XMAP215–EB1 Interaction Is Required for Proper Spindle Assembly and Chromosome Segregation in *Xenopus* Egg Extract

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In metaphase *Xenopus* egg extracts, global microtubule growth is mainly promoted by two unrelated microtubule stabilizers, end-binding protein 1 (EB1) and XMAP215. Here, we explore their role and potential redundancy in the regulation of spindle assembly and function. We find that at physiological expression levels, both proteins are required for proper spindle architecture: Spindles assembled in the absence of EB1 or at decreased XMAP215 levels are short and frequently multipolar. Moreover, the reduced density of microtubules at the equator of Δ EB1 or Δ XMAP215 spindles leads to faulty kinetochore–microtubule attachments. These spindles also display diminished poleward flux rates and, upon anaphase induction, they neither segregate chromosomes nor reorganize into interphasic microtubule arrays. However, EB1 and XMAP215 nonredundantly regulate spindle assembly because an excess of XMAP215 can compensate for the absence of EB1, whereas the overexpression of EB1 cannot substitute for reduced XMAP215 levels. Our data indicate that EB1 could positively regulate XMAP215 by promoting its binding to the microtubules. Finally, we show that disruption of the mitosis-specific XMAP215–EB1 interaction produces a phenotype similar to that of either EB1 or XMAP215 depletion. Therefore, the XMAP215–EB1 interaction is required for proper spindle organization and chromosome segregation in *Xenopus* egg extracts.

INTRODUCTION

Meiotic and mitotic spindles are microtubule (MT)-based structures that segregate chromosomes during cell division (Karsenti and Vernos, 2001; Scholey *et al.*, 2003; Kwon and Scholey, 2004). The architecture of the spindle is established and maintained by the action of molecular motors and microtubule-associated proteins (MAPs) that organize MTs and regulate their polymerization dynamics (Wittmann *et al.*, 2001; Howard and Hyman, 2007). Interestingly, despite fast turnover of spindle MTs by poleward translocation and dynamic instability of plus ends, the overall length and shape of the spindle are maintained throughout metaphase. Although it is accepted that a balance of MT-stabilizing and -destabilizing activities governs spindle length, it is still not understood how distinct MAPs act collectively to regulate MT dynamic instability (Tournéize *et al.*, 2000; Goshima *et al.*, 2005).

XMAP215 is the founding member of the XMAP215/Dis1/Tog protein family that is conserved from yeast to humans. It increases ~7- to 10-fold the MT growth rate in

vitro by acting as a processive tubulin polymerase, which while residing on MT ends, supports multiple rounds of addition of individual tubulin dimers (Vasquez *et al.*, 1999; Brouhard *et al.*, 2008). In accordance with the role of XMAP215 as an MT growth promoter, depletion of Dis1/XMAP215/Tog proteins leads to shorter spindles in *Xenopus* egg extracts and *Drosophila* S2 cells as well as to defects in spindle morphology in HeLa cells, *Schizosaccharomyces pombe*, and *Caenorhabditis elegans* (Matthews *et al.*, 1998; Tournéize *et al.*, 2000; Garcia *et al.*, 2001; Cassimeris and Morabito, 2004; Goshima *et al.*, 2005).

Although XMAP215 can autonomously promote MT growth, several other important and unrelated MT-stabilizing factors have been characterized in various model systems. In metaphase *Xenopus* egg extracts, XMAP215 requires other MAPs to oppose the strong MT-destabilizing activity of XKCM1. For example, XMAP215 interaction with TACC3/Maskin potentiates the growth of nascent MTs off centrosomes (Kinoshita *et al.*, 2005; Peset *et al.*, 2005). Also other MAPs regulate global and local MT dynamics. For example, EB1 promotes MT growth in the overall cytoplasm, whereas CLASP locally stabilizes MTs at the kinetochores (Tirnauer *et al.*, 2002; Hannak and Heald, 2006b). However, in most cases it is unclear whether these proteins work independently or in a hierarchical manner together with other MT growth promoters.

End-binding protein 1 (EB1) is an evolutionary-conserved MAP that belongs to a group of plus end-tracking proteins (+TIPs) because it binds to plus ends of growing MTs (Akhmanova and Steinmetz, 2008). Various analyses demonstrated that EB1 associates with most of MAPs discovered

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Abbreviations used: APC/C, anaphase-promoting complex/cyclosome; C-EB1, C-terminal fragment of EB1; C-XMAP215, C-terminal fragment of XMAP215; MAP, microtubule-associated protein; MT, microtubule; MAP, microtubule-associated protein.

to date, so it has been placed at the hub of the MAP interactome (Akhmanova and Steinmetz, 2008). Recent in vitro work revealed that EB1 proteins autonomously track growing MT plus and minus ends and also recruit other microtubule-stabilizing factors, such as CLIP-170 proteins to MT plus ends (Bieling *et al.*, 2007, 2008). Research in various experimental systems suggested that EB1 might also contribute to the MT plus-end tracking of its other interaction partners, such as adenomatous polyposis coli, p150^{glued}, and MCAK (Carvalho *et al.*, 2003; Watson and Stephens, 2006; Lee *et al.*, 2008). In addition to targeting other MAPs to MT plus ends, EB1 has also been implicated in MT dynamics regulation (Rogers *et al.*, 2002; Tirnauer *et al.*, 2002).

