Complementary activities of TPX2 and chTOG constitute an efficient importin-regulated microtubule nucleation module

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Spindle assembly and function require precise control of microtubule nucleation and dynamics. The chromatin-driven spindle assembly pathway exerts such control locally in the vicinity of chromosomes. One of the key targets of this pathway is TPX2. The molecular mechanism of how TPX2 stimulates microtubule nucleation is not understood. Using microscopy-based dynamic in vitro reconstitution assays with purified proteins, we find that human TPX2 directly stabilizes growing microtubule ends and stimulates microtubule nucleation by stabilizing early microtubule nucleation intermediates. Human microtubule polymerase chTOG (XMAP215/Msp5/Stu2p/Dis1/Alp14 homologue) only weakly promotes nucleation, but acts synergistically with TPX2. Hence, a combination of distinct and complementary activities is sufficient for efficient microtubule formation in vitro. Importins control the efficiency of the microtubule nucleation by selectively blocking the interaction of TPX2 with microtubule nucleation intermediates. This in vitro reconstitution reveals the molecular mechanism of regulated microtubule formation by a minimal nucleation module essential for chromatin-dependent microtubule nucleation in cells.

The mitotic spindle ensures faithful chromosome segregation during cell division. In animal cells, spindles consist of a large number of microtubules and associated proteins. Spindle function requires tight control of microtubule nucleation and dynamics.

One protein that contributes to controlling microtubule mass in spindles is XMAP215 from Xenopus laevis. In vitro, purified XMAP215 acts as a microtubule polymerase by accelerating the addition of tubulin subunits to growing microtubule plus ends. In Xenopus egg extract (XEE), absence of XMAP215 markedly reduces microtubule mass, causing severe spindle defects. chTOG, the human homologue of XMAP215, has not been biochemically characterized yet. Its role in human cells seems to be less critical for proper spindle morphology as its depletion leads only to a moderate decrease in spindle length and microtubule density, raising the question of whether the activities of chTOG and XMAP215 are conserved.

Compared with the regulation of microtubule growth, the molecular mechanisms underlying microtubule nucleation are poorly understood. In spindles, centrosome-, chromatin-, kinetochore- and microtubule-dependent nucleation pathways work in parallel controlling microtubule nucleation. The chromatin-dependent pathway drives local stimulation of nucleation around chromosomes. A central player is the small GTPase Ran, which in its GTP-bound form (RanGTP) dissociates spindle assembly factors from their inhibitory interaction with nuclear transport receptors importins. RanGTP is produced locally around chromosomes. The resulting RanGTP concentration gradient generates a gradient of spindle assembly factors released from importins triggering microtubule nucleation and spindle assembly in meiosis and mitosis. The microtubule-dependent (branching) nucleation pathway, mediated by the augmin complex, is also controlled by RanGTP. A major Ran pathway target required for chromatin-stimulated and augmin-mediated microtubule nucleation and spindle assembly is TPX2 (targeting protein for Xklp2; refs 17–19). Depletion of TPX2 from XEE abolishes chromatin- and microtubule-dependent microtubule formation. Its expression in cells is tightly regulated. Reduction of TPX2 levels increases aneuploidy and elevates the frequency of spontaneous tumour development in mice; overexpression causes defects in microtubule organization and correlates with poor cancer prognosis and high metastasis frequency in humans. TPX2 is a multifunctional protein. Its central domain mediates interactions with importin-α (refs 23,24), explaining its Ran dependence. One or more potential microtubule-binding sites reside either in the amino terminus or the central part of TPX2, possibly overlapping with the importin-binding site. TPX2 interacts with several other proteins involved in spindle assembly.

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The N terminus of TPX2 activates and stabilizes aurora A kinase (AurA; refs 33–35). Whether TPX2 acts exclusively through AurA on microtubule nucleation is unclear with contradicting results having been reported17,21,26,36−39. Another important factor for Ran-dependent microtubule nucleation is the γ-tubulin ring complex40 (γ-TuRC), which is thought to facilitate nucleation by templating initial microtubule assembly40. TPX2-dependent AurA activation can stimulate γ-TuRC activity in XEE (ref. 39). However, the lack of TPX2 or γ-TuRC in chromatin-driven microtubule formation can be partially compensated independently of AurA, most efficiently by the excess of XMAP215 (ref. 37). This suggests that parallel pathways may exist, where multiple factors act on nucleation with distinct, yet partly overlapping activities. TPX2 enhances branching microtubule nucleation independently of its AurA interaction, but depending on its microtubule-binding ability41 pointing to a direct role of TPX2 in nucleation.

Early in vitro work reported that high concentrations of purified TPX2 induced the formation of tubulin aggregates and interconnected microtubule bundles23,26. Yet, it remains unknown whether at physiological levels purified TPX2 is sufficient to nucleate microtubules efficiently, or if additional factors are required. Kinetic measurements of how TPX2 affects microtubule dynamics and nucleation alone or in combination with other proteins implicated in localized microtubule formation are lacking. Therefore, the molecular mechanisms underlying the functionality of the protein network controlling chromatin-dependent microtubule nucleation, and particularly the role of TPX2 herein, its interplay with XMAP215/chTOG and its regulation, are poorly understood.

Here we studied the direct effects of human TPX2 and chTOG on microtubule nucleation and dynamics using real-time fluorescence microscopy-monitored in vitro reconstitutions. We find that TPX2 interacts preferentially with growing microtubule ends at binding sites that are distinct from other autonomous microtubule end trackers. TPX2 prevents microtubule catastrophes and slows down shrinkage thereby increasing microtubule lifetimes. At physiological concentrations, TPX2 strongly promotes microtubule nucleation by stabilizing microtubule nucleation intermediates. Fast and efficient microtubule formation requires the additional microtubule polymerase activity of chTOG, which on its own exhibits only moderate microtubule nucleating activity. Importins inhibit combined TPX2/chTOG-mediated nucleation by blocking the ability of TPX2 to interact with nucleation intermediates. Together, our in vitro reconstitutions define the molecular mechanism underlying the functional synergy of a minimal TPX2/chTOG microtubule nucleation module and its regulation by importins.

RESULTS

chTOG is a microtubule polymerase

We produced human chTOG (Fig. 1a,b) and characterized the biochemical activity of this member of the XMAP215 protein

![Figure 1](https://example.com/figure1.png)

**Figure 1** Human chTOG is a microtubule polymerase. (a) Scheme illustrating the domain structure of human chTOG. (b) Coomassie blue-stained SDS–PAGE gel showing 1 μg of purified recombinant chTOG constructs. (c) Scheme of the experimental set-up. (d) Dual-colour TIRFM kymographs showing Atto647N-labelled microtubules (magenta) growing in the absence or presence of 4 nM full-length chTOG–mGFP (green). (e) Plot of the mean growth speed as a function of the chTOG concentration. Data points, black; error bars are s.e.m.; hyperbolic fit (one-site binding)—magenta curve. Number of 25-s-growth intervals used to measure individual velocities for each chTOG concentration: 0 nM—1,003; 5 nM—966; 20 nM—661; 50 nM—499; 100 nM—510; 200 nM—600. Atto647N-labelled tubulin concentration in d and e was 7.5 μM. Data were pooled from two data sets. (f) Kymographs showing GMPCPP-stabilized Atto647N-labelled microtubules in the absence of soluble tubulin and either in the absence (left) or presence (right) of 100 nM chTOG. Scale bars as indicated. Unprocessed original scans of gels are shown in Supplementary Fig. 2.
Figure 2 The central part of human TPX2 determines its binding preference for growing microtubule ends. (a) Scheme of the human TPX2 constructs used in this study: full-length TPX2, N-terminally truncated TPX2 containing amino acids 274–747 (TPX2\textsuperscript{1N}), and a minimal TPX2 construct containing amino acids 274–659 (TPX2\textsuperscript{mini}). Regions known to interact with aurora A (AurA), and importin-\(\alpha\) (Imp-\(\alpha\)) are indicated, together with predicted coil coils (CC) and nuclear localization signal (NLS). (b) Coomassie blue-stained SDS–PAGE gel showing 1 \(\mu\)g of purified recombinant TPX2 constructs. (c,d) Single-channel and merged TIRFM images showing how mGFP–TPX2 (green in merge) at 5 nM (c) and 0.35 nM (d) binds either all along (c) or preferentially to the growing ends and the GMPCPP segment (d) of a growing Atto647N-labelled microtubule (magenta in merge; ‘\(\Lambda\)’ and ‘\(\Gamma\)’ indicate end binding; the GMPCPP ‘seed’ is marked by an arrowhead). (e) Kymographs depicting the time course of binding of 5 nM mGFP–TPX2 all along a growing microtubule. Atto647N-labelled tubulin concentration was 7.5 \(\mu\)M. (f) Kymographs depicting the time course of 0.35 nM mGFP–TPX2 binding to a growing microtubule end and the GMPCPP ‘seed’. Atto647N-labelled tubulin concentration was 12.5 \(\mu\)M. (g,h) Kymographs showing binding of 10 nM mGFP–TPX2\textsuperscript{1N} (g) and 33 nM mGFP–TPX2\textsuperscript{mini} (h) to dynamic microtubules (merged channels on the left, mGFP–TPX2 on the right). Atto647N-labelled tubulin concentration is 12.5 and 15 \(\mu\)M, respectively. (i,j) Kymographs showing that neither 1 nM full-length mGFP–TPX2 (i) nor 10 nM mGFP–TPX2\textsuperscript{mini} (j) binds to shrinking microtubules. Atto647N-labelled tubulin concentrations were 5 \(\mu\)M and 7.5 \(\mu\)M, respectively. For all kymograph pairs: merged channel—left, mGFP–TPX2 channel—right. Scale bars as indicated. Unprocessed original scans of gels are shown in Supplementary Fig. 2.

