more tubulin dimers have to be removed per event. Despite this uncertainty, the data is consistent with a motor removing a few tubulin dimers per event distributed over a few protofilaments most likely the ones on the top.

The MT run length of Kip3 decreased exponentially with load

To gain further insight into the detachment process, we also measured the load-dependent Kip3 run length on the MT lattice (Fig. 3B). We found that also the mean run length decreased exponentially with assisting loads. The distance to the transition state was $x_{+}^{\ddagger} = 1.9 \pm 0.4$ nm, smaller compared to all other transition distances. The extrapolated zero-load run length of 3.3 ± 1.0 µm was significantly shorter compared to the directly measured one (TIRF microscopy and literature values on the order of 10 µm) but comparable to the value of 4.3 ± 0.2 µm reported for a Kip3 mutant, Kip3ΔT-LZ, that lacks the tail microtubule binding site (14). The agreement suggests that high loads dissociate the second microtubule binding site from the MT such that the force-dependent run length is not influenced by this site and extrapolates to the zero-load run length of the mutant that lacks this site. During run events, we observed slip events consistent with previous work (16). Even though slips last only for a few milliseconds (16), the slip distances accumulate to a significant amount during a run. We estimated the mean cumulative slip distance based on a multiplication of the force-dependent slip distance and frequency (16) with the fitted run time (solid line in Fig. 3B). When we subtracted this cumulative slip distance from the mean run length, the slip-corrected run length (dashed line in Fig. 3B) was more sensitive to force confirming the notion that the slip state acts as a molecular safety leash (16). The strong force dependence also suggests that motors on the lattice subjected to high assisting loads mostly translocate in the weakly-bound slip state and runs are consequently also terminated via this slip state in contrast to the observation that motors did not slip off the microtubule end (Fig. S4).

DISCUSSION AND CONCLUSION

At microtubule plus ends, we tracked the activity of single Kip3 motors with high precision under varying loads. Interestingly, Kip3 was still motile at the microtubule end taking several 8 nm forward and backward steps before detachment. The distance that the motor explored at the microtubule end decreased with applied loads, whereby the reduction was larger for assisting loads compared to hindering loads. For hindering loads exceeding the stall force, motors did not reach the

microtubule end. The reduction for assisting loads is expected since they suppress backward motion and the microtubule end terminates forward-biased slip events. Surprisingly, even at assisting loads that were in magnitude significantly above the stall force of single motors, Kip3 still took single 8-nm center of mass steps against the force. Since such steps were not observed on the MT lattice, the observation suggests a change in the binding affinity at the microtubule end consistent with the switching model (21). The role of the microtubule end exploration is unclear, but might enable sideward stepping (17, 19) to find a protofilament that protrudes beyond the last helix of tubulin dimers that all have neighbors (Fig. 4). Since such a protruding protofilament lacks stabilization through lateral tubulin bonds it is curved and might represent the "weakest" protofilament most suitable for depolymerization. If curved tubulins suppress ATPase activity according to the switching model, motors should not translocate beyond the first encountered curved tubulin dimer. Thus, Kip3 may not reach the end of a long curled protofilament. Instead, the front motor head should be in the ATP state and strongly bound to the first curved tubulin dimer. According to the consensus kinesin stepping model (33), the rear head should have initially ADP bound. Since hydrolysis in the front head is suppressed (21), ADP in the rear head might eventually be released leading to a rigor bound rear head. High assisting loads might induce stepping of the motor with both heads being in a strongly bound state (35). Another possible explanation for the steps at high forces, might be occasional ATP hydrolysis accompanied by nucleotide exchange.