EB1 was found to stabilize MTs in the global cytoplasm during metaphase but not during interphase in *Xenopus* egg extracts (Tirnauer *et al.*, 2002; Niethammer *et al.*, 2007). However, its role in spindle organization in *Xenopus* egg extracts has not been investigated so far. Spindle length is partly determined by global MT dynamics, which is locally modulated around chromosomes through the RanGTP pathway (Karsenti and Vernos, 2001; Goshima *et al.*, 2005). It is therefore not safe to assume that MAPs involved in the control of centrosomal MT dynamics also contribute to the regulation of spindle length. For example, TACC3/Maskin is required for the stabilization of centrosomal MTs, but its absence does not significantly affect spindle length or morphology (Kinoshita *et al.*, 2005). In metaphase *Xenopus* egg extracts, XMAP215 and EB1 positively affect global MT growth, because the depletion of either protein results in a drastic reduction in the average length of centrosome-nucleated MTs (Niethammer *et al.*, 2007). Furthermore, these two proteins interact with each other in metaphase, and their interaction is essential for physiological MT dynamics in this particular cell cycle state (Niethammer *et al.*, 2007). In this study, we determined how the combined action of these two global MT growth promoters affects spindle assembly and function in *Xenopus* egg extracts.

MATERIALS AND METHODS

Protein and Antibody Expression and Purification

The cDNA containing coding sequence for *Xenopus laevis* EB1 (clone ID IMAGp998A2414227Q, obtained from RZPD, Deutsches Ressourcenzentrum fuer Genomforschung, Berlin, Germany) was subcloned into pHAT2 vector in frame with N-terminal His-tag. Recombinant His-EB1 was expressed in *Escherichia coli* (BL21) and purified on TALON beads (Clontech, Mountain View, CA), according to manufacturer's instructions. On SDS-gels, recombinant His-EB1 is running a bit higher than the endogenous EB1 because it contains 6xHis-tag and seven additional amino acids before the EB1 start codon. Recombinant XMAP215, glutathione transferase (GST)-C-terminal (C)-EB1 (amino acids [aa] 193-268) as well as N-XMAP215 (aa 1-560), M-XMAP215 (aa 543-1167), and C-terminal fragment of XMAP215 (C-XMAP215) (aa 1168-2065) were expressed and purified as described previously (Tournébeize *et al.*, 2000; Popov *et al.*, 2001; Niethammer *et al.*, 2007). The full-length coding sequence of RanQ69L was polymerase chain reaction-amplified from a pre-existing construct, cloned into pETMz (gift from G. Stier, Umea University, Umea, Sweden) and purified as described previously (Ribbeck *et al.*, 2006). Antibodies against full-length EB1 and N-terminal fragment of XMAP215 (1-560 aa) were prepared as described previously (Niethammer *et al.*, 2007). Monoclonal anti-GST (GST 26H1), penta-His antibodies as well as Alexa-488-labeled goat anti-rabbit secondary antibody (used in immunofluorescence experiments) were purchased from Cell Signaling Technology (Danvers, MA), QIAGEN (Hilden, Germany), and Invitrogen (Carlsbad, CA), respectively.

Egg Extract Preparation, Immunodepletion, and Immunoprecipitation

Cytostatic factor (CSF)-arrested *Xenopus* egg extracts were prepared, and immunodepletions were performed as described previously (Hannak and Heald, 2006a). To deplete EB1 (or ~70% of XMAP215) from 50 μ l of extract, 3 $\times$ 30 μ l (or 1 $\times$ 12.5 μ l) of antibody-coated Dynal beads (Invitrogen) were incubated with extracts on a rotating wheel at 4°C for 30 min per round, respectively. Control depletion was performed with immunoglobulin G (IgG)

from rabbit serum (Sigma Chemie, Deisenhofen, Germany). Depletion efficiency was assayed by Western blotting 0.25 μ l of extract per condition with a polyclonal anti-EB1 antibody (1:10,000) or a polyclonal anti-XMAP215 antibody (1:5000), respectively. For rescue experiments, 1.5–2 μ M EB1 or 100 nM XMAP215 were added at reentry into mitosis to restore endogenous concentrations (as estimated by Western blot analysis). In overexpression experiments, we added the same amount of XMAP215 into Δ EB1 extracts that we added into $\sim$ Δ XMAP215 extracts in rescue experiments, whereas we added three times the amount of EB1 used in Δ EB1 rescue experiments into $\sim$ Δ XMAP215 extracts.

