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Functional Overlap of Microtubule Assembly Factors in Chromatin-Promoted Spindle Assembly

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Distinct pathways from centrosomes and chromatin are thought to contribute in parallel to microtubule nucleation and stabilization during animal cell mitotic spindle assembly, but their full mechanisms are not known. We investigated the function of three proposed nucleation/stabilization factors, TPX2, γ-tubulin and XMAP215, in chromatin-promoted assembly of anastral spindles in *Xenopus laevis* egg extract. In addition to conventional depletion-add back experiments, we tested whether factors could substitute for each other, indicative of functional redundancy. All three factors were required for microtubule polymerization and bipolar spindle assembly around chromatin beads. Depletion of TPX2 was partially rescued by the addition of excess XMAP215 or EB1, or inhibiting MCAK (a Kinesin-13). Depletion of either γ-tubulin or XMAP215 was partially rescued by adding back XMAP215, but not by adding any of the other factors. These data reveal functional redundancy between specific assembly factors in the chromatin pathway, suggesting individual proteins or pathways commonly viewed to be essential may not have entirely unique functions.

INTRODUCTION

It has long been recognized that both centrosomes and chromosomes nucleate and organize microtubules during spindle assembly (Inoue and Ritter, 1975; Inoue, 1981). Some systems (e.g., *Caenorhabditis elegans* zygote mitosis) appear to largely use the centrosome pathway, and others (e.g., egg meiosis in animals, and higher plant mitosis) rely upon the chromosomal pathway, but many cells may use both in parallel (Ozlu *et al.*, 2005; Basto *et al.*, 2006; Basto and Pines, 2007; Heald *et al.*, 1997; Khodjakov *et al.*, 2000; Binarova *et al.*, 2006).

In recent years, the field has tried to identify protein factors that function only, or at least primarily, in one of the two pathways. The centrosome pathway requires both γ-tubulin and XMAP215, both of which localize strongly to centrosomes (Stearns and Kirschner, 1994; Popov *et al.*, 2002). γ-tubulin is usually viewed as the central player in nucleating at centrosomes, while XMAP215 and its tumor overexpressed gene family homologues are thought to more generally regulate microtubule plus-end dynamics (Slep and Vale, 2007). The chromatin pathway is thought to depend on local activation of microtubule assembly-promoting factors near chromatin by RanGTP (Carazo-Salas *et al.*, 1999; Carazo-Salas *et al.*, 2001; Wilde *et al.*, 2001). TPX2 is released from sequestration by RanGTP and is considered central to microtubule assembly in this pathway (Gruss *et al.*, 2001), though the precise role in nucleation, plus-end dynamics and stabilization is still unclear. TPX2 was proposed to nucleate microtubules and activate Aurora-A kinase (Schatz *et al.*, 2003; Tsai *et al.*, 2003). A TPX2 related protein also functions at centrosomes in *C. elegans* (Ozlu *et al.*, 2005), but in general TPX2 has been regarded as a canonical player in the chromatin pathway.

To further dissect the chromatin pathway in the absence of centrosomes, we added chromatin-coated beads to *Xenopus* egg extracts arrested in metaphase of meiosis II (Heald *et al.*, 1996). We took the standard immunodepletion/add-back approach to investigate the function of different microtubule assembly factors, but with a new twist. In addition to adding back only the factor we depleted, we also tested the effect of adding extra amounts of other assembly factors. Surprisingly, we were able in some cases to rescue the function of a factor previously considered to play a unique and essential function with a different factor, providing new insights into the molecular function of each factor, and the process of chromatin-promoted spindle assembly.

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Abbreviations used: HuRP, hepatoma up-regulated protein; MCAK, mitotic centromere-associated kinesin; MT, microtubule; TOG, tumor overexpressed gene; TPX2, targeting protein of Xklp2; γ-tubulin, gamma tubulin.

MATERIALS AND METHODS

Xenopus *Egg Extracts*

CSF extracts (CSF) were prepared from *Xenopus laevis* eggs and spindles were assembled as described previously (Murray, 1991; Desai *et al.*, 1999a) except the extract was filtered through a 0.8 mm filter (Millipore, Billerica, MA) before use to remove large particles. Spindles were assembled by addition of 1–2 ml of DNA-coated beads cycled from interphase (with addition of 0.4 mM $CaCl_2$) for 120 min before addition of CSF to drive the extract back into metaphase (Heald *et al.*, 1996). After 45 min, chromatin-beads were isolated from the extract and resuspended in fresh CSF extract. All experiments were repeated 2–3 times using different extract preparations.

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Time-Lapse Fluorescence Microscopy

Tubulin was visualized with addition of purified bovine tubulin (20 μg/ml) directly labeled with X-rhodamine (Invitrogen, Carlsbad, CA) as described previously ((Hyman *et al.*, 1991), (Sawin and Mitchison, 1991)). TPX2 was imaged by addition of approximately 20 nM of purified human GFP-TPX2 to extracts before spindle assembly, as previous described (Groen *et al.*, 2008). γ-tubulin were imaged by addition of 10 μg/ml Alexa-488 (Invitrogen) directly-labeled antibody (Groen *et al.*, 2004) after spindle assembly.