Family. Using a total internal reflection microscopy (TIRFM)-based in vitro assay (Fig. 1c) we investigated how chTOG fused to monomeric GFP (chTOG–mGFP) localized to dynamic microtubules and how it affected their growth. Microtubules were grown from immobilized microtubule ‘seeds’, which were stabilized by the non-hydrolysable GTP analogue GMPCPP, and elongated in the presence
Figure 3 Effect of full-length TPX2 and TPX2\textsuperscript{mini} on microtubule dynamic instability parameters. (a) Modified box-and-whiskers graph for the microtubule growth speeds in the absence (control) and presence of different concentrations of full-length mGFP–TPX2 and mGFP–TPX2\textsuperscript{mini}, as indicated. Number of observed microtubule growth episodes per condition: control—n = 150; mGFP–TPX2: 0.2 nM—n = 160, 1 nM—n = 150, 5 nM—n = 114; mGFP–TPX2\textsuperscript{mini}: 10 nM—n = 153, 50 nM—n = 121, 250 nM—n = 154. All events are from one data set each. (b) TIRFM kymographs showing dynamic Atto647N-labelled microtubules in the absence (control) or presence of 5 nM mGFP–TPX2 or 250 nM mGFP–TPX2\textsuperscript{mini}. Scale bars as indicated. (c-f) Bar graphs showing microtubule catastrophe (c) and rescue (d) frequencies, and box-and-whiskers graphs showing microtubule growth (e) and depolymerization (f) speeds, for control, 5 nM mGFP–TPX2, and 250 nM mGFP–TPX2\textsuperscript{mini}, as indicated. (g-i) Box-and-whiskers graphs showing microtubule lifetimes (g), depolymerization times (h) and depolymerization lengths (i) for the same conditions. Data for c-i were pooled from three data sets each. Total number of analysed events per condition: (c) catastrophes (total growth time in brackets): control—n = 757 (457,280 s), mGFP–TPX2—n = 209 (267,644 s), mGFP–TPX2\textsuperscript{mini}—n = 385 (409,675 s); (d) rescues (total depolymerization time in brackets): control—n = 612 (22,062 s), mGFP–TPX2—n = 209 (10,708 s), mGFP–TPX2\textsuperscript{mini}—n = 376 (13,130 s); (e) growth episodes: control—n = 863, 5 nM mGFP–TPX2—n = 271, 250 nM mGFP–TPX2\textsuperscript{mini}—n = 477; (f) depolymerization episodes: control—n = 756, 5 nM mGFP–TPX2—n = 211, 250 nM mGFP–TPX2\textsuperscript{mini}—n = 383. The same data were used in g-i (see Supplementary Note). Atto647N-labelled tubulin concentration was always 7.5 μM. Errors in bar graphs are s.e.m. For the modified box-and-whiskers plots the boxes range from 25th to 75th percentile, the whiskers span from 10th to 90th percentile, and the horizontal line marks the mean value. *P < 0.05; **P < 0.01; ***P < 0.001 (only shown for comparisons with control); determined for the comparison of mean values analysing raw data (Tukey’s test in conjunction with one-way ANOVA).

of Atto647N-labelled tubulin and GTP (ref. 41). Kymographs of dynamic microtubules showed that chTOG–mGFP accumulated at both growing and shrinking microtubule plus ends (Fig. 1d and Supplementary Video 1). chTOG also strongly accelerated microtubule growth, like other XMAP215 family members,\textsuperscript{34-44} in a dose-dependent manner saturating at ~200 nM chTOG with a 17-fold faster growth velocity (Fig. 1e). Similarly to XMAP215 (ref. 3), chTOG facilitated the depolymerization of GMPCPP-stabilized microtubules
in the absence of free tubulin (Fig. 1f). This in vitro characterization of human chTOG establishes it as a bona fide microtubule polymerase.

**TPX2 binds to dynamic microtubules with a preference for growing ends**

We purified full-length and truncated versions of mGFP-tagged human TPX2 (Fig. 2a,b), which seemed to be monomeric under our experimental conditions (Supplementary Table 1). TIRFM showed full-length TPX2 accumulating all along growing microtubules, as expected. This accumulation was already evident at low nanomolar concentrations (Fig. 2c,e). At sub-nanomolar concentrations, TPX2 bound preferentially to growing microtubule ends and to the GMPcpp-stabilized microtubule part (Fig. 2d,f and Supplementary Video 2). The GMPcpp lattice seems to induce a high-affinity conformation of the TPX2-binding site that is normally present at growing microtubule ends.

A truncated construct (mGFP–TPX2ΔN) lacking the N-terminal region that contains the AurA interaction site (Fig. 2a,b) bound more weakly to microtubules, but with the same preferences as full-length TPX2 (Fig. 2g). An even shorter construct (mGFP–TPX2ΔC) additionally lacking the carboxy terminus (Fig. 2a,b) retained identical binding preferences, but bound even more weakly (Fig. 2h). Therefore, the central part of the molecule is sufficient for the microtubule-binding specificity of TPX2, whereas the peripheral parts contribute to increasing binding affinity consistent with earlier reports of multiple microtubule-binding regions in the protein.25,26 Neither full-length mGFP–TPX2 nor mGFP–TPX2ΔC tracked shrinking microtubule ends (Fig. 2i,j).

**TPX2 increases the lifetime of microtubules**

TIRFM revealed that TPX2 had almost no effect on the growth speed of dynamic microtubules at concentrations ranging from microtubule end tracking conditions to complete lattice decoration (Fig. 3a). Nevertheless, the microtubule lifetimes doubled in the presence of TPX2 (Fig. 3b,g). This was mostly a consequence of a reduced catastrophe frequency (growth-to-shrinkage transitions; Fig. 3c and Supplementary Fig. 1) and depolymerization speed (Fig. 3f) under conditions when TPX2 bound all along the microtubule lattice. This slowdown of depolymerization increases the depolymerization time (Fig. 3i) augmenting the likelihood of rescues (shrinkage-to-growth transitions) during shrinkage (Fig. 3b), and reducing depolymerization episode lengths (Fig. 3h). mGFP–TPX2ΔC also inhibited catastrophes resulting in increased microtubule lifetimes, however at higher concentrations (Fig. 3c,g and Supplementary Fig. 1); it had no major effect on other dynamic instability parameters (Fig. 3d–f,h,i). In summary, full-length TPX2 increases microtubule lifetimes by acting as an anti-catastrophe factor and by slowing down depolymerization, in agreement with a recent report.45

**TPX2 recognizes a unique binding site at growing microtubule ends**

We analyzed the preferential binding of TPX2 to microtubule ends, using mostly TPX2ΔC, which constitutes the functional core of TPX2 with respect to its microtubule interaction. Averaged mGFP–TPX2ΔC intensity profiles demonstrated that TPX2 accumulated in a short region at growing microtubule ends (Fig. 4a). The length of this region increased with increasing growth speed, up to ~1 μm for the fastest tested condition (highest tubulin concentration; Fig. 4a), reminiscent of the binding region of the established microtubule-end-binding protein EB1 (refs 46–48). However, different from EB1 and other autonomous end binders, the length of the TPX2-binding region extended with the overall time the microtubule spent growing (Fig. 4b) and with increasing mGFP–TPX2ΔC concentrations (Fig. 4c).

Single-molecule experiments either in the absence or presence of an excess of Alexa647-labelled SNAP–TPX2ΔC (‘spike’ experiment, Fig. 4d, left and right, Supplementary Figs 2b and 3a) revealed that the TPX2 binding/unbinding turnover at the growing microtubule end was fast with a mean dwell time of 0.27 ms (1/koff with koff = 3.7 s⁻¹, Supplementary Fig. 3b). This suggests that the increase of the average length of the TPX2ΔC accumulation region with increasing growth speed and time, or TPX2ΔC concentration directly reflects an increase of the length of the underlying TPX2-binding region and a structural property of the growing microtubule end. Single-molecule turnover measured at increasing distances from the microtubule end (covering the TPX2ΔC accumulation region) also reflected the microtubule conformation change away from the growing end (increasing dissociation constant koff and decreasing association rate ron, depending on the TPX2ΔC concentration (Fig. 4e) and the duration of microtubule growth (Supplementary Fig. 3c)). Single-molecule turnover of mGFP–TPX2ΔC on GMPcpp microtubules, to which TPX2 binds with high preference, was similarly fast as on growing microtubule ends (Supplementary Fig. 3d,e). Full-length TPX2 turned over more slowly agreeing with its higher binding strength (Supplementary Fig. 3f), yet dynamically, indicating an absence of oligomerization or aggregation, in contrast to previous observations.23,39 At microtubule ends growing in the presence of GTP the mGFP–TPX2ΔC intensity fluctuated strongly (Fig. 4f). This may be a consequence of dynamic changes of the end structure of the unfinished tube as microtubules grow.49 Interestingly, TPX2 also tended to accumulate on bent microtubules (Fig. 4g). Moreover, unlike EB1 (ref. 50), TPX2 did not bind strongly along microtubules grown in the presence of another GTP analogue, GTPγS (Fig. 4h). Nevertheless, TPX2 still accumulated at the growing ends of GTPγS microtubules (Fig. 4i), further suggesting that it recognizes a unique structural feature not strictly coupled to the nucleotide state at the distal end of the growing microtubule.

These data demonstrate that the TPX2-binding region at growing microtubule ends has distinct properties compared with other autonomous end tracking proteins.

