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Recognition of the Nucleotide-Dependent Feature Facilitates the Microtubule End-Binding of MCAK

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Abstract

The growing plus-end is a key regulatory site for microtubule dynamics. MCAK (mitotic centromere-associated kinesin), a microtubule depolymerizing kinesin, is an end-binding regulator of catastrophe frequency. It is intriguing how MCAK specifically binds to dynamic microtubule ends. Here, we measure the end-binding kinetics of MCAK using single-molecule imaging and reveal the end-binding preference. MCAK binds to the entire GTP cap, including the EB cap and the distalmost cap. Further analysis shows that MCAK strongly binds to GTPγS microtubules, suggesting that it could recognize the nucleotide-dependent feature of microtubules. Moreover, the binding preference is independent on the nucleotide state of MCAK, and this feature facilitates the high-affinity end-binding of MCAK. Finally, we show that despite partially sharing the binding regions, MCAK and XMAP215 function in an additive manner, demonstrating a simple logic of how the end-binding regulators work in co-ordination. In all, our results provide novel insights into understanding how MCAK regulates the dynamics of microtubule ends.

eLife assessment

This work presents **valuable** new information on the microtubule-binding mode of the microtubule kinesin-13, MCAK. The authors use quantitative single-molecule studies to propose that MCAK preferentially binds to a GDP-Pi-tubulin portion of the microtubule end. However, the evidence provided to support this claim remains **incomplete** and would benefit from a more rigorous methodology. Additionally, the physiological relevance of the proposed binding mode remains speculative.

Introduction

Microtubule is a major type of cytoskeleton in eukaryotic cells and supports many cellular processes, such as cell division, polarization and migration (Alberts, 2015). However, the properties of microtubules vary a lot in different cellular processes and structures (Janke and Magiera, 2020; Kapitein and Hoogenraad, 2015; Wittmann et al., 2001). The plasticity implies that cellular microtubules are under complex and accurate regulations. Because

microtubule-associated proteins (MAPs) are important components in the regulatory networks of microtubules, their functions and working mechanisms are key to understand cellular regulations on microtubules.

Not only do kinesin superfamily members move cargoes along microtubules, they also act as regulators of microtubule dynamics, for example, the depolymerizing kinesins (e.g. the kinesin-8 and kinesin-13 family members). Kinesin-13 was initially identified in studying spindle functions and neurons (Aizawa et al., 1992; Noda et al., 1995; Walczak et al., 1996; Wordeman and Mitchison, 1995). Later, the functions of kinesin-13 were revealed in more cellular processes, including spindle assembly (Walczak et al., 2002), chromosome segregation (Kline-Smith et al., 2004), directional migration (Zong et al., 2021), ciliary length control (Piao et al., 2009; Vasudevan et al., 2015), and neuronal polarization (Ghosh-Roy et al., 2012; Puri et al., 2021). The loss of kinesin-13 often alters structural and dynamic properties of cellular microtubules, implying that the kinesin-13 class members play regulatory roles. In addition, the subcellular localizations of kinesin-13, such as growing microtubule ends, centrosome and centromere, are active regulatory sites of microtubule dynamics in cells, further suggesting the functions of the kinesin-13 class members in controlling the length, mass and polarity of microtubules in cells.

As a representative model for the kinesin-13 family, MCAK was shown to be a microtubule depolymerase and a catastrophe factor (Desai et al., 1999; Gardner et al., 2011; Hunter et al., 2003). Biochemical and structural analysis suggests that MCAK could deconstruct the protective end structure of dynamic microtubules, and it does so by bending and then breaking off the underlying substrate (i.e. terminal tubulin dimers or protofilaments) (Moores and Milligan, 2006). It was proposed that MCAK targets microtubule ends by rapid lattice diffusion or binding to the end-binding proteins (EBs) (Gouveia et al., 2010; Helenius et al., 2006; Honnappa et al., 2009). Furthermore, the direct binding of MCAK to growing microtubule ends was also observed (Gouveia et al., 2010), implying the presence of specific end-binding sites. Previous studies on the structures of MCAK, propose that MCAK targets to microtubule ends by recognizing the curved protofilaments (Asenjo et al., 2013; Benoit et al., 2018; Tan et al., 2008; Trofimova et al., 2018; Wang et al., 2017). This was mostly based on the findings on stabilized microtubules, short protofilaments or tubulin dimers. It still remains elusive how MCAK binds to dynamic microtubule ends.

In the present study, we characterize the direct binding of MCAK to growing microtubule ends using single-molecule imaging. The key finding is that MCAK binds to the entire GTP cap, including both the EB cap, where GDP·Pi-tubulin dimers are thought to gather, and the distalmost cap, where XMAP215 binds to curved protofilament (mostly in GTP state). The high-affinity binding of MCAK to the EB cap was intriguing. Further analysis shows that MCAK strongly binds to GTPγS microtubules, thought to be the analogue of GDP·Pi-tubulins. Therefore, we think that MCAK could recognize the nucleotide-dependent feature at dynamic microtubule ends. Moreover, the mutant studies show that the binding-preference of MCAK for GDP·Pi-tubulins facilitates the end-binding and in turn the function of MCAK. Finally, we find that despite having partially overlapped binding regions, MCAK and XMAP215 could precisely specify their regulatory function at dynamic microtubule end, thereby providing novel insights in understanding how microtubule-associated proteins functionally orchestrate at growing microtubule ends.

Results

MCAK shows a binding preference for growing microtubule ends

We set off by measuring the single-molecule binding kinetics of MCAK at growing microtubule ends using the *in vitro* microtubule dynamics assay. This experiment was performed in BRB80 supplemented with 50 mM KCl and 1 mM ATP, providing a nearly physiological ion strength. In

this condition, the individual binding events of MCAK can be observed at both the end and the lattice (**Fig. 1A**). By measuring the fluorescence intensity of these binding events, we confirmed that they reflected individual MCAK dimers (**Fig. s1**). Using an in-house software (Song et al., 2020), we determined the binding position of individual MCAK molecules at subpixel resolution by fitting a Gaussian function to their intensity profile along the long axis of microtubules (**Fig. 1B**). Using the position of the plus-end as the spatial reference, we overlaid the end-binding of 152 MCAK molecules (**Fig. 1C**). The distribution showed that the binding regions of MCAK had a length of 162 nm (FWHM, the Full Width at Half Maximum), about 20 layers of tubulin dimers. We defined the binding events of MCAK within this region as "end binding" and those on the lattice as "lattice binding" (**Fig. 1A**).

To understand the binding kinetics, we first compared the apparent association rates of MCAK on the plus-end ($k_{on\text{-}P}$) and lattice ($k_{on\text{-}L}$) in the presence of ATP. The ratio ($R_{E/L}$) of $k_{on\text{-}P}$ ((22. 6 ± 1.4) $\times 10^{-6}$ s^{-1} nM^{-1}, per tubulin dimer, hereinafter) and $k_{on\text{-}L}$ ((2.8 ± 0.3) $\times 10^{-6}$ s^{-1} nM^{-1}) was 13 ± 1 (n=66 microtubules from 3 assays) (**Fig. 1 D-E**). We then measured the dwell time, which reflects the dissociation rate (k_{off}) (**Fig. s1**). The mean dwell time of MCAK on the lattice was shorter than that at the plus-end (plus end: 0.75 ± 0.02 s, n=966 events from 3 assays; 0.62 ± 0.02 s, n=702 events from 3 assays, p<0.001) (**Fig. 1F**). Using these kinetic parameters, we calculated the dissociation constant (K_d) of MCAK for the plus-end ($K_{d\text{-}P}$ =69 μM) and lattice ($K_{d\text{-}L}$=1057 μM), respectively. Therefore, MCAK shows a clear end-binding preference. We also observed single-molecule binding events of MCAK to the minus-end (**Fig. 1A**). Given the higher physiological relevance, we focused on the plus-end binding events (referred to as "end-binding" hereinafter) in the following analysis.

To understand the influence of the nucleotide state, we measured the end-binding kinetics of MCAK·AMPPNP, MCAK·ADP or MCAK·APO (the nucleotide-free state). First, the $k_{on\text{-}P}$ and $k_{on\text{-}L}$ of MCAK·AMPPNP were both reduced in comparison to that of MCAK·ATP (**Fig. 1 D-E**), resulting in an end-binding preference ($R_{E/L}$=11 ± 2, n=29 microtubules from 2 assays, p>1) that is similar to that of MCAK·ATP. Mean-while, the end-binding of MCAK·AMPPNP showed a longer dwell time (3.32 ± 0.24 s, n=231 events from 2 assays, p<0.001), indicating a lower k_{off} (**Fig. 1F and Fig. s1**). As a result of the proportionally reduced $k_{on\text{-}P}$ and k_{off}, the end-binding affinity ($K_{d\text{-}P}$=61 μM) of MCAK·AMPPNP was similar to that of MCAK·ATP. Second, MCAK·ADP showed a nearly unchanged $k_{on\text{-}P}$ and an increased $k_{on\text{-}L}$, leading to a smaller $R_{E/L}$ (2 ± 0.3, n=21 microtubules from 2 assays, p<0.001) and suggest a reduced, but not the absence of, end-binding preference (**Fig. 1 D-E**). Meanwhile, it showed a significantly shorter end-binding dwell time (0.46 ± 0.02 s, n=327 events from 2 assays, p<0.001) than MCAK·ATP (**Fig. 1 F**). Therefore, MCAK·ADP has a lower end-binding affinity ($K_{d\text{-}P}$=163 μM). Third, APO·MCAK showed an increase in $k_{on\text{-}P}$ and an even greater increase in $k_{on\text{-}L}$, thereby having a smaller $R_{E/L}$ (2 ± 0.2, n=36 microtubules from 3 assays, p<0.001) (**Fig. 1 D-E**). It also showed a significantly longer end-binding dwell time (1.89 ± 0.23 s, n=71 events from 3 assays, p<0.001) (**Fig. 1 F**), suggesting a lower k_{off} (**Fig. s1**). Therefore, in comparison to MCAK·ATP, MCAK·APO possesses a higher end-binding affinity ($K_{d\text{-}P}$=1.8 μM) but partially loses the end-binding preference. Based on these measurements, we conclude that the end-binding preference and affinity of MCAK depend on its nucleotide state (see **Discussion**).