Bead spindle assembly was imaged at ~18–20°C on an inverted Nikon Eclipse TE2000-U (Nikon, Melville, NY) microscope stand equipped with a cooled CCD Orca ER camera (Hamamatsu, Bridgewater, NJ) using a Nikon 40X/1.3 NA plan-Fluor differential interference contrast (DIC) objective at Five-seven μl of an assembly reaction was squashed under a 22 × 22 mm coverslip and sealed with valap (Desai *et al.*, 1999a). Chromatin beads were isolated, resuspended in fresh extract, and stored on ice for several hours before preparation of the squash. 500 ms exposures were acquired every 1 min.

Quantification of Fluorescence Intensity

X-rhodamine-labeled tubulin intensity was quantified for each frame of a timelapse of spindle assembly using MetaMorph (Molecular Devices, Sunnyvale, CA) software. In brief, a larger and smaller circular region was drawn manually, using the ellipse region tool, around the assembling spindle and moved accordingly for each frame so that the two regions always contained the structure. The area and integrated intensity of both regions for every frame were then exported from the "region measurements" window in Metamorph to an Excel (Microsoft, Redmond, WA) spreadsheet for calculating the background signal and the total fluorescence intensity. The following equations were applied: Backgound signal = (Integrated fluorescence intensity$_{\text{big area}}$ − Integrated fluorescence intensity$_{\text{small area)}}$/(Area$_{\text{big}}$ − Area$_{\text{small}}$). Total intensity = Integrated fluorescence intensity$_{\text{small area}}$ × (Background signal × Small Area). The total intensity for each frame of the time-lapse movie is reported in the graphs.

TPX2, γ-tubulin, and XMAP215 Depletions and Mitotic Centromere-associated Kinesin (MCAK) Inhibition

TPX2, γ-tubulin, XMAP215-depletion was carried out as previously described (Shirasu-Hiza *et al.*, 2003; Groen *et al.*, 2004). Briefly, 10 μg of affinity-purified antibody was coupled to protein-A Dynabeads (Invitrogen) and the extract was subjected to three one-hour rounds of incubation at 4°C. α-MCAK arrays were assembled by the addition of ~0.05 mg/ml inhibitory MCAK antibody to chromatin beads after fresh CSF addition (Walczak *et al.*, 1996). Excess EB1, XMAP215, or TPX2 was added to chromatin beads after fresh CSF addition.

XMAP215, EB1, TPX2, Ran(Q69L)GTP and Ran(T24N) Purification

His-XMAP215, His-EB1, GST-TPX2, GST-Ran(Q69L)GTP and His-Ran(T24N) were purified as previously described (Kinoshita *et al.*, 2001; Nachury *et al.*, 2001; Tirnauer *et al.*, 2002a; Tirnauer *et al.*, 2002b; Groen *et al.*, 2004). The concentrations of XMAP215 and TPX2 used during the assays were determined by the minimal concentration required to stimulate microtubule assembly in *Xenopus* egg extracts (without the presence of DNA). The concentration of EB1 was determined by titration (300 nM, 500 nM) in each of the DNA-Bead experiments, using the minimal concentration effective for rescuing spindle assembly. EB1 was labeled with Alexa-488 (Invitrogen) as described previously (Groen *et al.*, 2008).

RESULTS

Kinetics of Microtubule Accumulation

We used time-lapse fluorescence microscopy of spindle assembly around chromatin-coated beads, visualized with trace x-rhodamine tubulin, to determine the kinetics of microtubule assembly. A pronounced lag phase of 3–15 min was followed by a sigmoidal increase in microtubule mass, which plateaued at ~40min (Figure 1; Supplemental Movie 1). Microtubule organization transitioned from disordered to bipolar after 40 min by extrusion and focusing of poles beginning at 27 min, similar to the pole extrusion pathway previously described (Mitchison *et al.*, 2004). More specifically, we observed the following transitions: i. Initial tubulin polymerization (cloud), ii. Radial array of microtubules (MTs), iii. Appearance of a pole, iv. Extension/extrusion of poles, and v. Bipole structure (Figure 1). The specific timing of each transition varied with each spindle assembly reaction (Figure 1), most likely due to variations between extracts. A majority of tubulin polymer is generated around chromatin before the evident onset of bipolarity or assembly of organized poles. This indicates that neither pole assembly nor bipolarity promote a dramatic increase in microtubule polymerization during anastral spindle assembly. The lag phase in polymerization was not due to chromatin assembly, since chromatin was pre-assembled on the beads. It may arise from the time required to activate Ran-regulated factors near beads, or perhaps from microtubule-dependence of microtubule nucleation (Clausen and Ribbeck, 2007).

In bulk polymerization of pure tubulin, which also follows sigmoidal kinetics, nucleation tends to predominate early, and elongation late (Johnson and Borisy, 1975, 1977). To test if proposed nucleation factors are recruited early in the spindle assembly process, we coimaged two potential nucleation factors, TPX2 and γ-tubulin, with bulk tubulin, using a nonperturbing antibody probe for γ-tubulin, and a GFP fusion for TPX2 (Figure 1, C–D; Supplemental Movies 2–3). The localizations of the nucleation factors colocalized with tubulin, showing that both candidate nucleation factors were recruited to forming spindles with the same kinetics as bulk tubulin. The nucleation factors were concentrated onto nascent spindle poles coincident with pole assembly (after ~24–27 min; Figure 1, C–D). Our data suggest the γ-tubulin- and TPX2-dependent microtubule regulatory pathways are likely to begin contributing to microtubule polymerization early on in the spindle assembly process. The situation is different in centrosome-catalyzed spindle assembly, where γ-tubulin is pre-localized to centrosomes (Heald *et al.*, 1997).