**Surface-immobilized chTOG mildly stimulates microtubule nucleation**

To test whether these autonomous end binders, chTOG and TPX2, which have complementary effects on microtubule dynamics, promote microtubule nucleation we developed a TIRFM-based assay for the real-time observation of microtubule formation. We produced biotinylated full-length chTOG and TPX2 and, for controls, a biotinylated monomeric kinesin rigor mutant (Kin1ΔC; Supplementary Fig. 2a). The proteins were attached in an oriented manner through NeutrAvidin to functionalized and polyethylene glycol (PEG)-passivated glass slides41 (Fig. 5a and Supplementary Fig. 4a,b). After addition of fluorescently labelled tubulin and GTP...
Figure 4 TPX2 binds to a unique binding region at growing microtubule ends. (a) Right: Images of 33 nM mGFP–TPX2<sup>mini</sup> (green in merge) at growing Atto647N-labelled microtubule ends (magenta in merge) at the indicated tubulin concentrations. Left: Averaged mGFP–TPX2<sup>mini</sup> intensity profiles at the end regions of microtubules growing in the presence of 33 nM mGFP–TPX2<sup>mini</sup> and the indicated Atto647N-labelled tubulin concentrations. (b) Averaged mGFP–TPX2<sup>mini</sup> intensity profiles at the end regions of microtubules growing in the presence of 33 nM mGFP–TPX2<sup>mini</sup> and 20 μM Atto647N-labelled tubulin for three different time windows after the start of growth. (c) Averaged mGFP–TPX2<sup>mini</sup> intensity profiles for the indicated mGFP–TPX2<sup>mini</sup> concentrations at 25 μM Atto647N-labelled tubulin. (d) Schematics depicting the assay conditions and TIRFM kymographs showing single-molecule binding events of 5 nM mGFP–TPX2<sup>mini</sup> (green in merge) to the ends of growing Atto665-labelled microtubules (blue in merge) in the absence or presence of 195 nM Alexa647-labelled SNAP–TPX2<sup>mini</sup> (magenta in merge). (e) Dissociation rate constant $k_{off}$ and association rate $r_{on}$, respectively, as determined for the conditions in (d) from the dwell and waiting time distributions of the single mGFP–TPX2<sup>mini</sup> molecule binding and unbinding events (Supplementary Fig. 3a–c and Supplementary Note) as a function of the distance from the growing microtubule plus end. Note that the increase in $k_{off}$ values and decrease in $r_{on}$ values is less steep in the presence of excess Alexa647-labelled SNAP–TPX2<sup>mini</sup>, agreeing with (c). Lower panel: The normalized total fluorescence intensity of all mGFP–TPX2<sup>mini</sup> binding events shows their steady-state distribution. (f) Kymographs and instantaneous intensity line scans at different times, as indicated, showing strong temporal fluctuations of the fluorescence intensity of mGFP–TPX2<sup>mini</sup> (25 nM, green in merge) at growing Atto647N-labelled microtubule ends (magenta in merge, 12.5 μM tubulin). (g) TIRFM images showing 33 nM mGFP–TPX2<sup>mini</sup> (green in merge, lower panels) accumulating strongly at the curved microtubule plus end, agreeing with (c). Lower panel: The normalized total fluorescence intensity of all mGFP–TPX2<sup>mini</sup> binding events shows their steady-state distribution. (h) Schematics depicting the assay conditions and TIRFM kymographs showing single-molecule binding events of 5 nM mGFP–TPX2<sup>mini</sup> (green in merge) to the ends of growing Atto647N-labelled microtubules (magenta in merge, 25 μM tubulin) growing in the presence of the slowly hydrolysable GTP analogue GTPγS. Scale bars as indicated.
Figure 5 Surface-immobilized TPX2 arrests nucleation intermediates, but in combination with chTOG efficiently nucleates microtubules. (a) Scheme of the experimental set-up for the 'surface' nucleation assay. (b) TIRFM images from a time series showing Atto647N-labelled microtubules and/or tubulin particles on a glass surface with immobilized biotinylated Kin1\textsuperscript{rigor} mutant, chTOG or TPX2 after pre-incubation at the indicated concentrations. Insets on the right depict magnified images of the same surface at 7.5 min. (c) Time courses of the mean Atto647N–tubulin fluorescence intensities measured at the surface for the entire field of view for different chTOG, TPX2 and Kin1\textsuperscript{rigor} densities. Concentrations of biotinylated proteins used for surface incubation as indicated. The same Kin1\textsuperscript{rigor} curve is shown as a control for both chTOG and TPX2 plots. Note that the fluorescence signal typically represents the sum of several different tubulin species: soluble tubulin in the TIRF field (creating part of the background), immobilized individual tubulins (for example, bound to chTOG), tubulin ‘stubs’ (bound to TPX2) and microtubules (bound to chTOG or TPX2). Raw intensities including background are shown. (d) Time series of TIRFM images showing nucleation and growth of Atto647N-labelled microtubules on surfaces with immobilized biotinylated TPX2 (pre-incubated at 125 nM, top three rows) or, for controls, with a biotinylated Kin1\textsuperscript{rigor} (pre-incubated at 125 nM, bottom two rows) in either the absence or presence of 100 nM untagged chTOG, as indicated. As the microtubule density is high at the TPX2 surface in the presence of chTOG, the same time series is also shown with reduced contrast for better visualization of individual microtubules. Atto647N-labelled tubulin concentration was always 12.5 M. Scale bars as indicated. $t=0$ when the sample is placed at 30°C.
to surface-immobilized chTOG, a few microtubule nucleation and growth events were observed (Fig. 5b, second and third row and Supplementary Video 3), similar to previous observations with XMAP215 (ref. 51). This effect was specific, because no microtubules were observed on a Kin11<sup>1<sup>iso</sup></sup> control surface (Fig. 5b, top row and Supplementary Video 3), and it proportionally increased with chTOG concentration (Fig. 5c, left and Supplementary Fig. 4c).

**Surface-immobilized TPX2 stabilizes and arrests nucleation intermediates**

When biotinylated TPX2 was surface-immobilized, a granular mass of tubulin (`stubs') was recruited to the surface (Fig. 5b, bottom two rows and Supplementary Video 3), very different from the observations with immobilized chTOG. Only few microtubules were observed after more than 10 min. Accumulation of stubs occurred over several minutes (Fig. 5c, right) in a dose-dependent manner increasing with higher TPX2 densities (Fig. 5b,c, right and Supplementary Fig. 4b,c) and increasing tubulin concentrations (Supplementary Fig. 4d,e). As TPX2 does not interact strongly with soluble tubulin dimers (Supplementary Fig. 4f), it may specifically stabilize larger tubulin assemblies on the surface that could represent nucleation intermediates<sup>52-54</sup>. However, these structures failed to transform into elongating microtubules, raising the question of whether additional activities could overcome the apparent arrest of microtubule formation.

**The combined action of immobilized TPX2 and soluble chTOG promotes efficient microtubule nucleation**

Remarkably, addition of chTOG to the assay with surface-immobilized TPX2 caused strong microtubule nucleation and growth within minutes (Fig. 5d, second and third row and Supplementary Video 4), in marked contrast to the absence of chTOG (Fig. 5d, first row). When immobilized TPX2 was replaced by biotinylated Kin1<sup>1<sup>iso</sup></sup>, only few microtubules formed in the presence of soluble chTOG (Fig. 5d, fourth and fifth row and Supplementary Video 4), similar to the effect of immobilized chTOG alone (Fig. 5b, second and third row), emphasizing the specificity of TPX2 activity in combination with chTOG. The microtubule-end-binding protein EB1 could not functionally substitute for chTOG in the nucleation assay with surface-immobilized TPX2, potentially owing to lack of a growth promoting effect under these conditions, and mostly only stubs were observed (Supplementary Fig. 5).

These results demonstrate that the combined actions of immobilized TPX2 and chTOG synergistically stimulate efficient microtubule nucleation and growth. It seems that on the TPX2-coated surfaces, chTOG can overcome a hindered transformation of nucleation intermediates into microtubules.

**In solution TPX2 nucleates microtubules more efficiently than chTOG**

To test whether immobilization of TPX2 prevents the transformation of TPX2-induced nucleation intermediates, we modified the nucleation assay (Fig. 6a). We pre-incubated biotinylated TPX2 together with tubulin and GTP in solution before transferring the reaction mixture onto a functionalized NeutrAvidin-coated glass surface, followed by TIRFM imaging. Strikingly within minutes, biotinylated TPX2 induced massive nucleation of slowly growing microtubules (Fig. 6b, middle row and Supplementary Video 5). Hence, in solution, TPX2-induced nuclei can transform into microtubules, a conformational change that is apparently inhibited when nuclei form directly on a TPX2 surface (Fig. 5b, bottom two rows). In contrast to TPX2, biotinylated chTOG promoted the nucleation of only a few, fast growing microtubules in this ‘solution’ nucleation assay (Fig. 6b, top row and Supplementary Video 5), similar to observations in the ‘surface’ assay (Fig. 5b, second and third row). Nucleation promoting activities of both proteins were dose-dependent (Fig. 6c,d), with TPX2 promoting the appearance of an increasingly denser mass of shorter microtubules as opposed to fewer longer microtubules by chTOG (Fig. 6c). Hence, under these conditions in solution, TPX2 is considerably more efficient in promoting microtubule nucleation than chTOG, although TPX2 hardly accelerates microtubule growth.