Having measured the end-binding kinetics, we were able to calculate whether the binding of MCAK on dynamic microtubule ends is due to direct end-binding (3D diffusion) or 1D lattice-diffusion (Gouveia et al., 2010; Helenius et al., 2006). Note that these two mechanisms are not mutually exclusive. Using our dataset, we measured that the lattice-diffusion coefficient of MCAK (D) on the lattice of dynamic microtubules was 0.023 ± 0.001 $\mu m^2 \cdot s^{-1}$ (**Fig. 1G**), close to the previously reported value (Cooper et al., 2010). Because the dwell time of the lattice-binding events was ~0.6 s, the average length scanned by a MCAK molecule via 1D lattice-diffusion was ~160 nm. Note that this is close to the length the GTP cap. Using a previously reported Fick's first equation of diffusion (Helenius et al., 2006), we calculated the flux of MCAK reaching growing ends via 1D diffusion was 0.004 s^{-1}. This was significantly lower than the arrival rate of MCAK onto

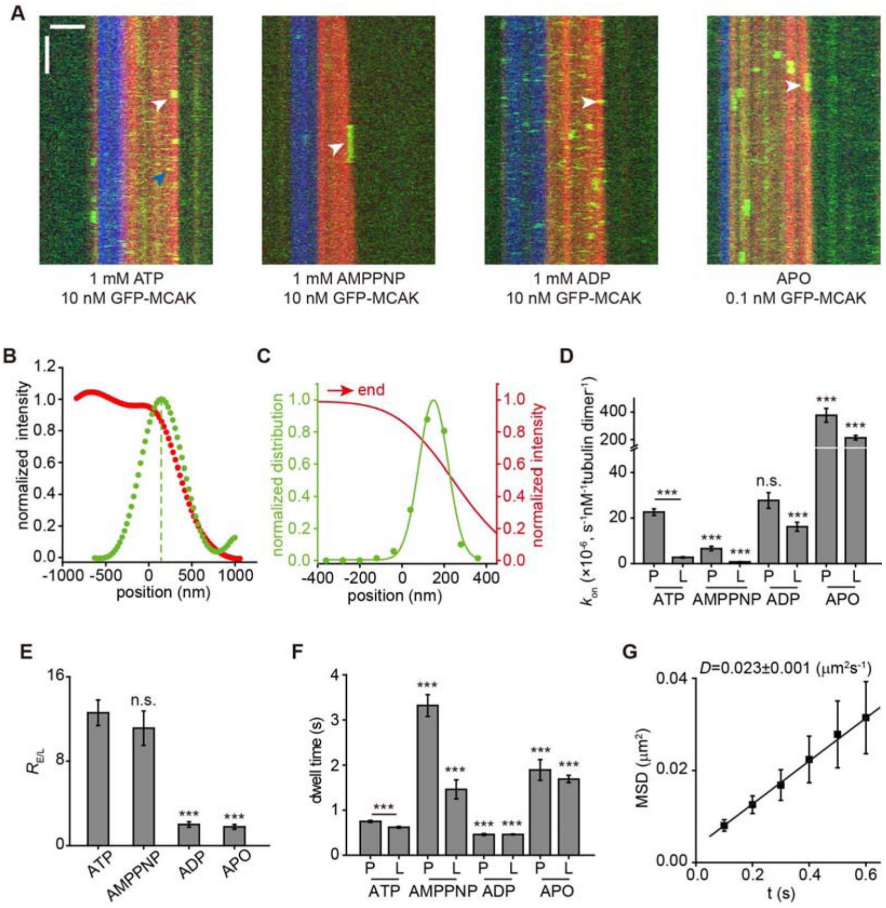

Figure 1.

MCAK preferentially binds to growing microtubule ends

(A) The representative kymographs of single-molecule GFP-MCAK (green) binding events on dynamic microtubules (red, tubulin: 16 μM) growing from GMPCPP microtubule seeds (blue) in the presence of 1 mM ATP, AMPPNP, ADP and APO (the nucleotide-free state). The binding events at the plus-end and lattice were indicated by a white arrowhead and a blue arrowhead, respectively. Vertical bar: 5 s; horizontal bar: 2 μm.

(B) The plot showing the representative intensity profiles of a single GFP-MCAK molecule (green) and a growing microtubule end (red). The peak position of a GFP-MCAK molecule was recorded as the binding location (dashed line in green).

(C) The spatial distributions of the binding sites of GFP-MCAK (green, n=152 events) along the long axis of microtubules. The averaged intensity profile of the growing microtubule ends (red) was shown as the positional reference.

(D) Statistical quantification of the apparent association constant (k_{on}) of GFP-MCAK on the plus-end and lattice of dynamic microtubules in the presence of 1 mM ATP (n=66 microtubules from 3 assays), AMPPNP (n=29 microtubules from 2 assays), ADP (n=21 microtubules from 2 assays) and APO (n=36 microtubules from 3 assays). The statistical comparison was made versus the k_{on} of MCAK on the corresponding location (i.e. plus end or lattice) in the ATP condition.

(E) Statistical quantification of $R_{E/L}$ in the presence of ATP, AMPPNP, ADP and APO. The statistical comparison was made versus the $R_{E/L}$ of the plus-end binding events in the ATP condition.

(F) Statistical quantification of the dwell time of GFP-MCAK on the growing end and lattice of dynamic microtubules in the presence of ATP (n=966 events from 3 assays for the plus end; n=702 events from 3 assays for lattice), AMPPNP (n=231 events from 2 assays for the plus end; n=142 events from 2 assays for lattice), ADP (n=327 events from 2 assays for the plus end; n=1184 events from 2 assays for lattice) and APO (n=71 events from 3 assays for the plus end; n=573 events from 3 assays for lattice). The statistical comparison was made versus dwell time of the binding events on the corresponding location (i.e. plus end or lattice) in the ATP condition.

(G) The mean-squared displacement (MSD) of GFP-MCAK was plotted against the time interval (t, 0.1s per frame). The diffusion coefficient (D) of 0. 023 $\mu m^2\ s^{-1}$ was obtained using a linear fitting ($<x^2>= 2Dt$). The error bar represented SEM (n=203 trajectories).

In panel **D**, **E** and **F**, all the data were presented as mean ± SEM. All the comparisons were performed using the two-tailed Mann-Whitney U test with Bonferroni correction, n.s., no significance; ***, p<0.001.

the plus end observed in our experiments ($N_p = k_{on} \cdot C = 0.067\ s^{-1}$. C: MCAK concentration). Therefore, in our experimental condition, 1D lattice-diffusion makes a minor contribution to the end-binding of MCAK (~ 6%), and the end-binding of MCAK can be thought of as the direct interaction between diffusive MCAK and the end structure.

MCAK binds to the entire GTP cap

Having recorded and analyzed the end-binding events of MCAK, we wondered how MCAK could recognize the end of dynamic microtubules. Previous studies, mostly based on stabilized microtubules, proposed the idea that MCAK binds to microtubule ends by recognizing curved protofilaments (Asenjo et al., 2013; Benoit et al., 2018; Trofimova et al., 2018). This model predicts that the binding site of MCAK is located to the distalmost tip of growing microtubule ends, where the XMAP215 family members are thought to bind (i.e. the distalmost cap) (Ayaz et al., 2012; Brouhard et al., 2008). We tested this prediction by measuring the end-binding regions of MCAK, EB1 and XMAP215 (**Fig. 2A**). To our surprise, the binding region of MCAK was longer than that of XMAP215. It extended proximally towards the lattice, and covered the binding region of EB1 (i.e. the EB cap) (Maurer et al., 2012). To further understand if MCAK binds to the distalmost cap and the EB cap with different kinetics, we compared the dwell time of the distally located MCAK to that of the proximally distributed ones (**Fig. 2B**). Note that we defined the MCAK molecules localizing to the left of the FWHM of XMAP215 binding region as the proximally distributed, while those localizing to the right of the FWHM of EB1 binding region was distally distributed. No significant difference was found (τ_{distal}=0.8 ± 0.5, n=28 binding events; $\tau_{proximal}$=0.7±0.6, n=45 binding events; p>0.3, the two-tailed Mann-Whitney U test with Bonferroni correction). Note that in all experiments, the morphologies of growing microtubule ends were comparable (**Fig. 2C**). Based on these results, we conclude that MCAK recognizes the entire GTP-cap. This result is intriguing as it suggests that in addition to the curved protofilaments, there are additional end-binding sites for MCAK. Moreover, this also provides an independent measurement for the size of the GTP cap at growing microtubule ends, which was previously determined using the tubulin dilution experiments or by measuring the binding region of EB1 (Duellberg et al., 2016).

MCAK strongly binds to GTPγS microtubules in a nucleotide-independent manner

How could MCAK bind to the EB cap? The tubulin dimers in the EB cap are thought to be different from those in the distalmost cap (Maurer et al., 2011). For example, the distalmost tubulin dimers (mostly GTP-tubulin) often lack lateral constrains (Vitre et al., 2008) and adopt a bent shape (Hoeoeg et al., 2011; McIntosh et al., 2018), while those in the EB cap (mostly GDP·Pi-tubulin) have formed at least some lateral contacts and had a more straight conformation (Guesdon et al., 2016; Maurer et al., 2012). To address this issue, we first examined that to what extent, MCAK may adopt a similar end-binding mechanism as EB1.

We tested the contribution of GDP·Pi-tubulins, which underlies the end-binding preference of EBs (Maurer et al., 2011; Maurer et al., 2012). Here, we compared the binding affinity of MCAK·AMPPNP on GTPγS, GMPCPP and GDP microtubules (**Fig. 3A**), which mimics tubulin dimers in different GTP hydrolysis states. Because MCAK in the concentration required for a full-lattice decoration would rapidly disassemble microtubules, we cannot directly compare the total binding of MCAK on microtubules as what was previously done for the EBs (Maurer et al., 2011; Maurer et al., 2012). Therefore, we took an alternative approach by recording the single-molecule binding of MCAK·AMPPNP (1 nM) on microtubules and measuring fluorescence intensity summation over a period of time (1000 frames, 0.3 s per frame) (**Fig. s2**). Strikingly, we found that MCAK·AMPPNP bound strongly to GTPγS microtubules (**Fig. 3 A-C**). Quantitative analysis revealed a significantly stronger binding on GTPγS microtubules than on GMPCPP or GDP