TPX2, γ-tubulin, and XMAP215 Are Required for Anastral Spindle Assembly

We next tested whether three factors previously implicated in microtubule nucleation were required for anastral spindle assembly around DNA beads by conventional immunodepletion/add-back experiments (Figure 2). All three were required; in the case of γ-tubulin we did not have pure complex for the add-back control, but were able to rescue assembly by adding back small amounts of undepleted extract (20%; Supplemental Figure 2, A–B). Western blots confirmed the depletion of each factor (Figure 2). These results provide an essential reference point for the unexpected data below. TPX2 was previously shown to be essential for spindle assembly around DNA beads, while γ-tubulin and XMAP215 were previously shown to be required for microtubule assembly from centrosomes (Stearns and Kirschner, 1994; Gruss *et al.*, 2001; Popov *et al.*, 2002). Despite the requirement for TPX2, γ-tubulin, or XMAP215 during microtubule assembly, it has been unclear how these factors function together. It is possible that all function in the same pathway, for example, as sequential factors in a linear pathway with each being absolutely required for microtubule assembly. To test this, we asked if other factors could substitute for one of the immunodepleted factors.

Excess TPX2 Does Not Rescue γ-tubulin or XMAP215 Depletion

We first tested whether excess TPX2 could substitute for γ-tubulin or XMAP215. Addition of recombinant TPX2 to TPX2-depleted extracts rescued bipolar spindle assembly as expected (Figure 3A–B). However, excess TPX2 (300 nM final extract concentration, which is ~3X endogenous concentration; Supplemental Figure 1A) failed to rescue the microtubule assembly defects of either γ-tubulin or XMAP215 depleted extracts (Figure 3, A–B) (Wittmann *et al.*, 2000). Addition of higher concentrations of TPX2 (up to 1 μM final concentration) had similar results (data not