Immunoprecipitation was performed by cross-linking 0.25 μ g/ μ l appropriate antibodies to 20 μ l of Dynal beads (Invitrogen) in the presence of dimethyl pimelimidate dihydrochloride (Sigma Chemie) as described by Harlow and Lane (1999). Beads were incubated at 4°C for 90 min with 50 μ l of CSF-arrested egg extracts in the absence of sperm nuclei (Supplemental Figure S1G). Finally, beads were washed twice with phosphate-buffered saline (PBS) buffer and twice with PBS + 0.5 M NaCl, before they were dissolved in SDS sample buffer and subjected to Western blot analysis.

Spindle Assembly, Spin-Downs, and Immunofluorescence

Spindles were assembled around replicated sperm chromosomes and chromatin beads as described previously (Hannak and Heald, 2006a). To visualize MTs, we supplemented extracts with 0.3 mg/ml Cy3-labeled tubulin. Tubulin was purified from pig brain (Castoldi and Popov, 2003) and labeled with Cy3 monoreactive dye (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom) according to Hyman *et al.* (1991). To assemble spindles in the presence of RanQ69L, extracts were first sent to interphase by addition of 0.4 mM CaCl₂ and incubation for 90 min at 20°C. Subsequently, interphase extract was mixed 1:1 with CSF-arrested extract and at the same time 15 μ M RanQ69L was added. Regardless of MT nucleation center used (sperm chromosomes, chromatin beads, or RanQ69L), spindles would form after 30–60 min of extract incubation at 20°C. Then, extracts were fixed in 1 ml of spindle dilution buffer [80 mM piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.8, 1 mM MgCl₂, 1 mM EGTA, 30% glycerol, 1% Triton X-100, and either 0.25% glutaraldehyde or 4% formaldehyde] and further processed for spin-downs. Formaldehyde fixation was used in immunofluorescence and MT density distribution experiments. Spindle spin-down and chromosome spin-down were performed as described previously (Maresca and Heald, 2006). In Figures 4, 7, and Supplemental Figure S2, coverslips were subjected to immunofluorescence that was performed according to Hannak and Heald (2006a). The XMAP215 antibody was used at a 1:3000 dilution, whereas anti-Mad2 antibody (a gift from R. H. Chen, Institute for Molecular Biology, Academia Sinica, Taipei, Taiwan) and anti-CENP-E antibody (a gift from A. Abrieu, Centre National de la Recherche Scientifique–Centre de Recherche En Biochimie Macromoléculaire, Montpellier, France) were used at a 1:200 dilution.

In mitotic exit experiments, cycled metaphase extracts were induced into anaphase by readdition of 0.4 mM CaCl₂ and incubation for 40 min at 20°C. Then, the extract was fixed in 1 ml of spindle dilution buffer and centrifuged onto coverslips for the subsequent fluorescence microscopy analysis of obtained structures. To assay the level of endogenous cyclin B before and 40 min after Ca²⁺ pulse in the mitotic exit experiment, we subjected 0.25 μ l of extract per condition to Western blot analysis using a polyclonal anti-cyclin B antibody (1:1000; a gift from O. Gruss, ZMBH, Heidelberg, Germany).

Fluorescence Speckle Microscopy

Fluorescence speckle microscopy was used to measure poleward MT flux. In this experiment, spindles were assembled in 20 μ l of cycled extracts incubated with 500 sperm/ μ l and 17.5 nM Cy3-labeled tubulin. As oxygen scavengers, we used a 1/40 dilution of saturated hemoglobin solution and 2/125 dilution of anti-fading mix (13.3 μ M catalase, 20.8 μ M glucose oxidase, and 0.3 M glucose). For spindle imaging, 3 μ l of spindle reaction was squashed under 22- $\times$ 22-mm coverslips. Images were acquired every 2 s for at least 1 min at 20°C by using an Axiovert 135 microscope (Carl Zeiss, Jena, Germany) controlled by MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) and equipped with a CoolSNAP camera (Roper Scientific, Trenton, NJ), a 100 $\times$ Plan-apochromat numerical aperture (NA) 1.4 oil immersion objective lens, and a long-pass rhodamine filter (Chroma Technology, Brattleboro, VT). Kymographs were obtained using the MetaMorph Imaging software, and flux rates were calculated as averages of at least 200 speckle trajectories from at least eight spindles per condition (see legend for Figure 5, B and D).