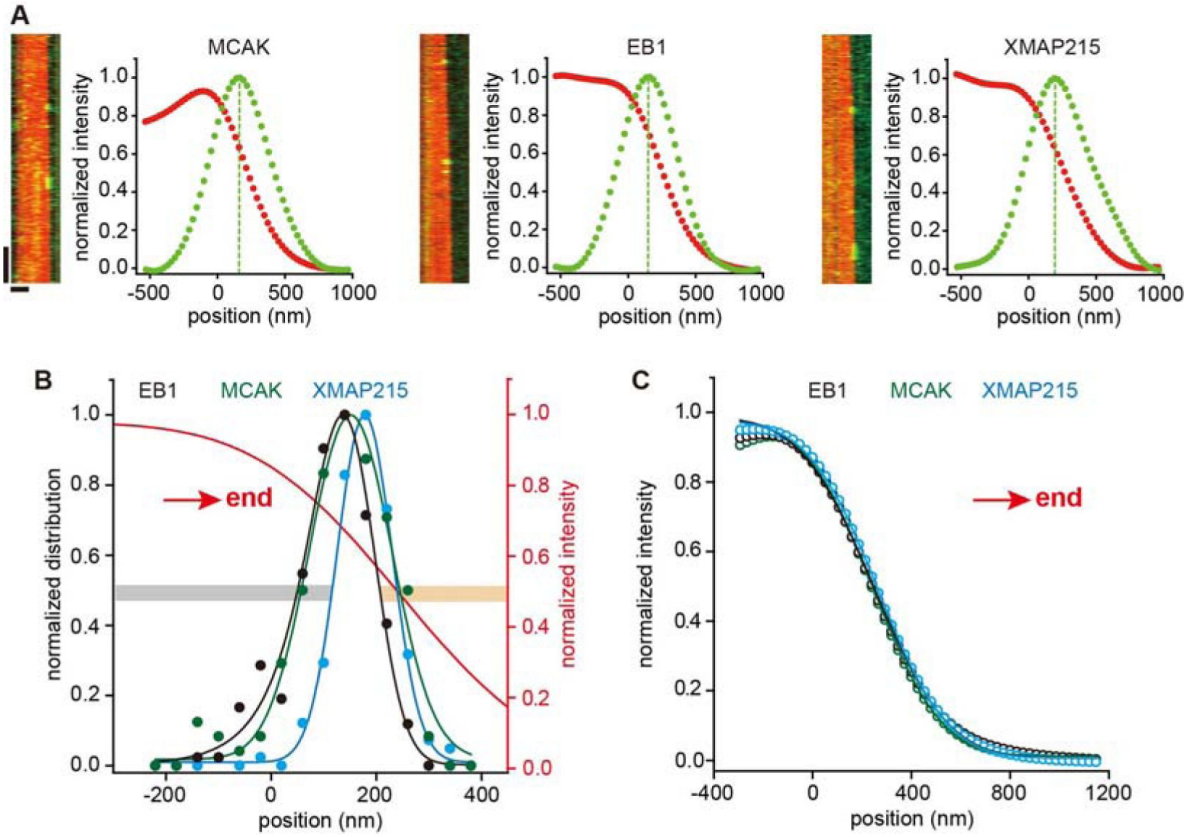

Figure 2.

MCAK binds to the entire GTP cap of growing microtubule ends

(A) The representative kymograph showing the individual binding events of GFP-MCAK (1 nM, with 1 mM ATP, left), EB1-GFP (10 nM, middle) or XMAP215-GFP (1 nM, right) at growing microtubule ends (red, tubulin: 12 μM). The plots showed the representative intensity profiles of a single molecule (green) and a growing microtubule end (red). The peak position of individual molecules was determined using a Gaussian fit (dashed line in green). Vertical bar: 5 s; horizontal bar: 2 μm.

(B) The spatial distributions of the binding sites of GFP-MCAK (green, n=123 events), EB1-GFP (black, n=184 events) and XMAP215-GFP (blue, 142 events) along the long axis of microtubules. The averaged intensity profile of the growing microtubule ends (red) was shown as the positional reference. The region within the FWHM of the blue curve was considered to be the binding region of XMAP215. The MCAK molecules localized to the left of this region was considered to be proximally distributed (grey bar). The region within the FWHM of the black curve was considered to be the binding region of EB1. The MCAK molecules localized to the right of this region was considered to be distally distributed (orange bar).

(C) The averaged intensity profiles of the growing microtubule ends were used to quantify the localization of GFP-MCAK (green, 123 microtubules), EB1-GFP (black, 184 microtubules) and XMAP215-GFP (blue, 142 microtubules). Note that the morphologies of microtubule ends in three conditions were nearly identical. In panels **B** and **C**, the red arrows indicated the direction of microtubule growth.

microtubules (**Fig. 3 B-C**). Because the tubulin dimers in GTPγS microtubules were found to be the analogue of GDP·Pi-tubulins (Maurer et al., 2011), this result suggests that MCAK·ATP has a binding preference for GDP·Pi-tubulins.

Because the end-binding affinity and preference of MCAK depends on the nucleotide state, we wondered if the preference for GTPγS microtubules is similar. Therefore, we performed the same experiments using MCAK·AMPPNP, MCAK·ATP, MCAK·ADP and MCAK·APO. Although the MCAK variants bound to microtubules with different affinities (MCAK·APO > MCAK·AMPPNP > MCAK·ADP > MCAK·ATP) (**Fig. 3B**), they all showed a clear binding preference for GTPγS microtubules over GDP or GMPCPP microtubules (**Fig. 3 B-C**). These observations suggest that the binding preference to GTPγS microtubules (i.e. GDP·Pi-tubulin) is, to a large extent, independent on the nucleotide state of MCAK.

MCAK binds to the end region of stabilized GMPCPP microtubules in a nucleotide state-dependent manner

We noted that MCAK showed a nucleotide state-dependence in the binding preference for dynamic microtubule ends, but the binding to GTPγS microtubules is nucleotide independent. Therefore, we wondered if the binding to curved protofilaments at the distalmost cap of dynamic microtubules could account for the nucleotide-state dependence. To address this issue, we measured the binding preference of MCAK at the end of stabilized GMPCPP microtubules (**Fig. 4A**), where the length and curvature of protofilaments are thought to be similar to those at the end of dynamic microtubules but has no GDP·Pi-tubulins (Manka and Moores, 2018; McIntosh et al., 2018). We found that the MCAK in ATP or AMPCPP state showed a binding preference for the end of GMPCPP microtubules, while MCAK·ADP and MCAK·APO did not (**Fig. 4B-C**). This is similar to the observation that MCAK·ADP and MCAK·APO had a lower binding preference for dynamic microtubule ends, thereby providing an explanation for the nucleotide state-dependence of the end-binding of MCAK.

The binding preference for GDP·Pi-tubulins facilitates the end-binding of MCAK

Intuitively, the binding preference for the EB cap, in addition to the previously established preference for the curved protofilaments, would provide more high-affinity sites for MCAK, thereby facilitating the end-binding of MCAK. To tested this idea, we studied two mutants, $MCAK^{K524A}$ (K524A in the α4 helix) and $MCAK^{V298S}$ (V298S in the L2 loop). The α4 helix of MCAK interacts with the intra-dimeric interface of tubulin heterodimer (**Fig. 5A**), and the K524A mutation is expected to disrupt the electrostatic interaction between MCAK and tubulin heterodimer (Patel et al., 2016; Wang et al., 2017). The L2 loop of MCAK has a direct contact with the inter-dimeric interface of tubulin heterodimer (**Fig. 5A**), and the V298S mutation is expected to disrupt the hydrophobic interaction between the L2 loop and α-tubulin (Wang et al., 2015). We confirmed that both mutants were depolymerization-deficient (**Fig. s3**), as previously reported (Wang et al., 2017; Wang et al., 2015).

Compared to MCAK, $MCAK^{K524A}$ showed a significantly reduced binding intensity on microtubules. Meanwhile, it still showed a binding preference for GTPγS microtubules (**Fig. 5B-C**), but the ratios between the binding intensity on GTPγS microtubules and that on GMPCPP or GDP microtubules were both reduced (**Fig. 5D**). This suggests that $MCAK^{K524A}$ had a lower preference for GTPγS microtubules. On the contrary, we found that $MCAK^{K524A}$ showed a higher binding to the end than to the lattice of GMPCPP microtubules (**Fig. 5 E-F**), suggesting that the binding preference for curved protofilament is maintained. Therefore, $MCAK^{K524A}$ could be a candidate variant to test how the preference for GDP·Pi-tubulin contributes to the end-binding preference of MCAK. We found that $MCAK^{K524A}$ could bind to dynamic microtubule ends, but its

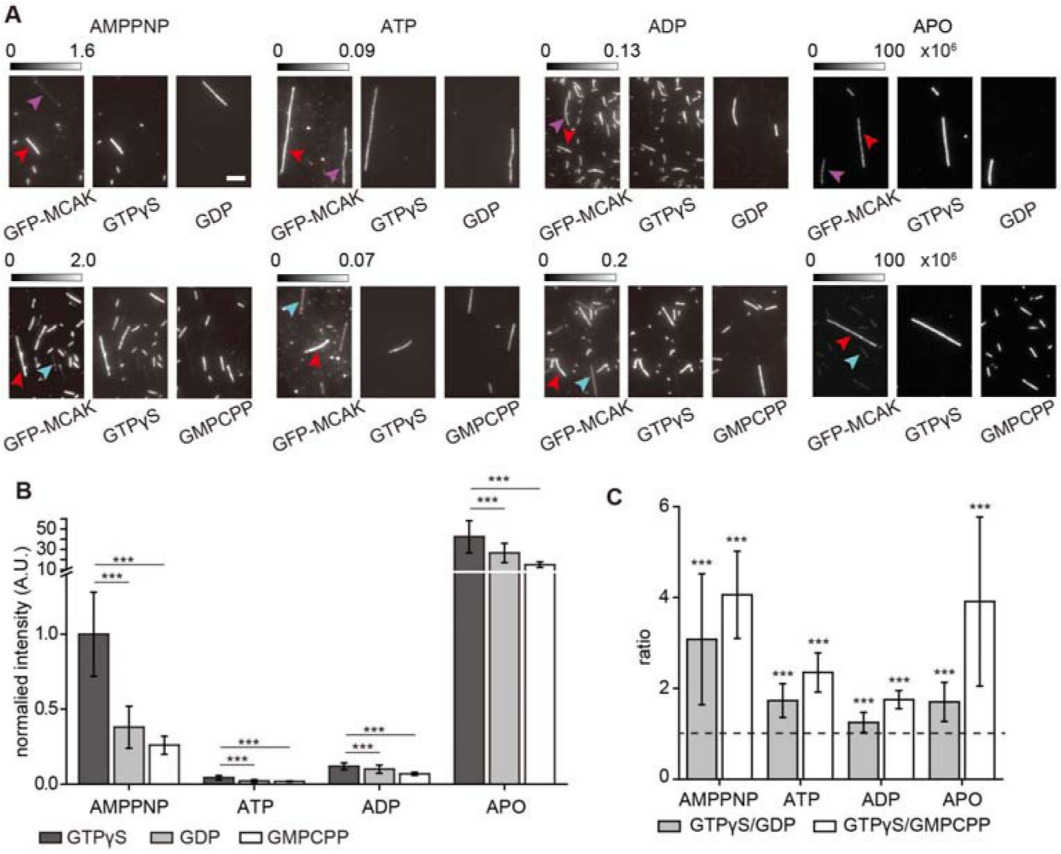

Figure 3.