**The central part of TPX2 is sufficient to stimulate microtubule nucleation**

TPX2<sup>mini</sup> also stimulated microtubule nucleation in solution in a dose-dependent manner (Fig. 7a, top two rows), however requiring higher concentrations than full-length TPX2, agreeing with its weaker microtubule binding (Fig. 2). Combining TPX2<sup>mini</sup> and chTOG again further amplified microtubule formation (Fig. 7a, bottom row). Moreover, similarly to full-length TPX2, the minimal construct as well as TPX2<sup>2N</sup> also seem to promote microtubule nucleation through stabilization of microtubule nucleation intermediates as suggested by the recruitment of ‘stubs’ when only surface-immobilized protein is present in the assay (Fig. 7b and Supplementary Fig. 7 and Supplementary Video 6). Therefore, the central part of TPX2 that contains its core microtubule-binding domain and the importin interaction region also harbours its basic microtubule nucleating activity.

**Importin-α/β regulates synergistic TPX2:chTOG-dependent microtubule nucleation by interfering with the binding of TPX2 to nucleation intermediates**

In XEEs, the nucleating activity of TPX2 is inhibited by importin-α/β heterodimer (refs 19,23,26). The mechanism of how this interaction inhibits TPX2 is still debated. Therefore, we produced human importin-α and -β (Supplementary Fig. 2d) and investigated whether importins are sufficient to regulate the efficiency of TPX2:chTOG-dependent microtubule nucleation.

In our nucleation assay with immobilized Kin1<sup>1<sup>ref</sup></sup> on the surface, the addition of importins to chTOG had no effect on the mild nucleation promoting effect of chTOG (Fig. 8a, first and second row and Supplementary Video 7). The combination of full-length mGFP–TPX2 and chTOG caused pronounced microtubule nucleation and growth (Fig. 8a, third row and Supplementary Video 7), as in the previous experiments (Figs 5d and 6b). mGFP–TPX2 decorated
Figure 6 In solution TPX2 nucleates microtubules more efficiently than chTOG. (a) Scheme of the experimental set-up for the ‘solution’ nucleation assay. (b) Time series of TIRFM images showing Atto647N-labelled microtubules that nucleated in solution for 1 min in the presence of nucleation factors as indicated, followed after binding to a NeutrAvidin-coated surface through the biotinylated protein present as indicated. (c) TIRFM images showing examples of the concentration-dependent nucleation efficiencies of chTOG- and TPX2-mediated microtubule nucleation in the ‘solution’ nucleation assay. (d) Time courses of mean Atto647N–tubulin fluorescence intensities measured for the entire field of view in ‘solution’ nucleation assay at different biotinylated chTOG and TPX2 concentrations as indicated. Note that the fluorescence signal typically represents the sum of several different tubulin species (see note in legend of Fig. 5c). Raw intensities including background are shown. Atto647N-labelled tubulin concentration was always 12.5 nM. Other protein concentrations and scale bar as indicated.

The nucleated microtubules (Fig. 8a, third row), as expected for this TPX2 concentration (Fig. 2c). Addition of excess importin-α/β to TPX2 and chTOG strongly suppressed nucleation (Fig. 8a, fourth row) down to levels observed with chTOG alone (Fig. 8a, first and second rows), demonstrating that importins indeed inhibit synergistic TPX2:chTOG-dependent microtubule nucleation. TPX2 association with the few nucleated microtubules was severely reduced (Fig. 8a, bottom row). Importins also blocked the interaction of TPX2 with nucleation intermediates as their recruitment by surface-immobilized TPX2 was severely suppressed by importins (Fig. 8b). Thus, the inhibition of TPX2:chTOG-mediated microtubule nucleation by importins is a consequence of interfering with the stabilization of nucleation intermediates by TPX2.

DISCUSSION

How microtubule nucleation is controlled during cell division is a major open question. Multiple proteins whose interrelations are poorly understood at present are required for the Ran-dependent chromatin- and augmin-stimulated nucleation pathways in spindle assembly. Our TIRFM-based in vitro reconstitutions show that the Ran
assembly efficient nucleation is combined with fast growth to achieve the rapid production and maintenance of a large dynamic microtubule mass. The minimal TPX2:chTOG microtubule nucleation module harbours these critical activities. Importins control this module by inhibiting TPX2-mediated stabilization of nucleation intermediates, providing a potential mechanism for Ran-dependent localization of microtubule nucleation in vivo.

Microtubule ‘nuclei’ may be viewed as growing microtubule end regions preceding the closed tube (Fig. 8c). Therefore, understanding how TPX2 and chTOG localize to and act on growing microtubule ends can provide insight into the molecular mechanism of how these two proteins facilitate microtubule nucleation. In contrast to short-lived nucleation intermediates growing ends can be readily observed at steady state. Whereas chTOG showed the conserved behaviour of a microtubule polymerase, TPX2 suppressed microtubule catastrophes and reduced the depolymerization speed, strongly stabilizing dynamic microtubules without having a strong effect on the growth speed. Hence, these proteins affect different microtubule dynamic instability parameters, acting in a complementary manner.

Autonomous microtubule end binding as observed for chTOG is well established for this protein family, however, it has not been described for TPX2 before. TPX2 behaved very differently from other end binders with characteristics that help to explain its role in microtubule nucleation. First, TPX2 stabilizes its own binding sites at growing microtubule end regions by slowing down their transformation into mature lattice sites. This is likely to be at the origin of its stabilizing effects on dynamic microtubules and nucleation intermediates. Second, TPX2 has a remarkably high preference for binding to GMPCPP microtubules. GMPCPP is considered the most GTP-like nucleotide analogue, and it strongly promotes microtubule nucleation and stabilization. The longitudinal intertubulin spacing in GMPCPP microtubules is longer than that of GDP microtubules. This spacing or another feature of a high-affinity conformation of the yet unknown TPX2-binding site exhibited by the GMPCPP lattice may possibly reflect a structure present at growing microtubule ends and nucleation intermediates with a favourable curvature, potentially different from that of the fully closed tube. This view is supported by the stronger binding of TPX2 to bent microtubules. Such curvature sensing was proposed for the neuronal microtubule-stabilizing protein doublecortin and XMAP215 family microtubule polymerases.

In summary, TPX2 preferentially recognizes a conformation that is absent in mature microtubules and may well be related to the curved tapered or sheet-like extensions observed at growing ends and to the still poorly understood structure of microtubule ‘nuclei’. Interestingly, immobilized TPX2 can arrest the transformation of stabilized nucleation intermediates into microtubules, suggesting that a large-scale conformational change such as tube closure is required. This closure can be promoted by the growth enhancing activity of chTOG, suggesting that longer protofilaments with extended lateral contacts could facilitate the curvature change from nucleation intermediate to a microtubule.

Our in vitro reconstitution of a synergistic nucleation module consisting of TPX2 and chTOG can explain why in XEE, both TPX2 and XMAP215 are required for Ran-dependent microtubule formation around chromatin and why TPX2 facilitates

Figure 7 The central region of TPX2 is sufficient to stimulate microtubule nucleation. (a) Time series of TIRFM images of the ‘solution’ nucleation assay where microtubules nucleated in solution for 1 min in the presence of different concentrations of biotinylated TPX2 and in the additional presence of untagged chTOG, followed after binding to a NeutrAvidin-coated surface. (b) Time series of TIRFM images of the ‘surface’ nucleation experiments with immobilized biotinylated TPX2 (pre-incubated at 500 nM) in the absence (top row) or presence (bottom row) of untagged chTOG. Atto647N-labelled tubulin concentration was always 12.5 µM. Other protein concentrations and scale bars as indicated. t = 0 when the sample is placed at 30 °C.

pathway target TPX2 and the Ran-independent chTOG are two key players that cooperate in efficient microtubule nucleation.

We showed here that chTOG supported microtubule formation in solution rather weakly, presumably by its growth promoting activity. TPX2 however strongly stimulated nucleation probably by directly stabilizing early nucleation intermediates. Differences from previous in vitro work may well be explained by different assay conditions. Here under real-time imaging conditions (no fixatives), physiological concentrations of TPX2 (100 nM in XEE; ref. 19) strongly stimulated nucleation albeit with slow microtubule growth. During spindle
Figure 8 Regulation of TPX2- and chTOG-stimulated microtubule nucleation by importins. (a) Time series of TIRFM images showing microtubule nucleation and growth on surfaces with immobilized biotinylated rigor kinesin (pre-incubated at 125 nM) always in the presence of 100 nM chTOG and Atto647N-labelled tubulin, without and with additional 500 nM importin-α/β complex (first and second row, respectively), 100 nM mGFP–TPX2 (third row) or both 500 nM importin-α/β and 100 nM mGFP–TPX2 (fourth row) as indicated. Plots showing the time course of the mean Atto647N-labelled microtubule (magenta) and mGFP–TPX2 (green) intensities for the same experiments are shown next to each individual times series of images. Note that the mGFP–TPX2 signal declines at later time points possibly owing to depletion of TPX2 from solution due to binding to the efficiently nucleated growing microtubules. (b) Time series of TIRFM images showing that compared with a control (left), inclusion of importin-α/β (500 nM) inhibits stabilization of Atto647N-labelled nucleation intermediates by surface-immobilized biotinylated TPX2 (pre-incubated at 125 nM; right image pair). Plot showing the time course of the mean Atto647N-labelled tubulin intensities on the surface (right). Atto647N-labelled tubulin concentration was always 12.5 μM. Scale bars as indicated. t = 0 when the sample is placed at 30°C. (c) Model of synergistic TPX2- and chTOG-stimulated microtubule nucleation and growth. Different reactions, all promoting nucleation and growth, are regulated by the distinct and complementary activities of TPX2 and chTOG.
microtubule nucleation from XMAP215 beads in this experimental system, Ran-GTP-driven microtubule nucleation in cells and in XEE also requires the master nucleator γ-TuRC (ref. 37), which provides a structural template for assembling the helical microtubule lattice. Post-translational modifications or interactions with binding/targeting partners inducing conformational changes in γ-TuRC have been proposed to modulate its activity. To integrate previous observations in a model, conceptually, the process of microtubule nucleation in cells may be divided into three phases: initial templated assembly of tubulins onto the γ-TuRC surface; growth of a nucleation intermediate consisting of many tubulins that might resemble the tapered or sheet-like structure of growing microtubule ends; closure of this intermediate assembly into a tube with typical GDP lattice characteristics.