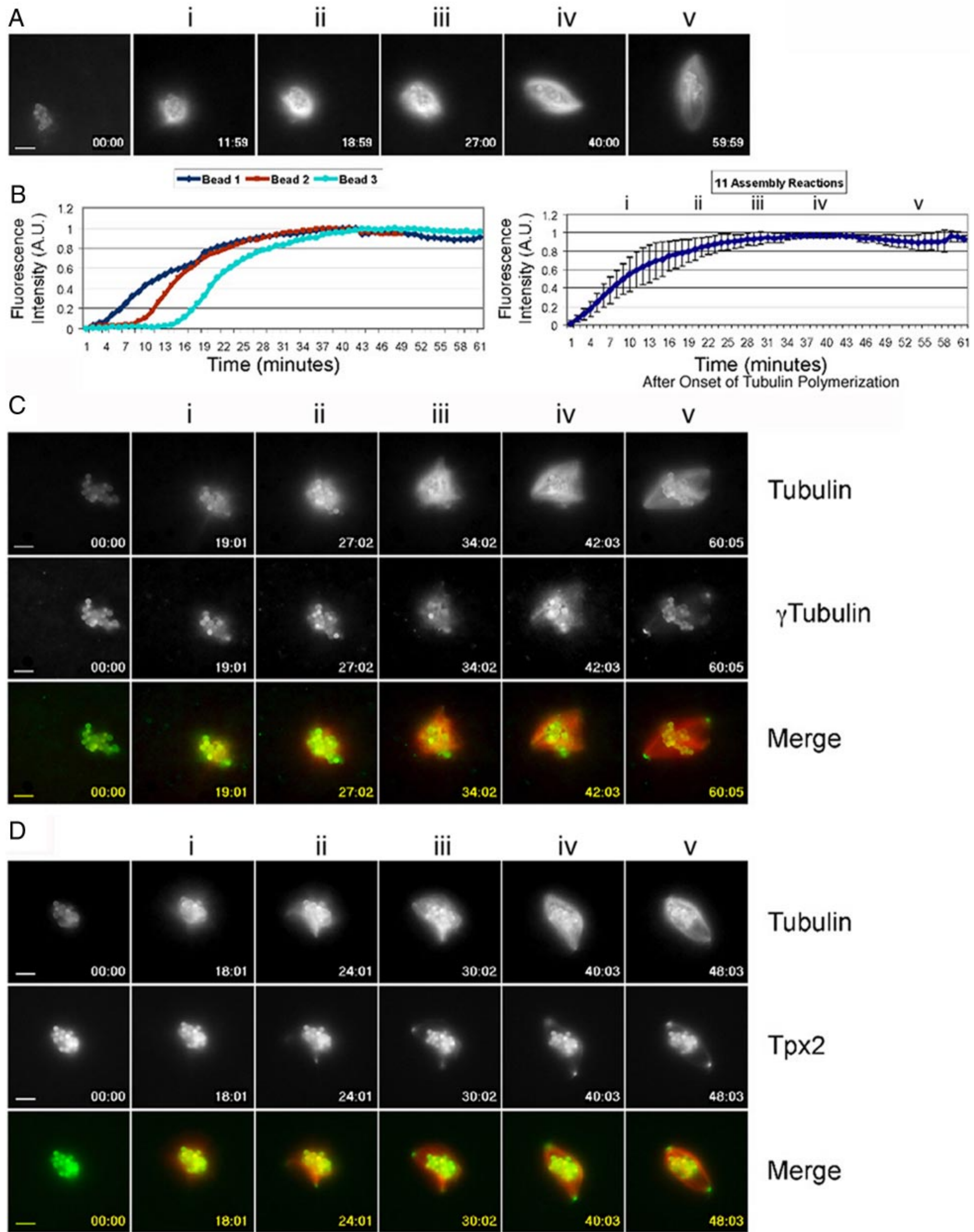

Figure 1. Time-Lapse Fluorescence Imaging of Anastal Spindle Assembly. (A) Selected frames from a time-lapse movie of spindle assembly around DNA-coated beads highlighting the following transitions (for A, C, and D): i. Initial tubulin polymerization (cloud), ii. Radial array of MTs, iii. Appearance of a pole, iv. Extension/extrusion of poles, and v. Bipole structure. The timing of each highlighted transition varied for each spindle assembly reaction (compare time stamps on lower right corner of frames for A, C, and D. (B) Quantification of tubulin intensity over time for 3 different bead spindle assembly reactions from 3 different extracts. The dark blue trace is from the assembly reaction shown in 1A. Note that maximum fluorescence intensity (max-FI) is reached ~25–30 min after onset of polymerization. Average tubulin

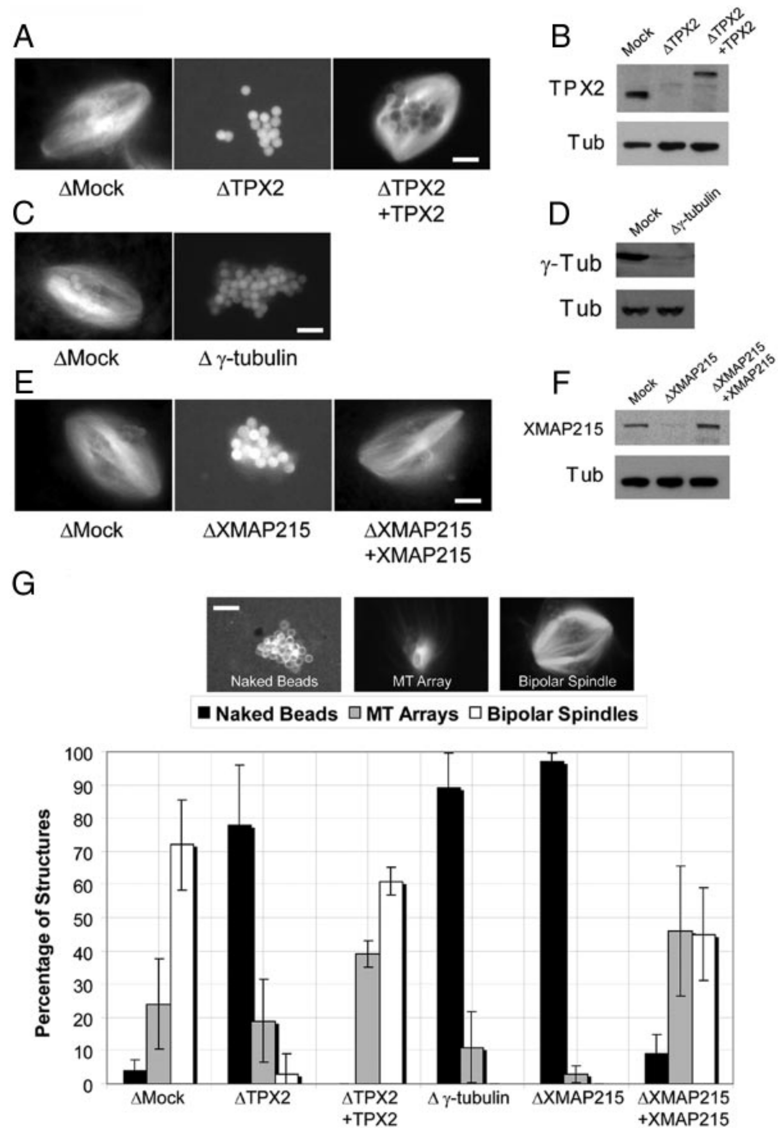

Figure 2. TPX2, γ-tubulin and XMAP215 are Essential for Anastral Spindle Assembly. (A) Anastral spindle assembly requires TPX2 and can be rescued by adding back purified TPX2. (B) Western blot analysis of TPX2 reveals efficient immunodepletion and addition of purified TPX2 to depleted extract at endogenous concentration (100 nM). The shift in molecular weight for the purified TPX2 is due to the presence of a GST tag. (C) γ-tubulin is required for spindle assembly around DNA beads. (D) γ-tubulin can be significantly (>90%) immunodepleted from assembly reactions. (E) XMAP215 is essential for bead spindle assembly and its depletion can be rescued by the addition of purified recombinant XMAP215. (F) XMAP215 is efficiently immunodepleted from extract and the purified protein is added back at endogenous levels (300 nM). (G) Quantification of the structures assembled around DNA-coated beads for conditions shown in A–F. The three types of structures, examples of which are shown in the inset, are naked beads, MT arrays, and bipolar spindles. Greater than 70% of structures in mock-depleted extracts are bipolar spindles while 78%, 85%, and 97% of bead structures lacked any associated microtubules in TPX2-, γ-tubulin- and XMAP215-depleted structures, respectively. Microtubule polymerization around beads (39% MT arrays and 61% bipolar spindles) was rescued by the addition of purified TPX2 to TPX2-depleted extracts. Microtubule polymerization around beads (46% MT arrays and 45% bipolar spindles) was rescued by the addition of purified XMAP215 to XMAP215-depleted reactions. (Scale bars: 10 μm; Error bars: SD for 4 different extracts; 100 structures counted per experiment).

shown). The addition of 3X the endogenous concentration of TPX2 to meiotic extract induces microtubule asters, but this activity also required γ-tubulin and XMAP215 (data not shown). We also found that hyper-activation of the Ran pathway by addition of Ran(Q69L)GTP did not rescue the TPX2-dependent microtubule assembly defects, showing that general up-regulation of other Ran-regulated activities cannot compensate for TPX2 depletion in spindle assembly (Supplemental Figure 4A, C). Thus, TPX2 promoted microtubule assembly depends upon γ-tubulin and XMAP215.