MCAK strongly binds to GTPγS microtubules in a nucleotide-independent manner

(A) The representative projection images of GFP-MCAK binding to GTPγS (red arrowhead), GDP (purple arrowhead) and GMPCPP microtubules (cyan arrowhead) in the presence of 1 mM AMPPNP (left), 1 mM ATP (left middle), 1 mM ADP (right middle) and at the APO state (right). Note that the binding on GDP or GMPCPP microtubules was compared to that on GTPgS microtubules in the same flow cell. Scale bar: 5 μm.

(B) Statistical comparison of the normalized fluorescence intensity of GFP-MCAK on different microtubules in the presence of 1 mM AMPPNP (112 GTPgS microtubules, 56 GMPCPP microtubules, 56 GDP microtubules from 3 assays), 1 mM ATP (88 GTPgS microtubules, 60 GMPCPP microtubules, 28 GDP microtubules from 3 assays), 1 mM ADP (95 GTPgS microtubules, 51 GMPCPP microtubules, 44 GDP microtubules from 3 assays) or at the APO state (66 GTPgS microtubules, 30 GMP-CPP microtubules, 36 GDP microtubules from 3 assays). All data were normalized to the binding intensity of GFP-MCAK (1 nM) on GTPgS microtubules in the AMPPNP condition.

(C) The ratios of the binding intensity of GFP-MCAK on GTPgS microtubules to that on GDP or GMPCPP microtubules in various nucleotide conditions. The dashed line on the plot represented 1. The statistical comparisons were performed between the ratios and 1.

In panel **B** and **C**, All the data were presented as mean ± std. All the comparisons were performed using the two-tailed Mann–Whitney U test with Bonferroni correction, ***, p<0.001.

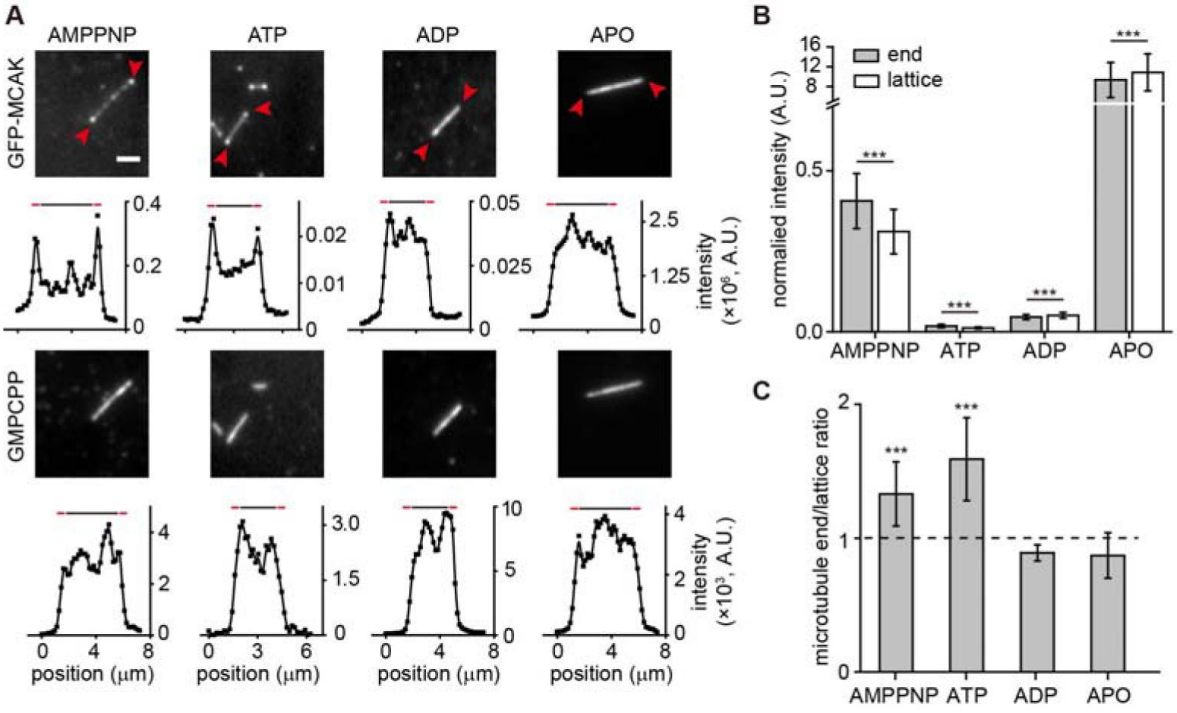

Figure 4.

MCAK strongly binds to the ends of GMPCPP microtubules in a nucleotide state-dependent manner

(A) The representative projection images and intensity profiles showing GFP-MCAK binding (upper) at the ends of GMPCPP microtubules (lower) in the presence of 1 mM AMPPNP (left), 1 mM ATP (left middle), 1 mM ADP (right middle) or at the APO state (right). Red arrowhead: the end-binding of MCAK. Red bar: ends. Black bar: lattice. Scale bar=2 μm.

(B) Statistical quantification of the binding intensity of GFP-MCAK on the lattice and end of GMPCPP microtubules in the presence of 1 mM AMPPNP (47 microtubules from 3 assays), 1 mM ATP (40 microtubules from 3 assays), 1 mM ADP (45 microtubules from 3 assays) or at the APO state (52 microtubules from 3 assays). All data were normalized to the binding of GFP-MCAK (1 nM) on GTPgS microtubules in the AMPPNP condition. All the data were presented as mean ± std. The statistical analysis was performed using the two-tailed pair *t*-test by Bonferroni correction, ***, p<0.001.

(C) The ratios between the binding intensity of GFP-MCAK at the end and the intensity on the lattice of GMPCPP microtubules in various nucleotide conditions. The ratios were presented as mean ± std. The dashed line on the plot represents 1. The statistical comparisons were performed between the ratios and 1 using the two-tailed Mann-Whitney U test with Bonferroni correction, ***, p<0.001.

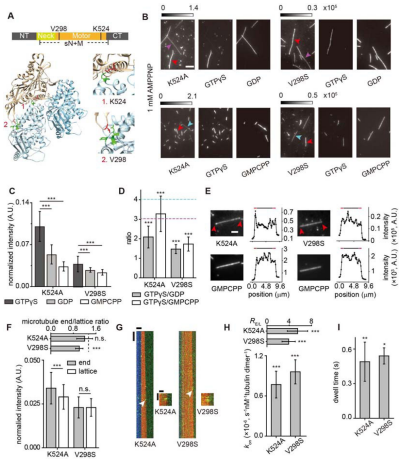

Figure 5.

The preference for GDP·Pi-tubulins facilitates the binding preference of MCAK for growing microtubule ends

(A) The structural model of the MCAK^{sN+M}-tubulin complex (PDB ID: 5MIO). The cartoon schematic in upper panel showed the domain organization of MCAK. Loop2 and α4 helix, two regions mediating the interaction between MCAK and tubulin, were enlarged and the key sites (K524, V298) were highlighted in red. The corresponding residues of tubulin that may interact with K524 and V298 were highlighted in green.

(B) The representative projection images of GFP-MCAK^{K524A} and GFP-MCAK^{V298S} binding to GTPγS (red arrowhead), GDP (purple arrowhead) and GMPCPP microtubules (cyan arrowhead) in the presence of 1 mM AMPPNP. Scale bar: 5 μm.

(C) Statistical quantification of the binding intensity of GFP-MCAK^{K524A} (144 GTPgS microtubules, 72 GMPCPP microtubules, 62 GDP microtubules from 3 assays) and GFP-MCAK^{V298S} (124 GTPgS microtubules, 65 GMPCPP microtubules, 59 GDP microtubules from 3 assays) on different microtubules. Note that all data were normalized to the binding intensity of GFP-MCAK on GTPgS microtubules in the AMPPNP condition. The data were presented as mean ± std.

(D) The ratios of the binding intensity of GFP-MCAK^{K524A} or GFP-MCAK^{V298S} on GTPgS microtubules to that on GDP or GMPCPP microtubules in the presence of 1 mM AMPPNP. Purple dashed line: The GTPgS/GDP ratio of GFP-MCAK. The cyan dashed line: the GTPgS/GMPCPP ratio of GFP-MCAK. The data were presented as mean ± std. The statistical comparisons were made versus the corresponding value of GFP-MCAK.

(E) The representative projection images and intensity profiles showing the binding of GFP-MCAK^{K524A} and GFP-MCAK^{V298S} to the end (red arrowhead in the upper panels) and lattice of GMPCPP microtubules (lower panels) in the presence of AMPPNP. Red bar: ends. Black bar: lattice. Scale bar=2 μm.

(F) Statistical quantification of the binding intensity of GFP-MCAK^{K524A} (88 microtubules from 3 assays) and GFP-MCAK^{V298S} (68 microtubules from 3 assays) on the lattice and end of GMPCPP microtubules in the presence of AMPPNP (lower panel). All the data were normalized to the binding of GFP-MCAK (1 nM) on GTPgS microtubules in the AMPPNP condition. All the binding intensity data were presented as mean ± std. The statistical analyses were performed using the two-tailed paired *t*-test by Bonferroni correction, n.s., no significance; ***, p<0.001. The inset (upper) showing the ratios of the end-binding intensity of GFP-MCAK^{K524A} or GFP-MCAK^{V298S} to the lattice-binding intensity on GMPCPP microtubules in the presence of AMPPNP. The ratios were presented as mean ± std. The statistical comparisons were made versus the ratio of GFP-MCAK (the dashed line shown in **Fig. 4C**).

(G) The representative kymographs of the single-molecule binding events of GFP-MCAK^{K524A} (10 nM, green) and GFP-MCAK^{V298S} (30 nM, green) on growing microtubules (red, tubulin: 16 μM) in the presence of 1 mM ATP. The end-binding events were indicated using white arrowheads and enlarged. For the original kymo-graphs, vertical bar: 2 s; horizontal bar: 2 μm. For the enlarged kymographs, vertical bar: 1 s; horizontal bar: 0.5 μm.

(H) Statistical quantification of the apparent association constant ($k_{on\text{-}P}$) of GFP-MCAK^{K524A} (33 microtubules from 3 assays) and GFP-MCAK^{V298S} (23 microtubules from 2 assays) on growing microtubule ends in the presence of ATP (lower). The statistical comparisons were made versus the $k_{on\text{-}P}$ of GFP-MCAK. The inset (upper) showing the statistical quantification of $R_{E/L}$ of GFP-MCAK^{K524A} and GFP-MCAK^{V298S}. The $R_{E/L}$ of both mutants were compared to that of GFP-MCAK in the ATP condition. All the data were presented as mean ± SEM.

(I) Statistical quantification of the dwell time of GFP-MCAK^{K524A} (19 binding events from 3 assays) and GFP-MCAK^{V298S} (43 binding events from 3 assays) on growing microtubule ends in the presence of ATP. The dwell time were presented as mean ± SEM. The dwell time of GFP-MCAK^{K524A} and GFP-MCAK^{V298S} were compared to that of GFP-MCAK.