This scenario would allow for multiple opportunities to regulate the nucleation efficiency: first, at the level of the γ-TuRC-mediated templating activity, for example by one of the indirect effects of TPX2—phosphorylation of γ-TuRC interaction partners by TPX2-activated AurA (ref. 39); second, at the level of the nucleation intermediate that extends from the γ-TuRC template by the direct stabilization of the unfinished lattice by TPX2 together with acceleration of its elongation by chTOG (Fig. 8c). Interestingly, both of these proteins were recently also reported to facilitate the initiation of growth from stable microtubule nucleation templates in vitro. Finally, microtubule nucleation efficiency could be controlled at the level of protofilament growth and initial tube closure. Different types of regulatory input from overlapping pathways could easily be integrated in such a model: global regulation by constitutively active players such as chTOG, local regulation from the Ran pathway through the direct and indirect activities of TPX2, and temporal control from cell cycle-dependent post-translational modifications.

In the future, the extension of the in vitro reconstitution of the minimal nucleation module studied here including additional regulatory factors will allow the systematic dissection of the individual contributions of the different proteins. Together, this will lead to a systems level understanding of the complex microtubule nucleation network and spindle assembly.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

J.R. and T.S. designed the study; J.R. generated the reagents and performed the experiments; J.R. and N.I.C. analysed the data; J.R. and T.S. wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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METHODS

Cloning and protein biochemistry. The full-length protein coding sequence of human TPX2 (amino acids 1–747) was amplified by PCR using its cDNA as a template (NM_012112.4, Origin), and cloned into modified pFastBacHis-based vectors as described previously. The His tag had been replaced with a StrepTagI (WSHPQFEK) (pFastBacSTREP), and either a monomeric green fluorescent protein (mGFP; refs 63,64), or a SNAP-tag coding sequence (NEB) had been inserted after the TEV protease cleavage site present in the original vector. This enabled the construction of expression plasmids for N-terminal fusion proteins of TPX2: StrepTagII–mGFP–G–A–TPX2 and StrepTagI–SNAP–G–A–TPX2 in which StrepTagII could be cleaved off by TEV protease treatment (Supplementary Table 2), final protein products being referred to as mGFP–TPX2, mGFP–TPX2°, and SNAP–TPX2 and SNAP–TPX2°, respectively. To generate TPX2, TPX2°, and TPX2° expression constructs that could be biotinylated, the respective coding regions were cloned into a modified pFastBacDual vector under the control of a polyclinoid promoter to form an N-terminal fusion with biotin acceptor peptide (BAP: GLNDIIFAEQK) followed by a G–linker, monomeric blue fluorescent protein coding sequence (mTagBP, Evrogen), and another G–linker resulting in BAP–G–mTagBP–G–A–TPX2 (or TPX2°, or TPX2°, respectively; Supplementary Table 2), referred to as biotinylated TPX2, biotinylated TPX2°, or biotinylated TPX2°, respectively (Supplementary Table 2). The F10 promoter in the same pFastBacDual vector drives the expression of the chTOG storage buffer. The cell lysate was added to the storage buffer with HiPrep 26/10 Desalting columns to remove the d-desthiobiotin. Next, the protein was again run over the StrepTrap HP column. The flow-through was pooled, concentrated with Vivaspin 15R concentrators (30,000 MWCO), ultrafiltrated (278,083,10 min, 4 °C), and separated by size-exclusion chromatography using a Superose 6 10/300 GL column. The peak fractions were pooled, concentrated, and ultracentrifuged, aliquoted, snap frozen, and stored in liquid nitrogen.

For the purification of in vivo biotinylated chTOG protein, the clarified lysate was buffer exchanged with HiPrep 26/60 Desalting columns to fresh lysis buffer to remove biotin. The lysate was then incubated with immobilized monomeric avidin resin (Thermo Scientific). Resin was washed with lysis buffer, followed by protein elution with lysis buffer supplemented with 2 mM biotin and protease inhibitors. The buffer was then exchanged back to chTOG storage buffer with HiPrep 26/10 Desalting columns to remove the d-desthiobiotin. Next, the resin was again run over the StrepTrap HP column. The flow-through was pooled, concentrated with Vivaspin 15R concentrators (30,000 MWCO), ultrafiltrated (278,083,10 min, 4 °C), and separated by size-exclusion chromatography using a Superose 6 10/300 GL column. The peak fractions were pooled, concentrated, and ultracentrifuged, aliquoted, snap frozen, and stored in liquid nitrogen.

The fusion protein was expressed in the Escherichia coli BL21 prl strain at 18 °C for 16 h induced by 0.1 mM IPTG in the presence of 7 μg ml−1 biotin to facilitate biotinylation of the protein. To purify biotinylated Kin18pro protein, the cells were resuspended in lysis buffer (50 mM Na-phosphate, 350 mM KCl, 5 mM MgCl2, 0.2 mM ATP, 1 mM EDTA, 1 mM 2-ME, pH 7.2) supplemented with protease inhibitors and DNAse 1, 0.2 mM ATP, 1 mM EDTA, 1 mM 2-ME, pH 7.2 supplemented with protease inhibitors and DNAse I and lysed using a microfluidizer. The lysate was centrifuged by ultracentrifugation (183,960 g, 45 min, 4 °C) and loaded on the StrepTrap HP column. After washing the column with chTOG storage buffer, proteins were eluted with the same buffer supplemented with 2.5 mM D-desthiobiotin. The N-terminal StrepTagII was cleaved off by overnight incubation with TEV protease on ice. The buffer was then changed back to chTOG storage buffer with HiPrep 26/10 Desalting columns to remove the D-desthiobiotin.

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Hiss-2tag-Importin and Hiss-2tag could be removed by TEV protease treatment (Supplementary Table 2).

Importin-α and β fusion proteins were expressed and purified in a similar manner to each other. Protein expression was induced in Escherichia coli BL21 pRIL by 0.1 mM IPTG at 18°C for 16 h. To purify either labelled proteins, cells were resuspended in lysis buffer (50 mM HEPES, 400 mM KCl, 10 mM MgCl₂, 2.5 mM EDTA, 25 mM sucrose, 2 mM imidazole, 5 mM 2-ME, pH 7.2) supplemented with protease inhibitors and DNase I and lysed using a microfluidizer. The lysate was clarified by ultracentrifugation (185,960g, 45 min, 4°C) and then incubated with Protoni Ni-TEC resin (Machery-Nagel). After washing the resin with lysis buffer, proteins were eluted with elution buffer (50 mM HEPES, 300 mM KCl, 2 mM MgCl₂, 2.5 mM EDTA, 25 mM sucrose, 400 mM imidazole, 5 mM 2-ME, pH 8.0). The protein-containing fractions were pooled and the buffer was immediately exchanged to importin storage buffer (50 mM HEPES, 300 mM KCl, 2 mM MgCl₂, 250 mM sucrose, 5 mM 2-ME) with PD-10 Desalting columns (GE Healthcare). The N-terminal His-2tag was cleaved off by overnight incubation with TEV protease on ice. The sample was subsequently concentrated using Vivaspin 15 concentrators, followed by ultracentrifugation (278,083 g, 10 min, 4°C) and separation by size-exclusion chromatography using a Superdex 200 16/60 column (GE Healthcare). The peak fractions were pooled, concentrated, ultracentrifuged, aliquoted, snap frozen, and stored in liquid nitrogen.

Porcine brain tubulin was purified as described previously and for fluorescence microscopy assays labelled with Atto656-N-hydroxysuccinimide ester ( NHS- Sigma-Aldrich), or Atto647-N- NHS ester (Sigma-Aldrich), or biotin-NHS ester (Thermo Scientific) according to published methods.

The sequences of all newly cloned constructs were verified by sequencing. All protein preparations were carried out at 4°C. Protein concentrations were determined either using a Bradford protein assay, or for tubulin, by measuring the absorbance at 280 nm. Protein concentrations refer to monomers, except for tubulin concentrations, which refer to dimers.

Size-exclusion chromatography multi-angle light scattering analysis (SEC-MALS) of TPX2 constructs. TPX2, TPX2N, TPX2C and bovine serum albumin (Sigma-Aldrich) were buffer exchanged into SEC-MALS buffer containing 50 mM HEPES (pH 7.5), 150 mM KCl, 2 mM MgCl₂, 50 mM arginine, 50 mM glutamate, 0.005% (vol./vol.) Brij-35, 5 mM 2-ME and ultracentrifuged (278,083 g, 10 min, 4°C). The samples (40 µl volume, at 2–3 g/ml concentration) were then subjected to size-exclusion chromatography using Superose 6 3.2 300 (GE Healthcare) in conjunction with MALS (Dawn+4+OptiLab t-REX; Wyatt) at room temperature.

Three independent runs were performed for each construct.