Loads decreased the end residence time of motors. For low assisting loads up to about 0.5 pN, the sensitivity to force was much larger compared to hindering loads of the same magnitude or higher assisting loads (Fig. 3A). This asymmetry may be due to the second microtubule binding site in the tail of Kip3. This site could still be in contact with the MT lattice for hindering and small assisting loads but not for high assisting loads. In the latter case, the Kip3 tail is most likely pulled away and beyond the end of the microtubule. Because the end residence times were generally smaller for assisting loads, this binding site may contribute to the long Kip3 microtubule end dwelling consistent with previous work (14). In support of this interpretation, the reported zero-load end residence time of Kip3 that lacks the tail microtubule binding site of 16.3 ± 0.6 s (14) is in excellent agreement with our high-assisting-load data extrapolated to a zero-load end residence time of 17 ± 2 s (Fig. 3A). Together with the extrapolation of the run length to the zero-force run length of the Kip3ΔT-LZ mutant (Fig. 3B), these findings indirectly confirm the relevance of the tail MT-binding site for Kip3's high processivity and end residence.

Is Kip3's microtubule end activity different from the normal stepping mechanism? To answer this question, we compared the end residence time with run times on the microtubule lattice. The run times of Kip3 on the lattice were consistently higher than end residence times at the same loads

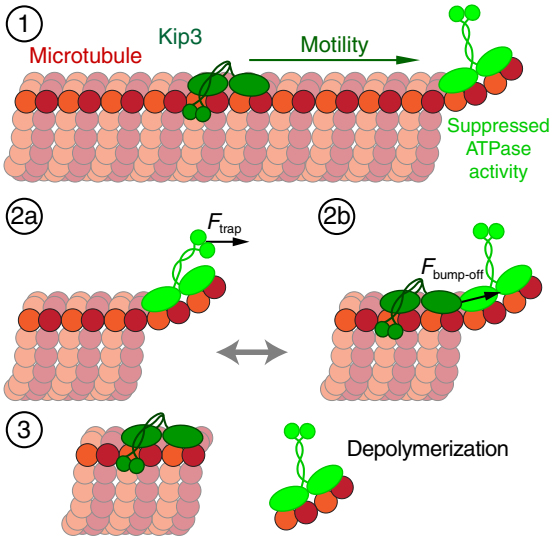

FIGURE 4 Model of collective, force-dependent microtubule depolymerization. (1) Kip3 (dark green) walks to the MT plus end with its processivity enhanced by the second microtubule binding domain in the tail and in contact with the microtubule lattice. At a curved single protofilament overhang, Kip3 switches to a strongly bound state with suppressed ATPase activity (bright green motor). A Kip3-tubulin complex may be removed either (2a) through an external force F_{trap}, e.g. applied via an optical trap or equivalently (2b) through an incoming Kip3 that applies an internal "bump-off" force $F_{bump\text{-}off}$. The second microtubule binding domain of the last motor on a protruding protofilament may not have access to the microtubule lattice or be "bumped-off" as well. (3) The strongly bound Kip3 was detached removing 2 tubulin dimers.

with a similar distance-to-transition-state. Shorter end residence times imply a mechanism at the microtubule end that is different from the motility mechanism on the lattice. This finding is consistent with the switching mechanism (21)—depolymerization by Kip3 does not require motile Kip3 but is coupled to a switch in the binding state with suppressed ATPase activity. In our experiments, we cannot distinguish whether motors at the microtubule end simply detached from the microtubule or whether a motor-tubulin complex dissociated. Based on the long attachment times in AMP-PNP and the low off-rate of the GFP-nanobody interaction (32), we do not expect that the GFP-tagged motor dissociated from the GFP nanobody or the microtubule during microtubule end events. If we assume that motors have switched to an ATP-bound, but hydrolysis incompetent state, motors should be strongly bound (Fig. 4). Therefore, we conclude that motors dissociated in a complex with tubulin and that force accelerated the breaking-off of tubulin-tubulin bonds. How many tubulin dimers are removed per dissociation event is unclear. In the absence of ATP hydrolysis, we expect that eventually the second head dissociates its bound ADP. Then, both heads are in a strongly bound state and the dissociated complex should contain at least two dimers. Potentially, more dimers might be removed because motors, according to the switching model, will stop at the encounter of the first curved tubulin dimer. Thus, if the