In panel **C**, **D**, **F** (the upper inset), **H** and **I**, all the comparisons were performed using the two-tailed Mann-Whitney U test with Bonferroni correction, n.s., no significance; *, p<0.05; **, p<0.01; ***, p<0.001.

$k_{on\text{-}P}$ and $R_{E/L}$ were both reduced (**Fig. 5 G-H**). Meanwhile, the end-binding dwell time of $MCAK^{K524A}$ was also reduced (**Fig. 5I**), suggesting a higher off-rate. These results are consistent with the idea that the higher binding affinity to GDP·Pi-tubulin directly contributes to the end-binding preference of MCAK at growing microtubule ends.

By comparison, the binding preferences of $MCAK^{V298S}$ for GTPγS microtubules and the end of GMPCPP microtubules were both reduced (**Fig. 5 B-F**), implying the reduced preferences for both the EB cap and the distalmost cap. In the dynamics assay, the $k_{on\text{-}P}$, $R_{E/L}$ and dwell time of $MCAK^{V298S}$ were decreased in comparison to those of MCAK (**Fig. 5 H-I**). We noted that the $k_{on\text{-}P}$, $R_{E/L}$ and dwell time of $MCAK^{V298S}$ were similar to those of $MCAK^{K524A}$ (two-tailed Mann-Whitney U test with Bonferroni correction, p>0.05), suggesting that the binding to the EB cap makes a major importance for the end-binding preference of MCAK at dynamic microtubule ends.

Functional specification of MCAK and XMAP215

Having revealed the novel binding sites of MCAK in dynamic microtubule ends, we wondered how MCAK may work in coordination with other microtubule plus tip-binding proteins, for example EB1 and XMAP215. It has been shown that EB1 interacts with and recruits MCAK to growing microtubule ends (Gouveia et al., 2010; Honnappa et al., 2009; Lee et al., 2008), so it is straightforward that EB1 would facilitate the function of MCAK as a catastrophe factor. On the contrary, MCAK and XMAP215 were shown to antagonistically regulate the assembly of microtubules, and it was thought that these two molecules may compete directly for the growing ends (Tournebize et al., 2000). Here, we showed that the binding regions of MCAK and XMAP215 in growing microtubule ends are partially overlapping. In addition, previously resolved structural models suggest that MCAK and XMAP215 likely bind to similar regions on tubulin dimers (**Fig. s4**). Therefore, these two molecules could indeed compete for the binding sites in the distalmost cap. However, it is not yet clear to what extent, the competition from XMAP215 would antagonize the function of MCAK, especially given the recent finding that XMAP215 itself could promote the catastrophe of microtubules (Farmer et al., 2021). Therefore, it is intriguing what would be the collective effect of MCAK and XMAP215.

To address this issue, we studied how they together regulate microtubule grow rate and catastrophe frequency. Based on our pilot experiments, we chose 20 nM for MCAK and 50 nM for XMAP215 as the representative concentrations. In the presence of 20 nM MCAK, the dynamic microtubules could grow but showed a significantly increased catastrophe frequency and shorter lifetime (**Fig. 6 A-B**). Meanwhile, 50 nM XMAP215 increased growth rate by more than four times (control: 0.8 ± 0.1 μm min^{-1}, n=97 microtubules from 6 assays; 50 nM XMAP215: 3.7 ± 0.4 μm min^{-1}, n=50 microtubules from 3 assays). We noted that XMAP215 alone caused a mild shortening in the lifetime of dynamic microtubules (**Fig. 6 A-B**). This is consistent with the previous report and suggesting the enhanced fluctuations in the end structures (Farmer et al., 2021). We then added both 20 nM MCAK and 50 nM XMAP215. In this case, microtubule grew in a similar rate as if there was no MCAK (3.9 ± 0.4 μm min^{-1}, n=52 microtubules from 3 assays, p=0.5, the two-tailed Mann-Whitney U test with Bonferroni correction), suggesting that MCAK has no major effect on the function of XMAP215 as a polymerase. Meanwhile, the lifetime of dynamic microtubules became shorter than that with only MCAK or XMAP215 (**Fig. 6B**). To understand if this reflects the simple summation of the separate contributions of MCAK and XMAP215 or their synergistic effect, we calculated the probability-based catastrophe frequency in all conditions (**Fig. 6C**) (Gardner et al., 2011).We found that the effects of MCAK and XMAP215 on the probability of microtubule catastrophe were only additive, showing that there was no synergic effect in regulating catastrophe frequency (**Fig. 6C**).

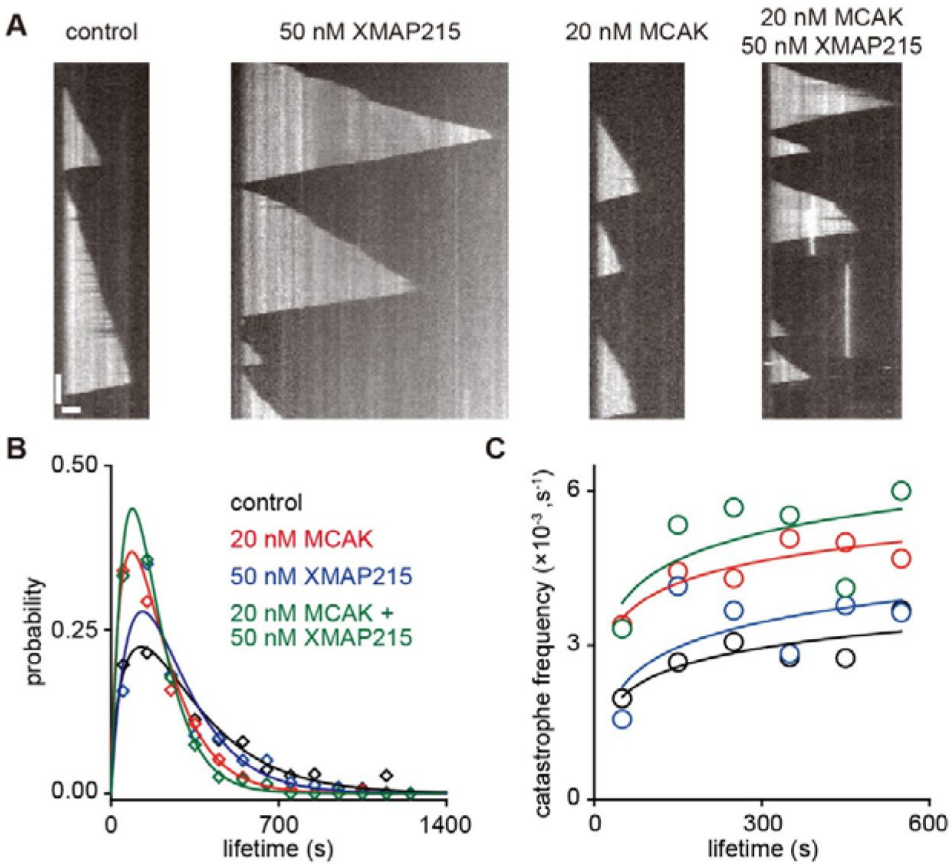

Figure 6.

Functional specification of MCAK and XMAP215 at growing microtubule ends

(A) The representative kymographs of dynamic microtubules (tubulin: 10 μM) in the control condition or in the presence of MCAK, XMAP215 or both. Vertical bar: 100 s; horizontal bar: 2 μm.

(B) The probability distribution of microtubule lifetime in the experimental conditions indicated in the panel **A**. The lines were gamma fitting curves. Control: 443 microtubules from 7 assays. 20 nM MCAK: 621 microtubules from 7 assays. 50 nM XMAP215: 237 microtubules from 3 assays. 20 nM MCAK+50 nM XMAP215: 283 microtubules from 3 assays.

(C) The plots of catastrophe frequency versus the lifetime of microtubules showing how the likelihood of catastrophe depended on the age of microtubules. The data were from the experiments shown in panel **A**.

Discussion

This study provides mechanistic insights into understanding the end-binding mechanism of MCAK. The previously established view on the end-binding mechanism of MCAK is based on specific recognition of curved protofilaments at the distalmost tip of growing microtubules (Asenjo et al., 2013; Benoit et al., 2018; Tan et al., 2008). The findings in the present study add to the model by showing that MCAK strongly binds to the EB cap, and this binding preference could facilitate the end-binding and in turn the depolymerizing activity of MCAK. We now discuss our main findings and their implications.

MCAK preferentially binds to the GDP·Pi-tubulin in a nucleotide state-independent manner

Our main finding is that MCAK preferentially binds to the EB cap where the GDP·Pi-tubulins gather, in addition to the previously known binding preference for the curved protofilaments (Asenjo et al., 2013; Benoit et al., 2018; Tan et al., 2008). This conclusion is based on several lines of evidence. First, MCAK·ADP or MCAK·APO shows a significant reduction but not a complete loss in the binding preference for dynamic microtubule ends. However, their binding preference for stabilized microtubule ends is completely absent. The different binding behavior to dynamic and stabilized microtubule ends suggests that other end features, in addition to the curved protofilament, also contribute to the end-binding of MCAK. Second, the localization analysis of MCAK shows that MCAK binds to the entire GTP cap, including the EB cap and the distalmost cap. Third, MCAK shows a binding preference for GTPγS microtubules over GDP or GMPCPP microtubules. Because the tubulin in GTPγS microtubules is considered to be the analogues to the GDP·Pi-tubulins (Maurer et al., 2011; Maurer et al., 2012), MCAK is expected to have a high affinity to the EB cap. Fourth, the loss of the binding preference of $MCAK^{K524A}$ for GTPγS microtubules, but not for the ends of GMPCPP microtubules, suggests that the binding preference for GDP·Pi-tubulins is required for the optimal end-binding affinity and preference.

Implications for the working mechanism of MCAK

We think that our findings add to the current working model of MCAK in four aspects. First, the nucleotide state-independent binding preference for GDP·Pi-tubulins provides a mechanism for MCAK staying bound to the GTP cap after ATP hydrolysis. For example, in a speculative scenario (**Fig. 7A**), when one of the motor domains of MCAK is converted to the ADP or APO state after the breakage of the terminal tubulin dimers, it loses the binding affinity to the curved protofilaments (**Fig. 7A**). However, it still preferentially binds to GDP·Pi-tubulins, so it has a larger chance binding back to the EB cap than to the lattice and thereby remains within the GTP cap (**Fig. 7A**). Then, while the ADP is being replaced by a new ATP, this motor domain could diffuse around to explore the working site for the next round of catalysis. In this way, the nucleotide state-independent binding feature of MCAK would facilitate the binding to the dynamic microtubule ends. This idea is consistent with the findings that $MCAK^{K524A}$, a mutant showing reduced preference for GDP·Pi-tubulins, had disrupted end-binding affinity, preference and dwell time.