Microscopy and Quantifications

The images presented in Figures 2, A and B; 3, D, E, J, and K; Supplemental Figure S1H; and kymographs displayed in the Figure 5, A and C, were taken using MetaMorph Imaging software controlled Axiovert 135 microscope (Carl Zeiss) equipped with a CoolSNAP camera (Roper Scientific), a 63 $\times$ (100 $\times$ for Figure 5, A and C) Plan-apochromat NA 1.4 oil immersion objective lens. The other images were acquired using an SP5 confocal microscope (Leica, Wetzlar, Germany) equipped with a 63 $\times$ Plan-apochromat NA 1.4 oil immersion objective lens.

Intensities of CENP-E and EB1 bands (Supplemental Figure S2, A and B) were measured using ImageJ 1.38m (National Institutes of Health, Bethesda,

MD). MATLAB macros used to quantify spindle length and pole-to-pole profiles of tubulin density in the spindles were described previously (Caudron *et al.*, 2005).

RESULTS

XMAP215 and EB1 Nonredundantly Regulate Spindle Length and Integrity

We examined, by immunodepletion and overexpression experiments, the potential functional redundancy of EB1 and XMAP215 in the assembly of *Xenopus* egg extract spindles. Spindles were assembled in extracts cycled once through interphase and sent back to metaphase in the presence of sperm nuclei (Figure 1). Those spindles contain MTs that are nucleated around replicated chromosomes through the RanGTP pathway as well as MTs nucleated from the duplicated centrosomes (Hannak and Heald, 2006a).

Spindles assembled in extracts depleted of EB1 (Δ EB1) were $\sim 40\%$ shorter than those assembled in control extracts, treated with nonspecific antibodies (Δ IgG) (Figure 1, A–C). This phenotype was fully rescued by adding back 1.5–2 μ M recombinant EB1 to Δ EB1 extracts (Figure 1, A–C). Interestingly, adding a onefold excess of XMAP215 (100 nM) to a Δ EB1 extract also rescued spindle length (Figure 1, A–C). In Δ EB1 extracts, we also detected $\sim 30\%$ more tripolar and multipolar spindles than in the control (Figure 1D). The number of tripolar and multipolar spindles was reduced to control levels when EB1 or an excess of XMAP215 were added to EB1-depleted extracts (Figure 1D).

We then examined spindle assembly in extracts from which $\sim 70\%$ of XMAP215 was depleted (referred to as $\sim \Delta$ XMAP215 extracts) (Figure 1, E and F). The residual XMAP215 was necessary to ensure a minimal level of MT stabilization, because full depletion of XMAP215 led to complete abrogation of MT growth (Tournébeze *et al.*, 2000; Niethammer *et al.*, 2007). This caused an $\sim 40\%$ reduction in average spindle length, similar to what we observed in Δ EB1 extract (Figure 1, C and G). The decrease in spindle length was rescued by adding back XMAP215 as well as by adding a threefold excess of EB1 into $\sim \Delta$ XMAP215 extracts (Figure 1G). The latter spindles, however, displayed strikingly aberrant morphology (Figure 1E). Moreover, $\sim 34\%$ of the spindles formed in $\sim \Delta$ XMAP215 extracts were tripolar or multipolar. This could be rescued by recombinant XMAP215 addition (Figure 1H). Interestingly, the addition of excess EB1 to $\sim \Delta$ XMAP215 extracts did not restore spindle bipolarity to control levels (Figure 1H).

These results show that the full depletion of EB1 or a reduction in XMAP215 levels cause strikingly similar defects in spindle architecture. Physiological concentrations of both EB1 and XMAP215 are necessary for the maintenance of spindle length and bipolarity, in agreement with observations made in other organisms (Srayko *et al.*, 2003; Cassimeris and Morabito, 2004; Goshima *et al.*, 2005; Buster *et al.*, 2007). However, although overexpression of XMAP215 could rescue the spindle defects caused by EB1 depletion, the overexpression of EB1 does not rescue the phenotype of partial XMAP215 depletion.

We then wondered whether the observed decrease in spindle length was a consequence of global MT growth inhibition or of the involvement of XMAP215 and EB1 in local, e.g., chromatin- or centrosome-mediated MT-stabilizing pathways. To examine this question, we formed spindle-like structures in the presence of chromatin-coated beads (Supplemental Figure S1, A and D) (Heald *et al.*, 1996) or an excess of RanQ69L (Supplemental Figure S1, B and E) (Wilde