Excess EB1 Partially Rescues Depletion of TPX2, but Not γ-tubulin or XMAP215

The tip-tracking protein EB1 is a potent promoter of microtubule polymerization in egg extracts, and so might substitute for loss of other factors (Tirnauer *et al.*, 2002a; Tirnauer *et al.*, 2002b; Tirnauer *et al.*, 2004). EB1 used in these assays was directly labeled with Alexa-488 and properly localized to kinetochores, tracked microtubule plus ends when added to extract spindles and induced microtubule asters (at ~3X endogenous concentration or 810 nM) in extract, confirming the functionality of EB1 (Tirnauer *et al.*, 2002b). Addition of excess EB1 (~3X endogenous concentration; Supplemental Figure 1B) largely rescued the microtubule assembly defects of TPX2 depletions (Figure 4), to the extent that many bipolar spindles were now observed (40% bipolar spindles with excess EB1 compared with 5% bipolar spindles in TPX2 depleted extracts; Figure 4, A–B). The addition of RanT24N, but not the Aurora A/B inhibitor VX-680 inhibited this effect, demonstrating that the ability to rescue TPX2 with

Figure 1 (cont). fluorescence intensity (N = 11) of bead spindle assembly over time with transitions i-v highlighted. (Samples were averaged after onset of tubulin polymerization because polymerization onset varied for each spindle). (C) Selected frames from a two-color time-lapse movie of x-rhodamine tubulin (top row), Alexa-488 labeled gamma-tubulin antibody (middle row) and color combine (lower row) during DNA bead spindle assembly with transitions i-v highlighted. (D) Frames from a two-color time-lapse movie of x-rhodamine tubulin (top row), TPX2-GFP (middle row), and color combine (lower row) during spindle assembly around DNA beads with transitions i-v highlighted. Scale bars: 10 μm. Time is shown in minutes:seconds.

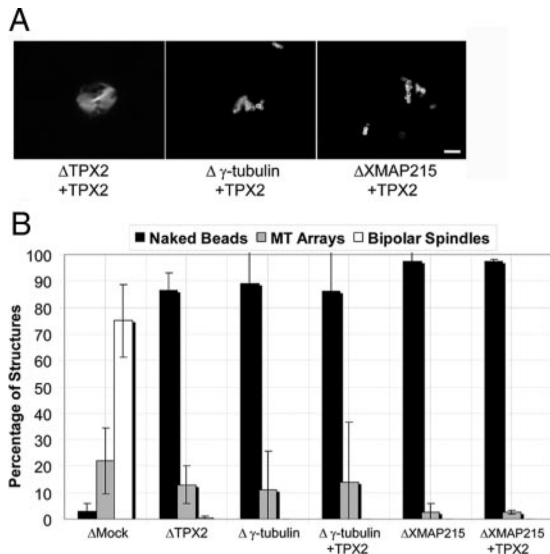

Figure 3. TPX2-dependent Microtubule Polymerization Requires γ-tubulin and XMAP215. (A) Addition of purified TPX2 (3X final extract concentration) rescues anastral spindle assembly in TPX2-depleted extracts but not γ-tubulin- or XMAP215-depleted reactions. (B) Quantification of the bead structures assembled in the conditions shown in 3B. While addition of purified TPX2 rescues TPX2 depletion, TPX2 addition did not rescue γ-tubulin- or XMAP215-depleted reactions (>80% naked beads in each case). (Scale bars: 10 μm; Error bars: SD for 4 different extracts; 100 structures counted per experiment).

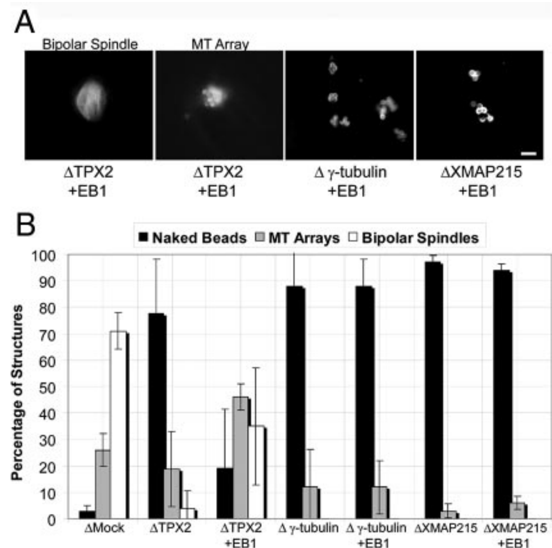

Figure 4. EB1 Rescues the Microtubule Assembly Defects of TPX2 Depletion but Not γ-tubulin or XMAP215 Depletions. (A) Addition of purified EB1 (3X final extract concentration) restores bead-associated microtubule polymerization to TPX2-depleted reactions but not γ-tubulin- or XMAP215-depleted extracts. Both bipolar spindles and microtubule arrays were commonly observed for the ΔTPX2 + EB1 condition. (B) Quantification of the structures assembled in the conditions shown in 4B. Addition of purified EB1 to TPX2-depleted extracts yielded a fourfold reduction in the percentage of naked beads relative to ΔTPX2 (46% microtubule arrays and 35% bipolar spindles). EB1 addition had no affect on the types of structures formed in either γ-tubulin or XMAP215-depleted extracts (>80% naked beads). (Scale bars: 10 μm; Error bars: SD for 4 different extracts; 100 structures counted per experiment).