Second, the previous studies showed that MCAK has a direct interaction with EB1, and EB1 increases the end-binding rate of MCAK (Gouveia et al., 2010; Honnappa et al., 2009; Lee et al., 2008). Intuitively, there is a gap here that after the arrival of MCAK at the binding region of EB1, how it could reach the high-affinity binding site or working site, previously known as the curved protofilament at the distalmost tip (Asenjo et al., 2013; Benoit et al., 2018; Tan et al., 2008). Our finding fills this gap by showing that MCAK also binds strongly to the GDP·Pi-tubulins where the EBs bind (**Fig. 7B**). Moreover, MCAK could explore the entire length of GTP cap (~160 nm) by

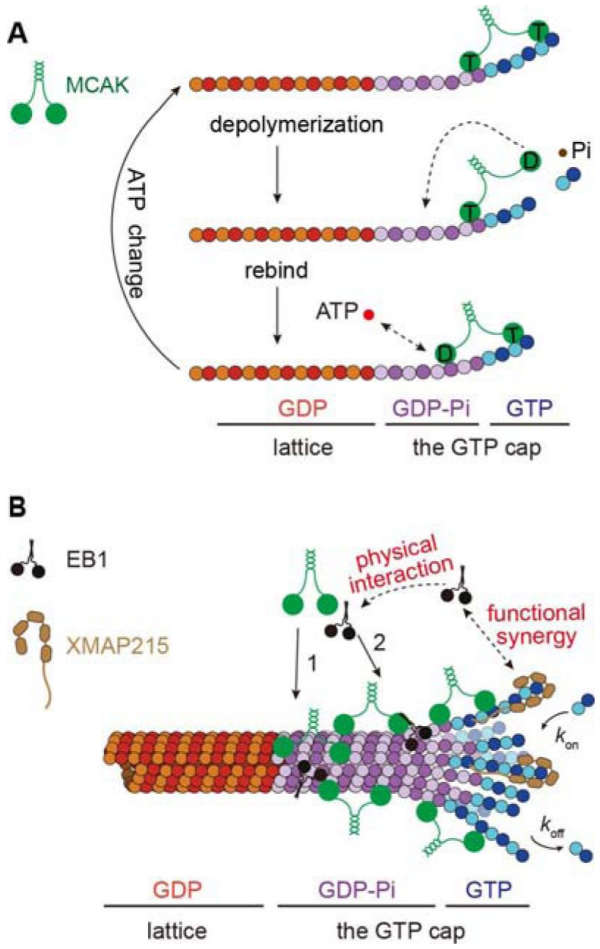

Figure 7.

Cartoon schematics depicting the working model of MCAK at growing microtubule-ends

(A) A hypothetic model for the binding cycle of MCAK, given its binding preference on the EB cap. T: MCAK·ATP. D: MCAK·ADP.

(B) The co-operation schematics of MCAK, EB1 and XMAP215 at growing microtubule ends. Pathway 1: the direct binding of MCAK to microtubule ends. Pathway 2: the indirect binding of MCAK to microtubule ends via EB1.

1D lattice diffusion within the binding dwell time. In this way, we argue that MCAK could find the precisely catalyzing site at dynamic microtubule ends by a combination of 3D diffusion (to first reach the GTP cap) and 1D lattice diffusion (short range, within the GTP cap).

Third, a previous study showed that the association on-rate is an important regulated parameter for the functions of MCAK (Cooper et al., 2010). The binding affinity and preference for the EB cap, in addition to the distalmost cap, would intuitively provide more binding sites, thereby further increasing the binding on-rate of MCAK. This idea is also consistent with the finding that $MCAK^{K524A}$, a mutant with a reduced preference for GDP·Pi-tubulins, had a reduced end-binding on-rate.

Fourth, despite having partially overlapped binding regions, MCAK and XMAP215 provide independent controls for catastrophe frequency and growth rate of microtubules, respectively (**Fig. 7B**). The free combination of these two regulators suggests a straightforward strategy in the regulations on the length and mass of microtubules, i.e., simply by controlling their amount (i.e. expression level) or activity (e.g. via phosphorylation). In this sense, the working scenario of MCAK and XMAP215 provides a molecular paradigm to understand how the regulators of microtubules orchestrate their behaviors at the nanoscopic end structure of dynamic microtubules and reflects a clear logic in the regulatory network of microtubule dynamics.

Materials and Methods

Protein expression and purification

MCAK purification

GFP-MCAK was expressed using a modified BAC-to-BAC protocol. The purification was performed as previously described (Helenius et al., 2006). Briefly, the cells were lysed by dounce homogenization in the lysis buffer. The lysate was centrifuged at 40,000 rpm for 60 min at 4°C (XPN-100 ultracentrifuge, Type 45 Ti rotor, Beckman, USA). The supernatant was filtered through a 0.45 μm filter (Pall, 66229) and then purified using a cation-exchange column (HiTrap SP-HP, Cat.17115101, GE, USA). The protein was eluted using the elution buffer supplemented with a continuous salt gradient (0.15~1.0 M NaCl). The peak fractions were pooled together and then loaded onto a Ni-sepharose column (QIAGEN, 30210). The column was then washed with the washing buffer I. After the washing step, 3C Protease (final concentration 10 μg/ml) (Thermo Fisher Scientific, 88946) was used to perform on-column his-tag cleavage overnight at 4°C. The eluate was further purified using a gel-filtration column (Superdex 200 increase 10/300 GL column, Cat. 28990944, GE, USA) and eluted using the washing buffer II. The purified MCAK was frozen in liquid N_2 and stored at −80°C. Lysis buffer: 50 mM HEPES pH 7.5, 150 mM NaCl, 5% glycerol, 0.1% Tween 20, 1.5 mM $MgCl_2$, 3 mM EGTA, 1 mM DTT, 0.5 mM Mg-ATP, 10 units/ml Benzonase. Elution buffer: 6.7 mM HEPES pH 7.5, 6.7 mM MES, 6.7 mM sodium acetate, 1.5 mM $MgCl_2$, 10 μM Mg-ATP. Washing buffer I: 50 mM $NaPO_4$ buffer pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM $MgCl_2$, 10 μM Mg-ATP. Washing buffer II: BRB80, 300 mM KCl, 1 mM DTT and 10 μM Mg-ATP. BRB80: 80 mM PIPES/KOH pH 6.9, 1 mM $MgCl_2$, 1 mM EGTA.

EB1 purification

EB1-GFP was expressed and purified as previously described (Song et al., 2020). Briefly, EB1-GFP was expressed in the *BL21 E. coli* strain and lysed using sonication in the lysis buffer. The lysate was centrifuged at 40,000 rpm for 60 min at 4°C. The supernatant was filtered through a 0.45 μm filter and loaded onto a Ni-sepharose column. The column was then washed using the washing buffer. After the washing step, 3C Protease (final concentration 10 μg/ml) was used to perform on-column his-tag cleavage overnight at 4°C. The eluate was desalted into the storage

buffer using the PD-10 desalting column (GE, 17085101). The final purified protein was frozen in liquid N_2 and stored at −80 °C. Lysis buffer: 50 mM $NaPO_4$ buffer pH 7.5, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 1 mM DTT, 0.1% Tween 20. Washing buffer: 50 mM $NaPO_4$ buffer pH 7.5, 300 mM NaCl, 10% glycerol, 30 mM imidazole, 1 mM DTT. Storage buffer: BRB80, 100 mM KCl, 10% glycerol and 1 mM DTT.

XMAP215 purification

XMAP215-GFP was expressed using a modified BAC-to-BAC protocol. The purification was performed as previously described (Brouhard et al., 2008). Briefly, the cells were lysed by dounce homogenization in the lysis buffer. The lysate was centrifuged at 40,000 rpm for 60 min at 4°C. The supernatant was filtered through a 0.45 μm filter and then purified using a cation-exchange column. The protein was eluted using the elution buffer supplemented with a continuous salt gradient (0.15~1.0 M NaCl). The peak fractions were pooled together and then loaded onto a Ni-sepharose column. The column was then washed with the washing buffer I. The protein was eluted using the washing buffer I supplemented with a continuous imidazole gradient (20-300 mM). The fractions with best purity were pooled together and further purified using a gel-filtration column (Superdex 200 increase 10/300 GL column, Cat. 28990944, GE, USA) and eluted using the washing buffer II. The final purified XMAP215 was frozen in liquid N_2 and stored at −80°C. Lysis buffer: 50 mM HEPES pH 7.5, 50 mM NaCl, 5% glycerol, 0.1% Tween 20, 1 mM DTT, 10 units/ml Benzonase. Elution buffer: 6.7 mM HEPES pH 7.5, 6.7 mM MES, 6.7 mM sodium acetate. Washing buffer I: 50 mM NaPO4 buffer pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 1 mM $MgCl_2$, 10 μM Mg-ATP. Washing buffer II: BRB80, 150 mM KCl, 1 mM DTT.

Tubulin preparation

Tubulin was purified from porcine brain tissues by two cycles of polymerization and depolymerization followed by affinity purification using the TOG-based column, as previously described (Gell et al., 2010; Widlund et al., 2012). Tubulin was labeled with biotin (Thermo Fisher Scientific, 20217), TAMRA (Thermo Fisher Scientific, C1171) and Alexa Fluor 647 (Thermo Fisher Scientific, A20106) using the NHS esters according to the standard protocols (Gell et al., 2010).

Microtubule depolymerization assay

Microtubules (5% Alexa Fluor 647 labeled and 20% biotin labeled) were polymerized in the presence of GMPCPP (JenaBioscience, NU-405L) as previously described (Song et al., 2020). The GMPCPP-stabilized microtubules were immobilized on the surface of a cover glass using the biotin-NeutrAvidin protein links (Thermo Fisher Scientific, 31000). GFP-MCAK was then added into the flow cell in the imaging buffer. The sample was kept at 35°C using a temperature controller (Tokai Hit, Japan). Images were recorded using a total internal reflection microscope (TIRFM) (Olympus, Japan) equipped with a 100× 1.49 N.A. oil TIRF objective (Olympus, Japan) and an Andor 897 Ultra EMCCD camera (Andor, Belfast, UK). Images were recorded every 5 s with a 100 ms exposure. Imaging buffer: BRB80 supplemented with 1 mM ATP, 50 mM KCl, 80 mM D-glucose, 0.4 mg/ml glucose oxidase, 0.2 mg/ml catalase, 0.8 mg/ml casein, 1% β-mercaptoethanol, 0.001% Tween 20.