Size-exclusion chromatography to test tubulin:TPX2 interaction. Tubulin and TPX2 buffer were exchanged to an ice-cold buffer containing 80 mM PIPES (pH 6.8), 60 mM KCl, 1 mM MgCl₂, 1 mM EGTA, 0.005% (vol./vol.) Brij-35, 5 mM 2-ME. The protein was then ultracentrifuged (278,083 g, 10 min, 4°C) and diluted in the same buffer either alone or in combination at the indicated concentrations. These samples were incubated on ice for 15 min before loading (400 µl volume) on a Superose 6 Increase column (GE Healthcare) at 4°C. The tubulin:TPX2 interaction experiment was performed once for each tubulin:TPX2 ratio.

Total internal reflection fluorescence (TIRF) microscopy. Flow chambers for assays were assembled from bovine–polyethylene glycol (PEG)-functionalized glass and poly(t-lysine)-PEG (SuSoS)-passivated corner glass as described previously. All experiments were imaged at 30°C ± 1°C on a TIRF microscope (iMIC, FEI Munich) described in detail previously unless indicated otherwise. Image acquisition and channel alignment were carried out as explained previously. Exposure times were between 60 ms (single molecule assays) and 150 ms (nucleation assays). Images were acquired either after every 60 ms (single-molecule imaging on dynamic microtubules), 100 ms (single-molecule imaging on GMPPCP-stabilized microtubules), 250 ms (localization studies, microtubule end tracking experiments) or 2 s (microtubule lifetime measurements, nucleation assays). Laser powers as well as exposure times and acquisition frame rate were kept constant within a set of experiments to allow for direct comparisons between different conditions.

GMPPCP-stabilized microtubules labelled microtubule ‘seeds’ for assays with dynamic microtubules were prepared as described previously (containing 12% of either Atto647N- or Atto565-labelled tubulin). The assay itself is a modification of the protocol developed earlier. In short, a flow chamber was sequentially incubated for 5 min with 5% Pluronic F-127 in MQ water (Sigma-Aldrich) at room temperature, washed with assay buffer (AB: 80 mM PIPES, 60 mM KCl, 1 mM EGTA, 1 mM MgCl₂, 1 mM EDTA, 1 mM GTP, 5 mM 2-ME, 0.15% (vol/vol) Brij-35, 0.02% (vol/vol) Brij-35) supplemented with 50 µg/ml κ-casein (Sigma-Aldrich), and then incubated for 2 min on a metal block on ice in the same buffer additionally containing 50 µg/ml of NeurAvidin (Life Technologies). The chamber was subsequently washed with AB and then incubated with AB containing an appropriate dilution of GMPPCP-stabilized microtubule ‘seeds’ for 3 min at room temperature. Following additional washes with AB, a final assay mix (below) was flowed into the chamber and imaging was started 1 min after placing the flow chamber on the microscope. Composition of the final assay mix: 75% (vol./vol.) AB, oxygen scavengers (180 µg ml⁻¹ catalase (Sigma-Aldrich), 750 µg ml⁻¹ glucose oxidase (Serva)) diluted in BRB80 (80 mM PIPES, 1 mM EGTA, 1 mM MgCl₂), varying concentrations of tubulin (containing 5% of either Atto647N- or Atto565labelled tubulin) in BRB80, and different mGFP–TPX2 or Alexa647-labelled SNAP–TPX2, or chTOG and chTOGmGFP constructs diluted in their storage buffers.

To keep the buffer composition of the final assay mix unchanged within a set of experiments and to allow for direct comparisons between experiments, the overall BRB80 and storage buffer content was kept constant within one set of experiments.

For single-molecule assays on GMPPCP microtubules samples were prepared as described above for the dynamic microtubule assays; however, GFP and tubulin were omitted from the final assay mix.

GMPPCP microtubule depolymerization assay was performed essentially as initially described elsewhere. Samples were prepared as summarized for the dynamic microtubule assays, using the same buffers and sequence of incubation steps; however, GFP and tubulin were omitted from the final assay mix.

For ‘surface’ microtubule nucleation assay a flow chamber was sequentially incubated for 5 min with 5% Pluronic F-127 in MQ water at room temperature, washed with AB (above) supplemented with 50 µg ml⁻¹ κ-casein, and then incubated for 2 min on a metal block on ice in the same buffer additionally containing 50 µg ml⁻¹ of NeurAvidin. The chamber was subsequently washed with TPX2 storage buffer (described above) and then incubated with an indicated concentration of protein constructs (bionitinated Kιin(1-595), bionitinated chTOG, bionitinated TPX2, bionitinated TPX2C, or bionitinated TPX2C(1-595)) diluted either in TPX2 storage buffer (Figs 5a and 8a and Supplementary Fig. 4a–e), or in AB (Figs 5d and 7b and Supplementary Fig. 7) for 10 min on a metal block on ice. Following washes with AB at room temperature, a final assay mix (below) was flowed into the chamber, which was then sealed with nail polish. Imaging was started 1 or 2 min after placing the chamber on the microscope.

Composition of the final assay mix: 75% (vol./vol.) AB, oxygen scavengers (180 µg ml⁻¹ catalase, 750 µg ml⁻¹ glucose oxidase) diluted in BRB80, 12.5 µM tubulin (containing 5% Atto647N-labelled tubulin) in BRB80, 1 mg ml⁻¹ bovine serum albumin (Sigma-Aldrich) in BRB80, and the indicated concentrations of mGFP–TPX2, chTOG, or EB1 diluted in their own storage buffers, and importin-α/β complexes, which had been individually buffer exchanged to AB using Zeba Micro Spin Desalting Columns (7K MWCO, Thermo Scientific), and then premixed in AB. When the effect of importins on microtubule nucleation was tested in the presence of surface-immobilized TPX2 (Fig. 8b), an additional 3 min incubation step with 500 nM importin-α/β complex (buffer exchanged as described above) at room temperature was included before adding the final assay mix. This additional step allows us to verify the absence or presence of 500 nM importin-α/β. Similarly to the dynamic microtubule assays, the buffer composition of the final assay mix remained unchanged to allow for direct comparisons between different experiments within one set of experiments.

The solution nucleation assay was based on the ‘surface’ nucleation assay with the following modifications. While the flow chamber was incubated with Pluronic F-127 for 10 min, the final assay mix, as in the ‘solution’ nucleation assay, but now also including the bionitinated protein (either chTOG or TPX2) at the indicated concentration, was mixed on ice and ultracentrifuged (278,083 g, 10 min, 4°C). In parallel, the flow chamber was washed with AB (described above) supplemented with 50 µg ml⁻¹ κ-casein, and then incubated for 2 min on a metal block on ice with the same buffer that additionally contained 50 µg ml⁻¹ of NeurAvidin. The flow chamber was then washed with AB at room temperature and placed in a microscope heating box at 30°C to initiate nucleation in solution. After 1 min this nucleation mix was quickly flowed through the pre-warmed flow chamber. The chamber was sealed with nail polish. Imaging was started 1.5 min after placing the chamber on the microscope.

Data analysis and image processing are described in detail in the Supplementary Note.

Reproducibility. Experiments were repeated three times, unless indicated otherwise. Representative images are shown. The number of events (number of microtubule growth and depolymerization episodes, rescues and catastrophes, total polymerization and depolymerization time) measured for calculating microtubule dynamic instability parameters for different conditions, as well as statistics information, is included in the figure legends. In cases in which data for quantifications from less than three data sets were used (Figs 1e and 3a and Supplementary Figs 1a and 5b), the data sets themselves were large and the described effects were observed reproducibly (dose-dependent growth...
promoting effect of chTOG, lack of growth promotion by TPX2 or EB1 under these conditions, and the prevention of microtubule minus end catastrophes by TPX2). Corresponding information about TPX2 microtubule-end-binding analysis (presented on Fig. 4 and Supplementary Fig. 3) can be found in the Supplementary Note.