curled protofilament extends beyond that dimer all of those tubulins might be removed along with the motor. On the other hand, if many motors are present at the microtubule end, we do not expect many long protruding curved protofilaments. Also, there might not be sufficient time for ADP dissociation such that only one head is strongly bound and consequently only one dimer is removed per motor consistent with our estimation of a few removed dimers per motor and the reported 1–2 dimers/motor (13). A direct observation of tubulin removal by Kip3 remains an open experimental challenge.

Since both load and increasing motor concentration accelerates microtubule end detachment, we can ask what load on a single motor has the same effect as having many motors at the microtubule end that depolymerize the microtubule collectively. According to the bump-off model (13), incoming Kip3 molecules exert such a force on a microtubule end-waiting Kip3 resulting in the dissociation of a Kip3-tubulin complex. Therefore, we calculated the average time needed to remove a tubulin dimer. Based on the maximal Kip3-induced microtubule depolymerization speed of about 15 nm/s (13) or 8 nm/s for the mutant that lacks the second MT binding site (14), this time is ≈0.5–1 s (τ_{end} = 8 nm / depolymerization speed). Based on the exponential decay of the end residence time for assisting loads, this removal time corresponds to a force of ≈4–5 pN, the lower force limit corresponding to the depolymerization rate without the second microtubule binding domain. This force range is much higher than the stall force of a single Kip3 suggesting that a single motor cannot break-off tubulins. While multiple motors can generate higher forces (17), it is unclear how such an augmented force might be transmitted to the last motor and contribute to the depolymerization. Instead, we expect that a single motor should stabilize the last dimer through the additional connection via the motor's neck linker to the previous tubulin dimer. If a second motor binds directly behind—either through its own motility or as in (21) as a monomer out of solution, steric hinderance—as suggested by structural modelling (18)—in particular, for a curved protofilament may generate sufficient force to bump-off the front motor-tubulin complex (Fig. 4). One requirement for the bump-off is that the motor heads are in a strongly bound state. Otherwise, heads might be only "bumped off" of tubulin resulting in a motor detachment without tubulin removal. This requirement seems plausible based on the current kinesin stepping model (33) and the Kip3 switch mechanism (21). One additional aspect of the search for the "weakest" tubulin at the microtubule end and the bump-off model, in particular, at high concentrations that imply a kind of "traffic jam", is that the second microtubule binding site of the last Kip3 might also be displaced from the microtubule, disconnecting the last, strongly bound Kip3 from the remaining microtubule lattice (Fig. 4). Thus, we conclude that both the switching and bump-off mechanism contribute to microtubule depolymerization.

In summary, the force dependence of the end residence time suggests a combination of the bump-off model (13) with the switching model (21) that also accounts for the binding states of the individual motor heads. Our combined model suggests that a single motor at the microtubule end—most likely having two strongly bound heads together with a tether to the remaining lattice via its second microtubule binding site—stabilizes microtubules. For efficient dissociation of a motor-tubulin complex, another motor is required to apply a force on the last motor. While the amount of force that is acting between an individual pair of consecutive motors will always be the same, the effective time-averaged force will depend on the motor concentration and, therefore, on the motor arrival rate at the end. In this sense, the concentration dependence of the end residence time is equivalent to its force dependence. Thus, collectively microtubules can be efficiently depolymerized in a force-dependent manner.

SUPPORTING MATERIAL

Supporting material is available at
http://www.biophysj.org/biophysj/supplemental/XXX

AUTHOR CONTRIBUTIONS

M.B. and A.J. performed measurements and analyzed the data. M.C. expressed and purified the Kip3. T.J.J. programmed the force clamp control software. M.B., A.J., and E.S. wrote the manuscript.

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