Microtubule dynamics assay

Microtubule dynamics assay was performed as previously described (Song et al., 2020). Briefly, the immobilized GMPCPP-stabilized microtubules were used as the template for microtubule growth. Tubulin dimers (13% TAMRA labeled) and the protein of interest were then added into the flow cell in the imaging buffer. The sample was kept at 35°C using a temperature controller (Tokai Hit, Japan). Images were recorded using a TIRFM (Olympus, Japan). To record microtubule dynamics, images were recorded every 5 s with a 100 ms exposure. To record single molecule

binding events, images were recorded every 100 ms with a 50 ms exposure. Imaging buffer: BRB80 supplemented with 1 mM ATP, 2 mM GTP, 50 mM KCl, 0.15% sodium carboxymethylcellulose, 80 mM D-glucose, 0.4 mg/ml glucose oxidase, 0.2 mg/ml catalase, 0.8 mg/ml casein, 1% β-mercaptoethanol, 0.001% Tween 20.

The diffusion coefficient of MCAK on dynamic microtubule lattices

The diffusion coefficient of MCAK on dynamic microtubule lattices was calculated as previously described (Helenius et al., 2006). Firstly, we gained the position coordinates of MCAK at its molecular trajectory on dynamic microtubule lattices using the plugin of imageJ (Fiji), TrackMate (Tinevez et al., 2017). Note that the minimum track length was kept at 400 ms (100 ms/frame). Then, the mean-squared displacement (MSD) of MCAK at every molecular trajectory (203 trajectories from 3 assays) was calculated against the interval (100 ms/frame) by an in-house script of MATLAB. Lastly, the MSDs of MCAK was plotted against the time interval, and the diffusion coefficient, D was obtained by a linear regression analysis ($<x^2>=2Dt$) in Origin 8.0 (OriginLab Corporation, USA).

The flux of MCAK reaching growing microtubule ends

The calculation of the flux of MCAK reaching growing microtubule ends by one-dimensional lattice-diffusion was performed according to Fick's first equation of diffusion (Helenius et al., 2006):

$$J = -D \cdot \partial c / \partial x \quad \text{(Eq.1)}$$

where J is the flux of MCAK reaching growing microtubule ends, $c(x, t)$ is the concentration of MCAK on microtubule lattice at position x from the growing microtubule end and time t.

$$\text{When } x = 0 \quad J_0 = -D \cdot c_\infty / x_0 \quad \text{(Eq. 2)}$$

Where

$$x_0 = (D/k_{\text{off}})^{1/2}$$

$$c(x \to \infty) = c_\infty = k_{\text{on}} \cdot C / k_{\text{off}}$$

Where C is the concentration of MCAK. Therefore, we can calculate the flux by taking the measured parameters (D, k_{on}, C and k_{off}) into the equation.

Localization analysis at growing microtubule ends

The analysis on the single-molecule localization at growing microtubule ends was performed as previously described (Song et al., 2020). Briefly, we determined the position of growing microtubule end by considering the microtubule lattice as a Gaussian wall and its end as a half-Gaussian. This model was used to fit the microtubule end and determined the position of microtubule end in every frame. The peak position of the half-Gaussian was used as the origin of the positional axis, which was used as a reference to measure the localization of individual single-molecule binding events. We estimated that the accuracy of microtubule end tracking was ~6 nm by measuring standard error of the distribution of the estimated error in microtubule end position (Demchouk et al., 2011). To measure the precise localization of individual binding events at growing microtubule ends, a Gaussian function was used to fit the intensity profile of individual molecules. We estimated that the accuracy of the measured position was ~2 nm by measuring standard error of the fitting peak location (Maurer et al., 2014). The peak location was recorded as the position of each molecule in every frame. The average peak location over the entire binding period was recorded as the position of the molecule. The positional distributions of GFP-MCAK, EB1-GFP, XMAP215-GFP at growing microtubule ends were plotted along the longitudinal axis of

microtubule. Finally, the plots of GFP-MCAK and XMAP215-GFP were fitted to a Gaussian function and the plots of EB1-GFP was fitted to an exponentially modified Gaussian function, from which we calculated the FWHM as an estimation for the length of the binding region of each molecule.

Microtubule polymerization

GMPCPP, GDP and GTPγS microtubules were polymerized as previously described (Manka and Moores, 2018). Briefly, GMCPP-microtubules were polymerized using a mixture of 10 μM tubulin, 1 mM GMPCPP (JenaBioscience, NU-405L) and 4 mM $MgCl_2$ in BRB80, which was incubated for 2 hr at 37°C. The polymerized microtubules were then collected using an Air-Driven Ultracentrifuge (Beckman, 340401), which were then resuspended in BRB80 and stored at 37°C. GDP-microtubules were polymerized using a mixture of 40 μM tubulin, 1 mM GTP (Roche, 10106399001), 4 mM $MgCl_2$ and 4% DMSO (Sigma, 276855). The mixture was incubated for 30 min at 37°C. The polymerized microtubules were collected using an Air-Driven Ultracentrifuge, resuspended in BRB80 with 20 μM taxol (Cell Signaling Technology, 9807) and stored at 37°C. GTPgS microtubules were obtained using two rounds of polymerizations. In the first round, a mixture of 40 μM tubulin, 2 mM GTPgS (Roche, 10220647001) and 4 mM $MgCl_2$ in BRB80 was incubated for 5 hours at 37°C. In the second round, 5 μl of the product from the first polymerization was mixed with additional 40 μM tubulin, 2 mM GTPgS and 4 mM $MgCl_2$ to make a final 25 μl reaction mixture. This mixture was incubated overnight at 37°C to obtain long GTPgS microtubules. The polymerized microtubules were collected using an Air-Driven Ultracentrifuge, resuspended in BRB80 with 20 mM taxol and stored at 37°C.

MCAK binding to different microtubules

Different nucleotide microtubules were first immobilized in flow cells. GFP-MCAK or its mutants were then added into the flow cell in the imaging buffer. Images were recorded every 300 ms with a 100 ms exposure. Imaging buffer: BRB80 supplemented with 1 mM ATP, ADP or AMPCPP, 20 μM taxol, 50 mM KCl, 80 mM D-glucose, 0.4 mg/ml glucose oxidase, 0.2 mg/ml catalase, 0.8 mg/ml casein, 1% β-mercaptoethanol, 0.001% Tween 20.

The binding intensity of GFP-MCAK on GMPCPP microtubule ends

To measure the binding intensity of GFP-MCAK on GMPCPP microtubule ends, an intensity profile of GFP-MCAK or the GMPCPP microtubule was plotted along the microtubule. The average binding intensity of GFP-MCAK of two pixels represents the end-binding intensity of GFP-MCAK at the corresponding position of the end, to the lattice from the midpoint of the microtubule intensity attenuation at the microtubule end.

Gamma fitting of microtubule lifetimes and catastrophe frequency analysis

The lifetime data of dynamic microtubules were fitted to the gamma density function using the dfittool toolbox in the MATLAB (Mathworks, USA). The probability-based catastrophe frequency is calculated as the ratio of the number of catastrophe events observed at a certain lifetime to the total number of microtubules that reached this lifetime (Gardner et al., 2011).

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Supplementary materials

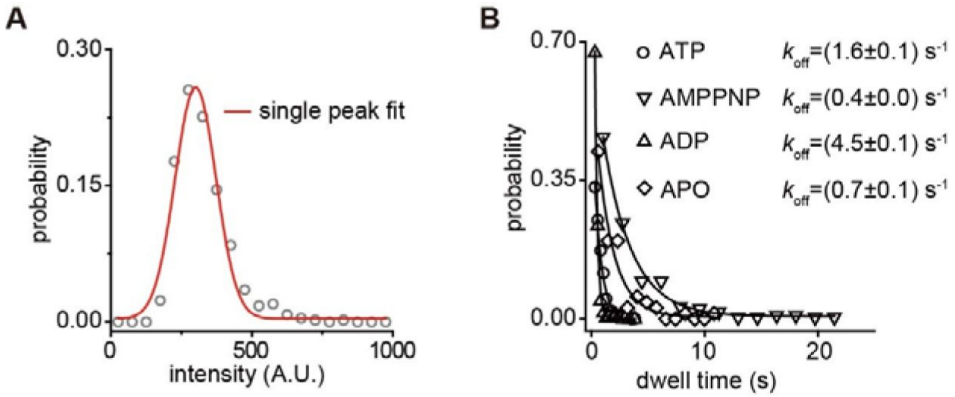

Figure s1.

Single-molecule fluorescence analysis and the apparent off-rates (k_{off}) of GFP-MCAK
(A) The fluorescence intensity distribution of GFP-MCAK (red, n=509 binding events) was fitted using a Gaussian function. The background intensity was subtracted. The intensity range (μ ± 2σ=300 ± 145 A.U.) was used to determine if a fluorescence spot represents a single dimer.
(B) The apparent off-rate (k_{off}) of GFP-MCAK on growing microtubule ends in the presence of ATP (minus end, square; plus end, circle), AMPPNP (down triangle), ADP (up triangle) and APO (diamond). k_{off} was calculated by fitting the dwell time of individual GFP-MCAK binding events to a single exponential function.

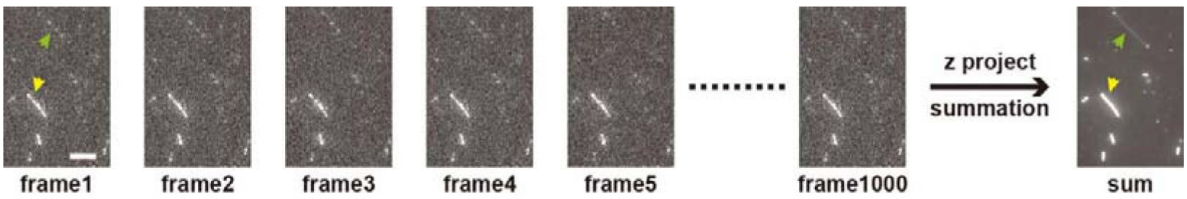

Figure s2.

Projection of single-molecule fluorescence images
The representative images showing how to generate a summation image using 1000 frames of raw images. The GDP and GTPγS microtubule were indicated using green and yellow arrowheads, respectively. The concentration of GFP-MCAK here was 1 nM. The experiment was performed in the presence of 1 mM AMPPNP. Scale bar: 5 μm.

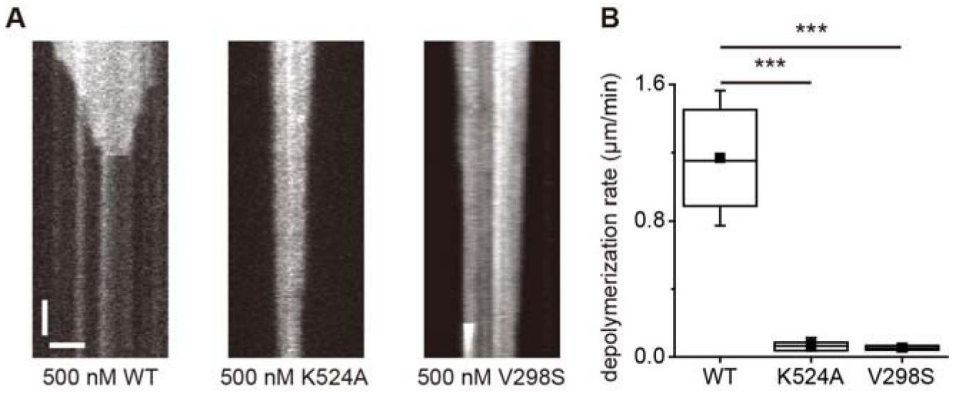

Figure s3.