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Supplementary Figure 1. TPX2 reduces catastrophes at the microtubule minus ends. Modified box-and-whiskers plot showing the microtubule minus end catastrophe frequencies in the absence (control) and presence of 5 nM full-length mGFP-TPX2 and 250 nM mGFP-TPX2\text{mini}, as indicated. Number of measured microtubule minus ends (total): control – n=93, mGFP-TPX2 – n=63, mGFP-TPX2\text{mini} – n=54. Number of catastrophes (total): control – n=608, mGFP-TPX2 – n=168, mGFP-TPX2\text{mini} – n=153. Microtubule growth time (total): control – 395,972 s, mGFP-TPX2 – 276,060 s, mGFP-TPX2\text{mini} – 235,408 s. For the modified box-and-whiskers plot the boxes range from 25th to 75th percentile, the whiskers span from 10th to 90th percentile, the horizontal line marks the mean value. Data were pooled from two datasets. Errors are SEM. * p ≤ 0.05; (only displayed for comparisons with control); determined for the comparison of mean values analysing raw data (Tukey’s test in conjunction with One Way ANOVA).
Supplementary Figure 2 Purified recombinant proteins used in this study. Uncropped Coomassie Blue-stained SDS-PAGE gels of (a) biotinylated human TPX2, TPX2\textsuperscript{N}, and TPX2\textsubscript{mini} and similarly tagged monomeric Drosophila kinesin-1 rigor mutant and human chTOG. Note that all biotinylated TPX2 constructs and the kinesin-1 mutant have a BAP-mTagBFP fused to their N-termini. The chTOG contains an mTagBFP-BAP tag at its C-terminus. The double bands visible for the constructs are not due to the protein degradation but a likely a consequence of different folding and/or maturation states of mTagBFP running with different molecular weights (as observed for mCherry, see for example\textsuperscript{65}). Mass-spectrometry analysis revealed very similar peptide coverage for faster and slower migrating forms of each protein displaying these double bands (data not shown). (b) Human SNAP-TPX2 and SNAP-TPX2\textsubscript{mini}. (c) Human chTOG, chTOG-mGFP, and human EB1. (d) Human importin α and importin β. 1 µg of protein is loaded in all cases. Note that parts of Supplementary Fig. 2c are also depicted on Fig. 1b.
Supplementary Figure 3 Single molecule characterisation of TPX2 binding to growing microtubule ends and to GMPCPP microtubules. (a) Example plots showing the time course of the measured fluorescence intensity, the calculated transition probability and the binarised probability of mGFP-TPX2\textsuperscript{mini} at a growing microtubule end. (b) Single molecule dwell time and waiting time distributions of 5 nM mGFP-TPX2\textsuperscript{mini} at growing microtubule ends (conditions as in Fig. 4e), with mono-exponential fits (magenta). (c) Average spatial distribution of SNAP-TPX2\textsuperscript{mini} single molecule fluorescence intensities for two different time windows after start of microtubule growth; this agrees with similar measurements performed using microtubule end tracking and comet analysis (Fig. 4b). Averages of 139,000 (< 4 minutes) and 160,000 (> 4 minutes) frames were used to generate the curves. (d) Kymographs showing 50 pM mGFP-TPX2\textsuperscript{mini} (green in merge) binding to GMPCPP-stabilised Atto565-labelled microtubules (blue in merge) either in the absence or presence of additional 181 nM Alexa647-labelled SNAP-TPX2\textsuperscript{mini} (magenta in merge), always in the absence of free tubulin. (e) The dissociation rate constant $k_{off}$ and association rate $r_{on}$ for the conditions shown in (d) demonstrate that also in the presence of excess TPX2, turnover remains dynamic (7,327 and 6,029 binding events, respectively). (f) Kymographs showing 10 pM of full-length mGFP-TPX2 (green in merge) binding to GMPCPP-stabilised Atto565-labelled microtubules (blue in merge) either in the absence (left) or presence (right) of additional 11 nM Alexa647-labelled SNAP-TPX2 (magenta in merge), both in the absence of free tubulin. Scale bars as indicated.
Supplementary Figure 4  Surface-immobilised TPX2 induces microtubule 'stub' formation in a TPX2 and tubulin concentration dependent manner. 
(a) TIRF microscopy images of flow chamber surfaces pre-incubated with 125 nM of biotinylated mTagBFP-tagged proteins visualised by mTagBFP fluorescence. These fields of view correspond to the ones depicting the Atto647N-labelled tubulin channel at the same protein concentrations (125 nM Kin1<sup>rigor</sup>, 125 nM chTOG, and 125 nM TPX2) on Fig. 5b. Scale bar as indicated. 
(b) Quantification of surface densities of biotinylated proteins at different concentrations based on mTagBFP-fluorescence. Three different fields of view were imaged for each condition after monitoring the microtubule nucleation using Atto647N-tubulin channel for experiments depicted on Fig. 5b-c. t = 0 when the sample is placed at 30°C. 
(c) Quantification of Atto647N-tubulin intensities on different biotinylated protein surfaces (same as Fig.-s 5c and Supplementary Fig. 4b) at 15 min time point. t = 0 when the sample is placed at 30°C. 
(d) Images of time series of TIRF microscopy images of Atto647N-labelled tubulin particles on a glass surfaces pre-incubated with 125 nM biotinylated TPX2 at increasing tubulin concentrations. Scale bar as indicated. 
(e) Plots of quantified time courses of the mean Atto647N-labelled tubulin intensities measured for the whole field of view at different tubulin concentrations as shown on Supplementary Fig. 4d. 
(f) Size-exclusion chromatography profiles showing TPX2, tubulin, and combinations of TPX2 and tubulin eluting from Superose 6 Increase column. Protein concentrations and peak elution volumes as indicated.
**Supplementary Figure 5** The combined action of TPX2 and EB1 does not stimulate efficient microtubule nucleation and growth in ‘surface’ nucleation assay. (a) Time series of TIRF microscopy images showing nucleation and growth of Atto647N-labelled microtubules on surfaces with immobilised biotinylated TPX2 (pre-incubated at 125 nM) in the absence (top row) or presence of 100 nM chTOG (middle row), or 100 nM human EB1 (bottom row). Atto647N-labelled tubulin concentration was 12.5 µM. Scale bar as indicated. t = 0 when the sample is placed at 30°C. (b) Modified box-and-whiskers graph showing the microtubule growth speeds measured for immobilised dynamic microtubules, as in Fig. 3, growing in microtubule nucleation assay buffer in the presence of 7.5 µM tubulin and microtubule binding proteins at the indicated concentrations, as also used in the microtubule nucleation assays in Supplementary Fig. 5a. Number of 25 s microtubule growth intervals observed to calculate the mean growth speeds for each condition: control – n=590, 100 nM EB1 – n=617, 100 nM TPX2 – n=379, 100 nM chTOG – n=213. All events are from one dataset each. For the modified box-and-whiskers plot boxes range from 25th to 75th percentile, the whiskers span from 10th to 90th percentile, the horizontal line marks the mean value. *** p ≤ 0.001 (only displayed for comparisons with control); determined for the comparison of mean values analysing raw data (Tukey’s test in conjunction with One Way ANOVA).
Supplementary Figure 6 EB1 activity does not synergise with TPX2 or chTOG in stimulating microtubule formation in the ‘solution’ nucleation assay. (a) Time series of TIRF microscopy images showing Atto647N-labelled microtubules that nucleated in solution in the presence of biotinylated TPX2 for 1 min followed by binding to neutravidin-coated surfaces via biotinylated TPX2 in the absence (first row) or presence (second row) of untagged EB1. (b) Time series of TIRF microscopy images as in (a), but now with biotinylated chTOG instead of biotinylated TPX2 in the absence (first row) or presence (second row) of untagged EB1. Atto647N-labelled tubulin concentration was always 12.5 µM. Other protein concentrations and scale bars as indicated.
**Supplementary Figure 7** N-terminally truncated TPX2 promotes microtubule nucleation when combined with chTOG in the 'surface' nucleation assay. Time series of TIRF microscopy images showing nucleation and growth of Atto647N-labelled microtubules on surfaces with immobilised biotinylated TPX2ΔN (pre-incubated at 125 nM) in the absence (first row) or presence of 100 nM untagged chTOG, as indicated. Tubulin concentration was 12.5 μM. Scale bar as indicated.
**Supplementary Table 1**

SEC-MALS analysis of recombinant TPX2 constructs

| Protein | Predicted size [kDa] (based on primary aa sequence) | Calculated size [kDa] (average of 3 SEC-MALS measurements)** |
|---------|-----------------------------------------------------|-------------------------------------------------------------|
| TPX2   | 88.8                                                | 98.3 ± 7.2                                                  |
| TPX2ΔN | 58.2                                                | 63.0 ± 2.6                                                  |
| TPX2mini | 47.8                                               | 53.7 ± 0.6                                                  |
| BSA    | 66.6                                                | 68.0 ± 2.0                                                  |

*all TPX2 constructs used in this assay contain an N-terminal StrepTagII and TEV protease cleavage site.

** errors are SD.
Supplementary Table 2
Protein expression constructs generated in this study

| Name           | Detailed description                                      |
|----------------|-----------------------------------------------------------|
| pJR218         | pETMz-HsImpβ                                               |
| pJR221         | pETMz-HsImpα                                               |
| pJR235         | pFastBacSTREP-HsTPX2                                       |
| pJR243         | pFastBacSTREP-mGFP-HsTPX2                                  |
| pJR245         | pFastBacSTREP-SNAP-HsTPX2                                  |
| pJR284         | pFastBacDual-BirA; BAP-mTagBFP-HsTPX2                      |
| pJR292         | pFastBacSTREP-mGFP-HsTPX2ΔN                               |
| pJR295         | pFastBacDual-BirA; BAP-mTagBFP-HsTPX2ΔN                    |
| pJR297         | pFastBacSTREP-HsTPX2ΔN                                     |
| pJR310         | pFastBacSTREP-mGFP-HsTPX2Δmini                            |
| pJR311         | pFastBacSTREP-SNAP-HsTPX2Δmini                            |
| pJR312         | pFastBacDual-BirA; BAP-mTagBFP-HsTPX2Δmini                 |
| pJR313         | pFastBacSTREP-HsTPX2Δmini                                  |
| pJR314         | pFastBacSTREP-HschTOG-mGFP                                |
| pJR315         | pFastBacSTREP-HschTOG                                      |
| pJR316         | pFastBacDual-BirA; HschTOG-mTagBFP-BAP                     |
| pCT001         | pRSFDuet1-BAP-mTagBFP-DmKin1rigor; BirA                    |
Supplementary Video Legends

Supplementary Video 1. Human chTOG is a microtubule polymerase. 4 nM human chTOG-mGFP (right, green) binds to the growing end of Atto647N-labelled microtubule (magenta) and increases its growth speed (right), compared to the control microtubule in the absence of chTOG-mGFP (left). Tubulin concentration was 7.5 μM. Time is in minutes. Scale bar is 3 μm. This movie relates to Fig. 1d.

Supplementary Video 2. Localisation of mGFP-TPX2 on dynamic microtubules. 5 nM mGFP-TPX2 (left, green in merge) binds all along the lattice of Atto647N-labelled growing microtubules (magenta in merge). 0.25 nM mGFP-TPX2 (right, green in merge) binds to the growing ends of Atto647N-labelled microtubules (magenta in merge) and GMPCPP stabilised microtubule “seed” regions. The lower panels show the mGFP-TPX2 channel only. Tubulin concentration was 7.5 μM. Time is in minutes. Scale bars are 3 μm. This movie relates to Fig. 2c-f.