and Zheng, 1999). Chromatin beads support spindle assembly in the absence of centrosomes and kinetochores, whereas RanQ69L is a constitutively active form of Ran that induces aster formation and spindle assembly in the absence of centrosomes and bulk chromatin (Heald *et al.*, 1996; Carazo-Salas *et al.*, 1999). To assemble spindles around chromatin beads, CSF-arrested Δ EB1 or $\sim \Delta$ XMAP215 extracts were incubated with DNA beads previously chromatinized in accordingly depleted interphase extracts (Supplemental Figure S1, A and D). RanQ69L spindles were assembled in cycled Δ EB1 or $\sim \Delta$ XMAP215 extracts to which 15 μ M RanQ69L was added at reentry into mitosis (Supplemental Figure S1, B and E). Spindles formed around chromatin beads or by addition of RanQ69L in Δ EB1 extracts were $\sim 20\%$ shorter than corresponding control spindles (Supplemental Figure S1, A–C). Similarly, $\sim \Delta$ XMAP215 spindles assembled in the presence of chromatin beads or RanQ69L were ~ 40 and $\sim 30\%$ shorter than control spindles, respectively (Supplemental Figure S1, D–F). Together, these results show that complete depletion of EB1 or partial depletion of XMAP215 reduced the length of spindles assembled either in the presence of sperm, chromatin beads or RanQ69L. We conclude that the requirement for EB1 and XMAP215 in maintaining proper spindle length and integrity results from their global stabilizing effect on MTs that is exerted independently of centrosomes, kinetochores or chromatin-regulated stabilizing cues.

Δ EB1 and $\sim \Delta$ XMAP215 Spindles Display Reduced MT Density at Their Equator

We noticed that Δ EB1 and $\sim \Delta$ XMAP215 spindles were affected in their spatial organization of MTs (Figure 1, A and E). To analyze in detail how the distribution of MT density was compromised, we aligned 50 bipolar spindles per condition and then averaged the fluorescence intensity of Cy3-labeled tubulin incorporated by those spindles (Figure 2, A and B). The control spindles displayed uniform MT density, whereas in Δ EB1 spindles we observed that the tubulin density around the equator was decreased by $\sim 40\%$ relative to the poles (Figure 2A). Adding EB1 or an excess of XMAP215 to Δ EB1 extracts rescued this phenotype (Figure 2A).

MT density at the equator was also drastically reduced in $\sim \Delta$ XMAP215 spindles (Figure 2B). This phenotype was rescued by the add back of XMAP215 but not by addition of EB1 in excess (Figures 1F and 2B). Therefore, an excess of EB1 cannot fully compensate for reduced levels of XMAP215, indicating that EB1 and XMAP215 regulate spindle assembly in a nonredundant manner.

Interaction of EB1 and XMAP215 Is Required for Spindle Assembly

Because morphology defects are similar for Δ EB1 and $\sim \Delta$ XMAP215 spindles, we suspected that EB1 and XMAP215 contribute to the regulation of spindle assembly through their interaction. We have previously shown that EB1 and XMAP215 interact specifically in metaphase *Xenopus* egg extracts and that this interaction involves the C-EB1 (193–268 amino acids) (Niethammer *et al.*, 2007). This fragment contains a dimerization domain of EB1 and an EB1-like domain, which is conserved among the EB1 family members across species and mediates EB1 interaction with other MAPs. When added into metaphase extracts, C-EB1 sequesters the endogenous XMAP215 from its complex with endogenous EB1 and inhibits MT growth from the centrosome, thus conferring a dominant-negative effect comparable with the depletion of either EB1 or XMAP215 (Niethammer *et al.*, 2007).

Figure 1. Nonredundant roles of EB1 and XMAP215 in the regulation of spindle length and bipolarity. (A) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in control depleted (Δ IgG) or EB1 depleted (Δ EB1) extracts supplemented with either recombinant EB1 (wild-type levels, Δ EB1 + 1 $\times$ EB1) or a onefold excess of XMAP215 (Δ EB1 + 1 $\times$ XMAP215). Microtubules (red) are visualized by Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (B) Accompanying immunoblots of extracts shown in A. (C) Average length of sperm-spindles (conditions as indicated in A). Error bars represent SD (n = 50 bipolar spindles per condition; one representative experiment is shown). (D) Quantification of pole numbers in the spindles assembled around sperm nuclei in extracts treated as described in A. Error bars represent SD (n = 100 structures per condition; average of two independent experiments). (E) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in control depleted (Δ IgG) or $\sim$ 70% depleted XMAP215 ($\sim\Delta$ XMAP215) extracts supplemented either with recombinant XMAP215 (wild-type levels, $\sim\Delta$ XMAP215 + 1 $\times$ XMAP215) or a threefold excess EB1 ($\sim\Delta$ XMAP215 + 3 $\times$ EB1). Microtubules (red) are visualized by incorporated Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (F) Accompanying immunoblots of extracts shown in E. (G) Average length of sperm-spindles under the conditions described in E. Error bars represent SD (n = 50 bipolar spindles per condition; one representative experiment is shown). (H) Quantification of pole numbers in spindles formed around sperm nuclei in extracts treated as described in E. Error bars represent SD (n = 100 structures per condition; average of two independent experiments).