GFP-MCAK^{K525A} and GFP-MCAK^{V298S} are depolymerizing-deficient mutants
(A) The representative kymographs showing the depolymerizing effect of GFP-MCAK, GFP-MCAK^{K525A} and GFP-MCAK^{V298S} on GMPCPP-stabilized microtubules in the presence of 1 mM ATP. Vertical bar: 100 s, horizontal bar: 2 μm.
(B) Statistical quantification of the depolymerization rates of GFP-MCAK (*n*=21 microtubules from 3 assays), GFP-MCAK^{K525A} (*n*=11 microtubules from 2 assays) and GFP-MCAK^{V298S} (*n*=52 microtubules from 3 assays). The data were presented as mean ± std. The statistical analysis was performed using the two-tailed Mann-Whitney U test with Bonferroni correction, ***, p<0.001.

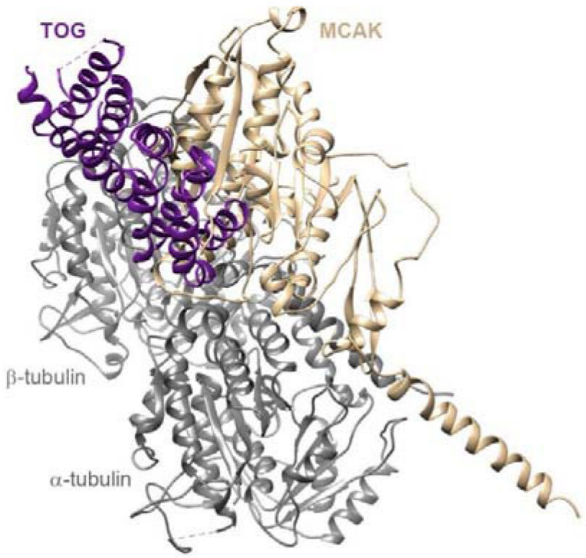

Figure s4.

Structural models showing the kinesin-13-tubulin contact site and the TOG-tubulin contact site
The structural model showing that the TOG domain (PDB ID: 4FFB) and the motor domain of MCAK (PDB ID: 6BBN) shared, to some extent, the binding site on a tubulin dimer.

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Reviewer #1 (Public Review):

Major concerns:

1. Is the direct binding of MCAK to the microtubule cap important for its in vivo function?

a. The authors claim that their "study provides mechanistic insights into understanding the end-binding mechanism of MCAK". I respectfully disagree. My concern is that the paper offers limited insights into the physiological significance of direct end-binding for MCAK activity, even in vitro. The authors estimate that in the absence of other proteins in vitro, ~95% of MCAK molecules arrive at the tip by direct binding in the presence of ~ physiological ATP concentration (1 mM). In cells, however, the major end-binding pathway may be mediated by EB, with the direct binding pathway contributing little to none. This is a reasonable concern because the apparent dissociation constant measured by the authors shows that MCAK binding to microtubules in the presence of ATP is very weak (69 uM). This concern should be addressed by 1) calculating relative contributions of direct and EB-dependent pathways based on the affinities measured in this and other published papers and estimated intracellular concentrations. Although there are many unknowns about these interactions in cells, a modeling-based analysis may be revealing. 2) the recapitulation of these pathways using purifying proteins in vitro is also feasible. Ideally, some direct evidence should be provided, e.g. based on MCAK function-separating mutants (GDP-Pi tubulin binding vs. catalytic activity at the curled protofilaments) that contribution from the direct binding of MCAK to microtubule cap in EB presence is significant.

b. As mentioned in the Discussion, preferential MCAK binding to tubulins near the MT tip may enhance MCAK targeting of terminal tubulins AFTER the MCAK has been "delivered" to the distal cap via the EB-dependent mechanism. This is a different targeting mechanism than the direct MCAK-binding. However, the measured binding affinity between MCAK and GMPCPP tubulins is so weak (69 uM), that this effect is also unlikely to have any impact because the binding events between MCAK and microtubule should be extremely rare. Without hard evidence, the arguments for this enhancement are very speculative.

2. The authors do not provide sufficient justification and explanation for their investigation of the effects of different nucleotides in MCAK binding affinity. A clear summary of the nucleotide-dependent function of MCAK (introduction with references to prior affinity measurements and corresponding MCAK affinities), the justifications for this investigation, and what has been learned from using different nucleotides (discussion) should be provided. My take on these results is that by far the strongest effect on microtubule wall and tip binding is achieved by adding any adenosine, whereas differences between different nucleotides are relatively minor. Was this expected? What can be learned from the apparent similarity between ATP and AMPPNP effects in some assays (Fig 1E, 4C, etc) but not others (Fig 1D,F, etc)?

3. It is not clear why the authors decided to use these specific mutant MCAK proteins to advance their arguments about the importance of direct tip binding. Both mutants are

enzymatically inactive. Both show roughly similar tip interactions, with some (minor) differences. Without a clear understanding of what these mutants represent, the provided interpretations of the corresponding results are not convincing.

4. GMPCPP microtubules are used in the current study to represent normal dynamic microtubule ends, based on some published studies. However, there is no consensus in the field regarding the structure of growing vs. GMPCPP-stabilized microtubule ends, which additionally may be sensitive to specific experimental conditions (buffers, temperature, age of microtubules, etc). To strengthen the authors' argument, Taxol-stabilized microtubules should be used as a control to test if the effects are specific. Additionally, the authors should consider the possibility that stronger MCAK binding to the ends of different types of microtubules may reflect MCAK-dependent depolymerization events on a very small scale (several tubulin rows). These nano-scale changes to tubulins and the microtubule end may lead to the accumulation of small tubulin-MCAK aggregates, as is seen with other MAPs and slowly depolymerizing microtubules. These effects for MCAK may also depend on specific nucleotides, further complicating the interpretation. This possibility should be addressed because it provides a different interpretation than presented in the manuscript.

5. It would be helpful if the authors provided microtubule polymerization rates and catastrophe frequencies for assays with dynamic microtubules and MCAK in the presence of different nucleotides. The video recordings of microtubules under these conditions are already available to the authors, so it should not be difficult to provide these quantifications. They may reveal that microtubule ends are different (or not) under the examined conditions. It would also help to increase the overall credibility of this study by providing data that are easy to compare between different labs.

6. Are there other published studies that report MCAK binding affinity to microtubules? I find it quite surprising that the authors have reported the apparent dissociation constant for MCAK as 1mM. Such a high Kd value suggests no interaction under normal conditions, given that the intracellular concentrations of most proteins are orders of magnitude lower. If this information is inaccurate, it raises questions about the accuracy of other quantifications in the study.

7. Experimental and data analysis techniques are described superficially, and in some cases, only references to the prior work by others are provided. More direct evidence for these techniques and the corresponding controls should be provided.

Reviewer #2 (Public Review):

Summary:
In this manuscript, Chen et al. investigate the localization of microtubule kinesin-13 MCAK to the microtubule ends. MCAK is a prominent microtubule depolymerase whose molecular mechanisms of action have been extensively studied by a number of labs over the last ~twenty years. Here, the authors use single-molecule approaches to investigate the precise localization of MCAK on growing microtubules and conclude that MCAK preferentially binds to a GDP-Pi-tubulin portion of the microtubule end. The conclusions are speculative and not well substantiated by the data, making the impact of the study in its current form rather limited. Specifically, greater effort should be made to define the region of MCAK binding on microtubule ends, as well as its structural characteristics. Given that MCAK has been previously shown to effectively tip-track growing microtubule ends through an established interaction with EB proteins, the physiological relevance of the present study is unclear. Finally, the manuscript does not cite or properly discuss a number of relevant literature references, the results of which should be directly compared and contrasted to those presented here.

Reviewer #3 (Public Review):

The authors revisit an old question of how MCAK goes to microtubule ends, partially answered by many groups over the years. The authors seem to have omitted the literature on MCAK in the past 10-15 years. The novelty is limited due to what has previously been done on the question. Previous work showed MCAK targets to microtubule plus-ends in cells through association with EB proteins and Kif18b (work from Wordeman, Medema, Walczak, Welburn, Akhmanova) but none of their work is cited.

It is not obvious in the paper that these in vitro studies only reveal microtubule end targeting, rather than plus end targeting. MCAK diffuses on the lattice to both ends and its conformation and association with the lattice and ends has also been addressed by other groups-not cited here. I want to particularly highlight the work from Friel's lab where they identified a CDK phosphomimetic mutant close to helix4 which reduces the end preference of MCAK. This residue is very close to the one mutated in this study and is highly relevant because it is a site that is phosphorylated in vivo. This study and the mutant produced here suggest a charge-based recognition of the end of microtubules.

Here the authors analyze this MCAK recognition of the lattice and microtubule ends, with different nucleotide states of MCAK and in the presence of different nucleotide states for the microtubule lattice. The main conclusion is that MCAK affinity for microtubules varies in the presence of different nucleotides (ATP and analogs) which was partially known already. How different nucleotide states of the microtubule lattice influence MCAK binding is novel. This information will be interesting to researchers working on the mechanism of motors and microtubules. However, there are some issues with some experiments. In the paper, the authors say they measure MCAK residency of growing end microtubules, but in the kymographs, the microtubules don't appear dynamic- in addition, in Figure 1A, MCAK is at microtubule ends and does not cause depolymerization. I would have expected to see depolymerization of the microtubule after MCAK targeting. The MCAK mutants are not well characterized. Do they still have ATPase activity? Are they folded? Can the authors also highlight T537 and discuss this?

Finally, a few experiments are done with MCAK and XMAP215, after the authors say they have demonstrated the binding sites overlap. The data supporting this statement were not obvious and the conclusions that the effect of the two molecules are additive would argue against competing binding sites. Overall, while there are some interesting quantitative measurements of MCAK on microtubules - in particular in relation to the nucleotide state of the microtubule lattice - the insights into end-recognition are modest and do not address or discuss how it might happen in cells. Often the number of events is not recorded. Histograms with large SEM bars are presented, so it is hard to get a good idea of data distribution and robustness. Figures lack annotations. This compromises therefore their quantifications and conclusions. The discussion was hard to follow and needs streamlining, as well as putting their work in the context of what is known from other groups who produced work on this in the past few years.