EB1 requires additional Ran-regulated factors but not Aurora kinase A/B activity (Supplemental Figure 4, A–B). Excess EB1 failed to rescue the microtubule assembly defects of either γ-tubulin or XMAP215 depletion (~90% naked beads in each extract; Figure 4A–B). Thus, promotion of microtubule polymerization by EB1 can partially substitute for TPX2, but the ability of either EB1 or TPX2 to promote polymerization depends on both XMAP215 and γ-tubulin.

MCAK Inhibition Partially Rescues Depletion of TPX2, but Not γ-tubulin or XMAP215

We next tested an independent method of stabilizing microtubules in extracts depleted of TPX2, γ-tubulin or XMAP215. MCAK is a microtubule destabilizing kinesin that promotes depolymerization of stabilized microtubules from both ends, and is a major plus-end catastrophe factor in egg extracts (Walczak *et al.*, 1996; Desai *et al.*, 1999b). Because inhibition of MCAK tends to make pre-existing MT assemblies larger, rather than promote nucleation of new ones, MCAK is probably more important for inhibiting elongation than nucleation in egg extracts. Previous work shows XMAP215 antagonizes the catastrophe-promoting activity of MCAK (Tournebize *et al.*, 2000; Kinoshita *et al.*, 2001), suggesting inhibition of MCAK might rescue loss of XMAP215, if regulation of plus-end dynamics was the only function of XMAP215. Inhibition of MCAK by function-blocking antibody (Ohi *et al.*, 2004) resulted in assembly of very large aster-like microtubule arrays around the DNA-beads in mock depleted extract, as previously described for sperm spindles (Walczak *et al.*, 1996). Large arrays were also observed when anti-MCAK was added to TPX2-depleted extracts (60% of mock depleted; Figure 5A–B). When anti-MCAK was added to either XMAP215 or γ-tubulin depleted extract, no microtubules assembled around beads. If we assume that MCAK regulates elongation and not nucleation, these data suggest that XMAP215 and γ-tubulin are required for nucleation around DNA beads, while TPX2 is not.

Excess XMAP215 Partially Rescues Depletions of TPX2 or γ-tubulin

Addition of pure XMAP215 rescued the microtubule assembly defects of XMAP215 depleted extracts as expected (<20% naked beads compared with >90% naked beads in XMAP215 depletion alone; Figure 6A–B). Unexpectedly, excess XMAP215 (1.2 μM final extract concentration, ~4X endogenous concentration; Supplemental Figure 1C; (Popov *et al.*, 2001)) partially rescued the microtubule assembly defects of both TPX2 (<30% naked beads compared with >80% naked beads in TPX2 depletion alone; Figure 6, A–B) and γ-tubulin depletion (<20% naked beads compared with >80% naked beads in γ-tubulin depletion alone; Figure 6, A–B). Remarkably, we observed bipolar spindle assembly without TPX2, or without γ-tubulin, when excess XMAP215 was added (~12% for TPX2 and ~29% for γ-tubulin; Figure 6C). However, similar to EB1 addition, these observations depended on Ran-regulated factors and not Aurora A/B kinase activity, as spindle assembly was inhibited by RanT24N and not affected by addition of VX-680 (data not shown; Supplemental Figure 4A–B). Thus, XMAP215 can promote microtubule assembly in the absence of TPX2 and γ-tubulin, and can even partially substitute in bipolar spindle assembly. These data suggest a particularly important role for XMAP215 in microtubule nucleation and/or stabilization in anastral spindles.

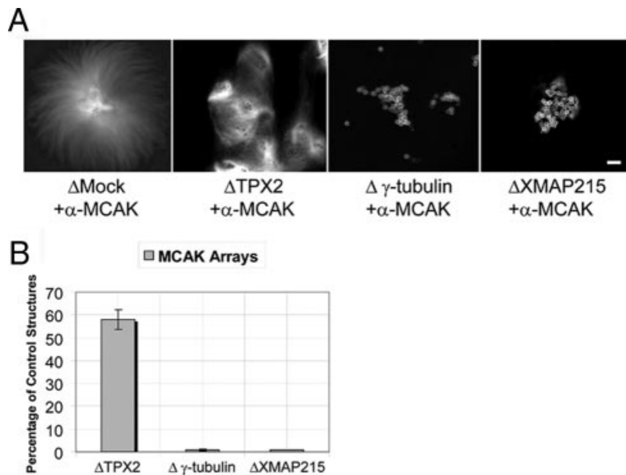

Figure 5. Microtubule Polymerization Stimulated by MCAK inhibition Requires γ-tubulin and XMAP215 But Not TPX2. (A) Inhibition of MCAK (with α-MCAK) stimulates the assembly of large microtubule arrays around beads in both mock- and TPX2-depleted extracts but not in Δγ-tubulin or ΔXMAP215 extracts. (B) The number of α-MCAK arrays were counted in 2 μl samples for mock-, TPX2, γ-tubulin- and XMAP215-depleted extracts. Each condition is reported as a percentage of the number of microtubule arrays in ΔMock + α-MCAK reactions. ΔTPX2 supported the assembly of 58% of control α-MCAK arrays while both γ-tubulin and XMAP215 depleted extracts supported assembly of ~1% the number of control structures. (Scale bars: 10 μm; Error bars: SD of for 3 different extracts; all the microtubule arrays in 2 μl of each reaction sample were counted per experiment).