Therefore, we wondered how the perturbation of XMAP215–EB1 interaction by C-EB1 would affect spindle assembly. To this aim, we assembled spindles in cycled

metaphase extracts to which we added either 2 μ M GST-tagged C-EB1 or GST as a control. As expected, spindles formed in the presence of C-EB1 seemed very similar to

Figure 2. EB1 and XMAP215 are required for stable MT overlaps at the spindle equator. (A and B) Right (average spindle), spindle images obtained through aligning and averaging 50 bipolar sperm-spindles assembled under indicated conditions. The spindles were aligned by a custom-written MATLAB macro by using their center as a reference. Left (central longitudinal scan), a plot of the fluorescence intensity of Cy3-tubulin along spindle's pole-to-pole axis. a.u., arbitrary units.

Δ EB1 or $\sim\Delta$ XMAP215 spindles (Figure 3A, 1A, and 1E). We again observed characteristic defects in spindle length, stability and MT distribution (Figure 3, A–D). Namely, C-EB1-treated spindles were $\sim 34\%$ shorter than the control spindles (Figure 3B). The frequency of tripolar and multipolar spindles was also increased by $\sim 25\%$ comparing to the control and Cy3-tubulin fluorescence intensity at the spindle equator was dramatically decreased (Figure 3, C and D). Interestingly, chromatin-bead spindles assembled in the presence of C-EB1 were also $\sim 20\%$ shorter than the control spindles, although in contrast to the sperm-spindles but similarly to Δ EB1 chromatin bead spindles, they did not display reduced density of MTs at their equator (Supplemental Figure S1, H and I). If C-EB1 impairs spindle assembly by sequestering endogenous XMAP215, we would expect to rescue spindle assembly defects by adding a one or twofold excess of recombinant XMAP215 to extracts that contained C-EB1 (Figure 3E). Indeed, those spindles were comparable with control spindles, both in terms of their length and overall morphology (Figure 3, E–F). This observation supports our assumption that C-EB1 might destabilize spindles by competing away endogenous XMAP215 from its complex with the full-length EB1.

To further interfere with the interaction between EB1 and XMAP215, we have identified a fragment of XMAP215 that binds EB1. We prepared three His-tagged fragments of XMAP215, i.e., N-XMAP215 (1–560 aa), M-XMAP215 (543–1167 aa), and C-XMAP215 (1168–2065 aa), which we added at $1\ \mu\text{M}$ concentration to metaphase extracts from which we subsequently immunoprecipitated EB1. Western blot analysis of the immunoprecipitates revealed that only C-XMAP215 binds EB1 and simultaneously precludes the interaction between the endogenous XMAP215 and EB1 (Supplemental Figure S1G). Addition of $1\ \mu\text{M}$ C-XMAP215 to the extract had the same effect on spindle assembly as EB1 or XMAP215 depletion or C-EB1 addition (Figure 3, G–J).

The same amount of C-XMAP215 also led to a $\sim 25\%$ reduction in length of chromatin bead spindles, whereas their equatorial zone seemed unaffected, which was comparable with $\sim\Delta$ XMAP215 chromatin bead spindles (Supplemental Figure S1, H and I). We assumed that C-XMAP215 could perturb the assembly of spindles by obstructing endogenous EB1 from interacting with full-length XMAP215. Therefore, we reasoned that addition of onefold or twofold excess of recombinant EB1, which would interact with endogenous XMAP215, could rescue the spindle defects caused by the presence of C-XMAP215 (Figure 3K). Indeed, length reduction of C-XMAP215-spindles was partially rescued in the presence of excess EB1, although those spindles also displayed splayed poles (Figure 3, K–M). Therefore, we conclude from this experiment that EB1 and XMAP215 interaction participates in spindle length regulation (Figure 3, K and L). Together, the above-described findings indicate that at physiological levels XMAP215 and EB1 as a complex contribute to regulation of proper spindle architecture in *Xenopus* egg extracts.

EB1 Promotes Binding of XMAP215 to Spindle MTs

It has recently been shown that EB1 targets CLIP170 onto MT plus ends in *Xenopus* egg extracts (Bieling *et al.*, 2008). Because the interaction between XMAP215 and EB1 is required for MT dynamics regulation and spindle assembly in *Xenopus* extract, we wanted to test whether EB1 is required for proper localization of XMAP215 to the spindle MTs. By immunofluorescence, we visualized XMAP215 on spindles assembled in cycled *Xenopus* egg extracts, which were control depleted, EB1 depleted, or EB1 depleted and then supplemented with a onefold excess of XMAP215 (Figure 4, A and B). Under all tested conditions, XMAP215 was binding to the spindle MTs, to the spindle poles, and to the kinetochores (Figure 4A). However, the average ratio of spindle-associated XMAP215 to polymerized tubulin (measured as