Supplementary Video 3. Surface-immobilised TPX2 arrests nucleation intermediates. Microtubule nucleation and growth of Atto647N-labelled microtubules on a surface with immobilised biotinylated Kin1\textsuperscript{Rig} control (pre-incubated at 125 nM, left), with biotinylated chTOG (pre-incubated at 125 nM, middle), or with biotinylated TPX2 (pre-incubated at 125 nM, right), as indicated. Atto647N-labelled tubulin concentration was always 12.5 μM. Time is in minutes. t = 0 when the sample is placed on the microscope at 30°C. Scale bars are 6 μm. This movie relates to Fig. 5b.

Supplementary Video 4. The combined action of TPX2 and chTOG synergistically stimulates efficient microtubule nucleation and growth. Microtubule nucleation and growth of Atto647N-labelled microtubules that were nucleated in solution in the presence of 25 nM biotinylated chTOG (left), 25 nM biotinylated TPX2 (middle), and 25 nM biotinylated TPX2 and 100 nM untagged chTOG (right) at 30°C for 1 min, followed by binding to neutravidin-coated surfaces via the biotinylated protein. Atto647N-labelled tubulin concentration was always 12.5 μM. Time is in minutes. t = 0 when the sample is placed on the microscope at 30°C. Scale bars are 6 μm. This movie relates to Fig. 5d.

Supplementary Video 5. In solution TPX2 nucleates microtubules more efficiently than chTOG. Nucleation and growth of Atto647N-labelled microtubules on a surface with immobilised biotinylated TPX2\textsuperscript{ΔN} (pre-incubated at 125 nM) in the absence (left) or presence (right) of 100 nM chTOG, as indicated. Atto647N-labelled tubulin concentration was 12.5 μM. Time is in minutes. t = 0 when the sample is placed on the microscope at 30°C. Scale bars are 6 μm. This movie relates to Fig. 7b and Supplementary Fig. 7.

Supplementary Video 6. N-terminal region of TPX2 is not required for synergistic TPX2/chTOG-dependent efficient microtubule nucleation and growth. Nucleation and growth of Atto647N-labelled microtubules on a surface with immobilised biotinylated TPX2\textsuperscript{ΔN} (pre-incubated at 125 nM) in the absence (left) or presence (right) of 100 nM chTOG, as indicated. Atto647N-labelled tubulin concentration was 12.5 μM. Time is in minutes. t = 0 when the sample is placed on the microscope at 30°C. Scale bars are 6 μm. This movie relates to Fig. 8a.

Supplementary Video 7. Regulation of TPX2 and chTOG-stimulated microtubule nucleation by importins. Microtubule nucleation and growth on a surface with immobilised biotinylated rigor kinesin (pre-incubated at 125 nM) always in the presence of 100 nM chTOG and 12.5 μM Atto647N-labelled tubulin (magenta), without (first movie from left) and with additional 500 nM importin α/β complex (second movie from left), 100 nM mGFP-TPX2 (green, third movie from left), or both 500 nM importin α/β and 100 nM mGFP-TPX2 (green, 4th movie from left), as indicated. Time is in minutes. t = 0 when the sample is placed on the microscope at 30°C. Scale bars are 6 μm. This movie relates to Fig. 8a.
SUPPLEMENTARY NOTE

Images and kymographs
Images were processed and analysed using the Fiji package of ImageJ, and MATLAB. If necessary, raw images were corrected for microscope stage drift using the Image Stabilizer plugin (K. Li). Individual frames of time-lapse movies, kymographs (space-time plots) and intensity profiles are presented in the figures. To generate the kymographs shown in Fig. 3b, groups of five consecutive image frames were first averaged and then used for kymograph analysis. Background subtracted movies were used to generate the microtubule channel kymographs in Fig. 4d, and for individual frames of microtubules and mGFP-TPX2\textsuperscript{mini} in Fig. 4g. To generate the kymographs in Supplementary Fig.-s 3d and 3f, five image frames were acquired for the Atto647N-tubulin channel, followed by a continuous stream of images in the mGFP-TPX2 channel. For the display of the kymograph in the tubulin channel, the frames were averaged and this average was used for the entire kymograph to indicate the initial microtubule position. To facilitate the visualisation of the forming dense microtubule mass images and movies displaying ‘solution’ nucleation (Fig.-s 6, 7a, Supplementary Fig. 6), a background image was generated from the first frame of each movie using Fiji (5 or 10 pixel rolling ball radius, sliding paraboloid). This background was subsequently subtracted from each frame of the movie. Mean Atto647N-labelled tubulin intensities for these experiments (Fig. 6d) were measured from untreated movies.

Analysis of microtubule dynamics
Microtubule dynamic instability parameters were determined by kymograph analysis. Mean microtubule growth speeds were calculated as the average of the speeds of either individual growth episodes (from start of growth to catastrophe) (Fig. 3), or of 25 s segments of continuous growth in the case of more variable growth (Fig. 1e, Supplementary Fig. 5b). Mean microtubule depolymerisation speeds were calculated as the average of the speeds of individual depolymerisation episodes (from catastrophe to the end of depolymerisation). Catastrophe frequencies were calculated as the number of events per total microtubule growth time. Rescue frequencies were calculated as the number of rescue events per total microtubule depolymerisation time. Mean microtubule lifetimes and mean depolymerisation times were calculated as the average of the duration of individual growth or shrinkage episodes, respectively. Mean depolymerisation lengths were calculated as the average of the distance of individual depolymerisation episodes. The total number of growth and
depolymerisation episodes, their total times (durations), and the total number of catastrophes and rescues measured in each case is stated in the respective figure legend.

**Microtubule end tracking and averaged fluorescence intensity comets**

Averaged fluorescence intensity profiles of mGFP-TPX2mini at microtubule ends as shown in Fig.-s 4a-c were generated as described previously. In brief, growing microtubules were tracked using an automated routine to identify the growing microtubule end position using a two-dimensional fitting procedure. Images acquired simultaneously in the Atto647N-labelled microtubule and the mGFP-TPX2mini channel were cropped around the position of the microtubule end and averaged. Averaged one-dimensional line profiles along the microtubule axis were created for the TPX2mini intensity from these cropped images (Fig. 4a-c). Number of frames averaged for each individual condition: Fig. 4a – 3,963 frames for 10 µM tubulin, 3,509 frames for 15 µM tubulin, 7,593 frames for 20 µM tubulin; Fig. 4b – 4,000 frames for 2 – 4 min time period, 5,000 frames for 4 – 6 min time period, 5,500 frames for 6 – 8 min time period; Fig. 4c – 17,000 frames for 25 nM mGFP-TPX2mini concentration; 18,000 frames for 75 nM mGFP-TPX2mini concentration.

**Single molecule analysis**

Single molecule binding/unbinding rate analysis was performed as follows. For each movie, a 50 pixel rolling-ball background subtraction was applied to each frame. A 3 pixel wide line was drawn along microtubules to generate kymographs of each channel. To generate single molecule intensity traces at different defined distances from the microtubule end, the position of the growing microtubule end was first marked in the kymographs by eye with a 3 pixel wide line; this line was then shifted down the microtubule away from the end by 2 pixels, and the GFP intensity trace with time was measured along the line. This was repeated with the line at 6 pixels (720 nm), 16 pixels (1920 nm), and 26 pixels (3120 nm) from the microtubule end (Fig. 4e, grey boxes), generating a separate time trace in each case. This procedure was repeated for at least 40 microtubules for each sample, using 7 different samples. The intensity traces for each profile position were combined, giving final traces 17,400 s long at a sampling rate of 16.3 fps (Supplementary Fig. 3a, top).

Each trace was analysed using the SMART single molecule analysis program, assuming a two state system with Poisson noise statistics; this program uses a Hidden Markov Model to produce a transition probability trace (Supplementary Fig. 3a, middle). A MATLAB script was then used to extract histograms of the dwell times of each binding event (Supplementary...
Fig. 3b, left) and the waiting times between successive binding events (Supplementary Fig. 3b, right). The total numbers of detected events in Fig. 4e for the mGFP-TPX2<sup>mini</sup> were 10,185; 9,100; 4,080; and 1,700; at successive positions from the microtubule end (Fig. 4e, upper panel). For the ‘spike’ experiment (in the presence of Alexa647-labelled SNAP-TPX2<sup>mini</sup>), the corresponding numbers were 9,786; 8,577; 8,911; and 4,695 (Fig. 4e, middle panel). The event histograms are well fit with mono-exponential decays, as expected for stochastic Poisson unbinding and binding processes, giving the characteristic rates $k_{\text{off}}$ and $r_{\text{on}}$, respectively. The measured on-rate was corrected for missed events with an additional factor $\exp(r_{\text{on}}*t_0)$, where $t_0$ is the lower time threshold for detecting events, taken to be $\sim1$ s from the graphs.

Average TPX2 spatial intensity profiles were generated from single molecule data of mGFP-TPX2<sup>mini</sup> and Alexa647-labelled SNAP-TPX2<sup>mini</sup> at the growing microtubule end (Fig. 4e, Supplementary Fig. 3c): each kymograph was straightened and re-centred using the previously marked microtubule end positions; all kymographs were then grouped corresponding to times pre- and post- 4 min of growth, and each group aligned and averaged. The intensities were averaged for all time points at each position along the resulting kymographs, giving the time-averaged spatial intensity profiles.

SUPPLEMENTARY NOTE REFERENCES

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