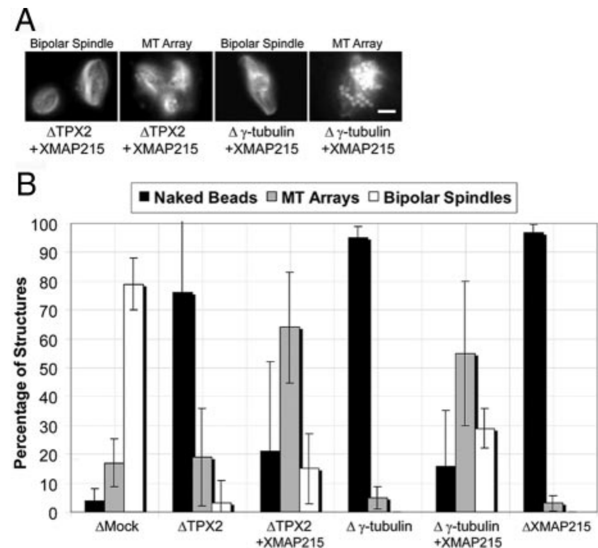

Figure 6. XMAP215 Rescues the Microtubule Assembly Defects of TPX2 and XMAP215 Depletion. (A) Addition of purified XMAP215 (4X final extract concentration) restores localized microtubule polymerization around DNA beads in TPX2-, γ-tubulin- and XMAP215 depleted extracts. Representative images of microtubule arrays and bipolar spindles observed in all the depletion + XMAP215 conditions. (B) Quantification of the structures assembled in the conditions shown in 6A. Addition of XMAP215 to ΔTPX2 extracts led to a ~fourfold reduction in the prevalence of naked beads compared with ΔTPX2. The ΔTPX2 + XMAP215 treatment resulted in 64% microtubule arrays and 15% bipolar spindles. XMAP215 also rescued γ-tubulin depletion with nearly a sixfold reduction in the percentage of naked beads (55% microtubule arrays and 29% bipolar spindles). Purified XMAP215 addition to XMAP215-depleted extract resulted in a nearly 11-fold reduction in the percentage of naked beads (46% microtubule arrays and 45% bipolar spindles). (Scale bars: 10 μm; Error bars: SD for 3 different extracts; 100 structures counted per experiment).

Given that γ-tubulin is often considered the main, or only, microtubule nucleating complex, we were concerned that addition of XMAP215 might potentiate the recruitment or activation of small amounts of γ-tubulin complex still present in extract after immunodepletion. We could not detect γ-tubulin in immunoblots of depleted extracts (Figure 2; Supplemental Figure 1B) and estimate the residual amount to be <5% of endogenous. To test if residual γ-tubulin is concentrated onto the bipolar spindles that assemble when excess XMAP215 is added to γ-tubulin depleted extracts, we stained fixed spindles with anti- γ-tubulin antibody. γ-tubulin was undetectable by immunofluorescence on these depleted, XMAP215 rescued spindles, whereas it was brightly stained on control spindles (Supplemental Figure 1D). We conclude that XMAP215 most likely rescues spindle assembly by substituting for γ-tubulin function, and not by promoting recruitment of residual γ-tubulin.

DISCUSSION

In summary, we confirm that TPX2, γ-tubulin, and XMAP215 are each essential for anastral spindle assembly around DNA beads in *Xenopus* egg extracts. Excess EB1 cannot compensate for loss of γ-tubulin and XMAP215, but excess XMAP215 can partially compensate for loss of TPX2 or γ-tubulin (Supplemental Figure 3A). These data contradict standard views on the functions of these proteins, and suggest new ideas, with XMAP215 assuming a particularly important role. Overall, our experiments suggest that TPX2, EB1, XMAP215, and γ-tubulin do not function in a simple linear pathway for microtubule assembly.

It is important to understand that when we state that A compensates for loss of B in this study, we do not mean to imply true complementation in the genetic sense. All of the proteins we studied have been shown to be essential by knockdown experiments in living cells; presumably the spindle-like assemblies we observe in compensation experiments are not fully functional in the sense of being able to segregate chromosomes normally, though it will be interesting to test this in future genetic experiments.

TPX2 has been viewed as a central factor in promoting microtubule nucleation by the RanGTP pathway (Gruss *et al.,* 2001), and one study suggested it is the actual nucleating factor in this pathway (Schatz *et al.,* 2003). We show that TPX2 function can be partially substituted by addition of excess XMAP215 or EB1, or by inhibition of MCAK. In each case, we see relatively normal amounts of microtubules formed, and they are spatially constrained to the vicinity of chromatin. XMAP215, EB1, and MCAK have been implicated more in promoting microtubule elongation/stability than nucleation in the literature, so our observation of functional overlap with TPX2 suggest that TPX2's primary function may be to promote microtubule elongation or stabilization, rather than nucleation. Furthermore, the molecular function of TPX2 does not seem to be unique, at least in anastral assembly. This may explain why the function of TPX2 homologues in astral spindle assembly in *C. elegans* seem very different from those proposed in the anastral pathway in *Xenopus* (Ozlu *et al.,* 2005).