Figure 3. Interaction between EB1 and XMAP215 is required for proper spindle assembly. (A) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in the presence of GST as a control or GST-tagged C-terminal fragment of EB1 (GST-C-EB1). Microtubules (red) are visualized by Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (B) Average length of metaphase sperm-spindles in response to treatments as indicated in A. Error bars represent SD ($n = 50$ bipolar spindles per condition; one representative experiment). (C) Quantification of pole numbers in spindles assembled around replicated sperm in the presence of GST as a control or GST-tagged C-terminal fragment of EB1 (GST-C-EB1). Error bars represent SD ($n = 100$ structures per condition; average of two independent experiments). (D) Average spindle image (right) and averaged Cy3-tubulin fluorescence intensity along spindle's pole-to-pole axis (central longitudinal scan, left). Analyzed spindles were assembled as described in A. (E) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in the presence of GST as a control or GST-C-EB1 and excess of XMAP215. Microtubules (red) are visualized by Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (F) Average length of metaphase sperm-spindles in response to treatments as indicated in E. Error bars represent SD ($n = 50$ bipolar spindles per condition; one representative experiment). (G) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in untreated extracts or in presence of C-terminal fragment of XMAP215 (C-XMAP215). Microtubules (red) are visualized by Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (H) Average lengths of spindles shown in G. Error bars represent SD ($n = 50$ bipolar spindles per condition; one representative experiment). (I) Quantification of pole numbers in the spindles shown in G. Error bars represent SD ($n = 100$ structures per condition; average of two independent experiments). (J) Average spindle image (right panel) and the plot of averaged Cy3-tubulin fluorescence along spindle's pole-to-pole axis (central longitudinal scan, left). Analyzed spindles were assembled as described in G. (K) Representative images of fixed metaphase-arrested spindles assembled around replicated sperm in untreated extracts or in the presence of C-XMAP215 and excess of EB1. Microtubules (red) are visualized by Cy3-tubulin and DNA (blue) is stained with Hoechst. Bar, 10 μ m. (L) Average length of metaphase sperm-spindles in response to treatments indicated in K. Error bars represent SD ($n = 50$ bipolar spindles per condition; one representative experiment). (M) Quantification of percentage of bipolar, multipolar, and unfocused spindles among those assembled under conditions shown in K. Error bars represent SD ($n = 50$ structures per condition; average of three independent experiments).

the ratio of the XMAP215 fluorescence in the spindle to Cy3-tubulin fluorescence intensity in the spindle) indicated that the absence of EB1 caused a strong decrease (by ~52%)

in the amount of spindle-associated XMAP215 (Figure 4C). The presence of XMAP215 was also reduced at the poles in Δ EB1 spindles, whereas its association with the kinetochores

Figure 4. EB1 increases binding of XMAP215 to the spindle microtubules. (A) Representative, maximum intensity Z-projections of fixed spindles assembled around sperm nuclei in cycled metaphase-arrested extracts that were control-depleted (Δ IgG), EB1-depleted (Δ EB1), or EB1-depleted and supplemented with a onefold excess of XMAP215 (Δ EB1 + 1x XMAP215). Microtubules are red (Cy3-tubulin), DNA is blue (Hoechst), and XMAP215 is shown in green (indirect immunofluorescence). Bar, 10 μ m. (B) Immunoblots of extracts treated as described above and probed with anti-XMAP215 and anti-EB1 antibody. (C) Average ratio of XMAP215 indirect fluorescence intensity and Cy3-tubulin fluorescence intensity calculated from maximum intensity Z-projections of spindles treated as described above. Error bars represent SD (n = 30 spindles per condition; one representative experiment).

Figure 5. Inhibition of poleward microtubule flux in spindles lacking overlapping microtubules at their equator. (A) Representative kymographs of spindles treated as indicated. The vertical axis of the kymograph corresponds to the time line of spindle imaging. The slope of speckle trajectories corresponds to the poleward microtubule flux rate. Bar, 5 μm . (B) Poleward microtubule flux rates measured for spindles assembled in control (ΔIgG) extracts (8 spindles, 2 different extracts, 222 speckle trajectories), ΔEB1 extracts ($n = 14$ spindles, 2 different extracts, 346 speckle trajectories), and ΔEB1 extracts supplemented either with EB1 (10 spindles, 2 different extracts, 290 speckle trajectories) or a onefold excess of XMAP215 (12 spindles, 2 different extracts, 298 speckle trajectories). Error bars show SD. (C) Representative kymographs of spindles treated as indicated. Bar, 5 μm . (D) Poleward microtubule flux rates measured for spindles assembled in ΔIgG control extracts ($n = 8$ spindles, 2 different extracts, 281 speckle trajectories), $\sim\Delta\text{XMAP215}$ extracts ($n = 11$ spindles, 2 different extracts, 329 speckle trajectories), and $\sim\Delta\text{XMAP215}$ extracts supplemented either with XMAP215 ($n = 8$ spindles, 2 different extracts, 202 speckle trajectories) or a threefold excess EB1 ($n = 11$ spindles, 2 different extracts, 354 speckle trajectories). Error bars show SD.

remained unchanged (Figure 4A). The reduced amount of spindle-associated XMAP215 in ΔEB1 spindles could partially be restored by the addition of a onefold excess XMAP215 (Figure 4, A and C). In conclusion, these data show that EB1 enhances binding of XMAP215 to the spindle MTs as well as that addition of excess XMAP215 is able to compensate for the reduced amount of XMAP215 on the ΔEB1 spindles.