γ-tubulin has been viewed as the only microtubule nucleating factor in many systems, though it may also function to

cap and perhaps stabilize minus ends (Zheng *et al.,* 1995; Moritz *et al.,* 2000; Moritz and Agard, 2001). It was thus surprising to find that excess XMAP215 can partially substitute for loss of γ-tubulin. Previous work has shown that microtubules can assemble without γ-tubulin in interphase Drosophila cells (Rogers *et al.,* 2008). To our knowledge, our study is the first example for γ-tubulin independent microtubule assembly during meiosis.

XMAP215 is shown by our data to have a particularly important role in microtubule assembly in our system. A simple interpretation of our data are that XMAP215 can also nucleate microtubules, at least in anastral spindle assembly. However, other interpretations are possible. For example, some upstream factor might promote nucleation, and either γ-tubulin or excess XMAP215 may be required to stabilize nascent microtubules, one acting at the minus end, and the other at the plus. Neither γ-tubulin nor XMAP215 are known to be regulated by RanGTP, and the hypothetical upstream factor might be. Another interpretation is that it can function to both nucleate (substituting for γ-tubulin) or elongate/stabilize microtubules (substituting for TPX2). This dual function is consistent with the molecular mechanism of XMAP215, as a microtubule polymerase (Gard and Kirschner, 1987; Brouhard *et al.,* 2008).

Astral versus Anastral Spindle Assembly

Current views of metazoan cell spindle assembly distinguish two major pathways of microtubule assembly, promoted by centrosomes and chromosomes (Carazo-Salas *et al.,* 1999; Carazo-Salas *et al.,* 2001; Sampath *et al.,* 2004; Basto *et al.,* 2006; Basto and Pines, 2007). A third mechanism may operate at kinetochores (Tulu *et al.,* 2006; Torosantucci *et al.,* 2008). γ-tubulin has been viewed as the microtubule nucleating factor at centrosomes (Stearns and Kirschner, 1994), while TPX2 is implicated in nucleating and/or stabilizing microtubules in the chromosomal pathway (Schatz *et al.,* 2003; Casanova *et al.,* 2008). This simple view is not consistent with all published data, for example, in *C. elegans* embryos that are thought to largely lack the chromosomal pathway, TPX2 functions in the centrosomal pathway (Ozlu *et al.,* 2005). Our data shows that both TPX2 and γ-tubulin function in the chromosomal pathway in *Xenopus* extract anastral spindles, as does XMAP215. Recent work suggests TPX2 and HuRP (hepatoma-up-regulated protein) play nonredundant roles in anastral spindle assembly, suggesting that HuRP may function either with γ-tubulin and/or XMAP215 or independently (Sillje *et al.,* 2006; Casanova *et al.,* 2008).

Our data do not clearly distinguish which factor(s) actually nucleate microtubules. Recent work suggests there are multiple factors in the nucleation pathway (Rogers *et al.,* 2008). Both γ-tubulin and XMAP215 could be nucleators, but the data do not rule out the possibility that there is an upstream nucleator, potentially regulated by RanGTP and that γ-tubulin and/or XMAP215 are required for stabilization of newly nucleated microtubules. We now favor a model for nucleation/stabilization during anastral spindle assembly where either γ-tubulin functions through XMAP215 or each function in parallel pathways. Further experiments are required to determine how HURP and other factors in the chromosomal pathway fit in this model.

How is Microtubule Assembly Spatially Targeted to Chromatin?

TPX2, locally de-sequestered from importins by RanGTP, has been accorded a central role in spatial regulation of microtubule assembly by chromatin (Carazo-Salas *et al.,* 1999; Carazo-Salas *et al.,* 2001) It is thus surprising that spatial regulation is rescued in TPX2-depleted extracts when microtubule assembly is promoted by several treatments (excess EB1 or XMAP215, loss of MCAK function). How is spatial control exerted in the absence of TPX2? One possibility is that other RanGTP-regulated cargos, such as HURP, act redundantly to maintain spatial control. We favor this hypothesis because of the fact that shutting down the activation of all Ran-regulated cargos by the addition of RanT24N completely prevented EB1 or XMAP215 from rescuing TPX2 depletion (Supplemental Figure 4B). However, another possibilty is that upstream nucleation factors such as γ-tubulin and/or XMAP215 are spatially controlled by proximity to chromatin, using yet-to-be-discovered regulatory mechanisms that depend upon RanGTP, Aurora-B kinase, or some other localized signals. We argue new biochemistry is required to understand how nucleation is spatially regulated, and furthermore, that elucidating local control of nucleation will be the key to understanding spatial organization of anastral spindles.

Uniqueness Versus Functional Overlap in Microtubule Regulators

Ordinary cell biological experiments, be they depletion-add back, genetic deletion, or pure protein reconstitution, tend to suggest unique functions for every important protein in a system. However, systems-level analysis of complex biological networks often reveals functional overlaps between different proteins, or between different subnetworks (Shtil and Azare, 2005). The whole, intact network is required for optimal fitness of the organism, but from the perspective of inputs and outputs of the system, individual proteins or pathways might not have unique functions. Here, we modified the conventional depletion-add-back strategy that has been the backbone of research in the *Xenopus* extract system only slightly, adding back a protein that is different from the one we depleted. We argue that this more inclusive approach to analyzing protein function in cell biology will have wide applicability, and will reveal mechanistic insights that were missed by more restrictive approaches.

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