Inhibition of Poleward MT Flux in ΔEB1 and $\sim\Delta\text{XMAP215}$ Spindles

In the spindle, MT plus ends point toward the spindle center, whereas their minus ends are directed to the poles. Antiparallel MTs overlap at the spindle equator and slide with respect to each other by the action of the bipolar kinesin Eg5 in a process called poleward MT flux (Sawin and Mitchison, 1991; Waterman-Storer *et al.*, 1998; Miyamoto *et al.*, 2004). Because spindles assembled in the absence of EB1 or at reduced level of XMAP215 showed a decreased MT density around their equator, we tested by fluorescence speckle microscopy whether the rate of poleward MT flux was also affected (Waterman-Storer *et al.*, 1998). Acquired spindle movies were used to prepare kymographs whose horizontal axis represents a line connecting two spindle poles, whereas their vertical axis corresponds to a period during which spindles were imaged (Figure 5, A and C). The

slope of concerted speckle movement in kymographs reflects the rate of poleward MT flux (Kapoor and Mitchison, 2001). Tubulin speckles in the control spindles displayed continuous poleward movement with an average speed of $2.3 \pm 0.8 \mu\text{m}/\text{min}$ (Figure 5B). Strikingly, in ΔEB1 spindles most of the speckle trajectories were vertical. This indicated an absence of MT poleward movement (Figure 5A). Indeed, we found an $\sim 70\%$ slower poleward flux in ΔEB1 spindles ($0.7 \pm 0.6 \mu\text{m}/\text{min}$) than in the control (Figure 5B). Addition of endogenous levels of EB1 or of an excess of XMAP215 to ΔEB1 extracts brought flux speed closer to the control (1.5 ± 0.6 and $1.5 \pm 0.9 \mu\text{m}/\text{min}$, respectively) (Figure 5, A and B). Poleward MT flux rate was also $\sim 78\%$ slower in $\sim\Delta\text{XMAP215}$ spindles ($0.4 \pm 0.3 \mu\text{m}/\text{min}$) than in the control ($1.8 \pm 0.8 \mu\text{m}/\text{min}$) (Figure 5, C and D). Adding back XMAP215 to $\sim\Delta\text{XMAP215}$ -depleted extract rescued poleward MT flux speed to control values ($1.5 \pm 0.7 \mu\text{m}/\text{min}$), whereas addition of EB1 in excess did not ($0.4 \pm 0.3 \mu\text{m}/\text{min}$) (Figure 5, C and D). In conclusion, we observed a strong reduction of poleward MT flux in spindles with diminished MT density at the equator (Figure 5, A–D).

Chromosome Segregation and Spindle Disassembly Are Inhibited in ΔEB1 and $\sim\Delta\text{XMAP215}$ Spindles

It is considered that poleward MT flux facilitates chromosome movement during anaphase in meiotic systems (Desai

Figure 6. EB1 and XMAP215 are required for chromosome segregation and spindle disassembly in *Xenopus* egg extracts. (A and B) Representative images of structures fixed 40 min after Ca^{2+} addition to metaphase-arrested extracts. Microtubules are visualized by Cy3-tubulin (red) and DNA is stained with Hoechst (blue). Bar, 10 μm . (C and D) Anti-cyclin B immunoblot of extracts after indicated treatments in metaphase (0 min, 0') and 40 min after anaphase onset was induced by Ca^{2+} addition (40').

et al., 1998). Because we observed an inhibition of poleward flux in Δ EB1 and $\sim\Delta$ XMAP215 spindles (Figure 5, A–D), we decided to follow them through anaphase by adding Ca^{2+} to the extracts (Figure 6, A and B). Under control conditions, 40 min after a Ca^{2+} pulse, chromatid segregation occurred and the observed elongation of centrosomal MTs indicated that the interphasic MT array started to reassemble (Figure 6A). Spindles persisted in a metaphase-like state without splitting of the chromosomal mass 40 min after Ca^{2+} addition to Δ EB1 extracts (Figure 6A). Those spindles were only $\sim 50\%$ of their metaphase length and consisted typically of fewer, strongly bundled MTs. In Δ EB1 extracts to which either EB1 or an excess of XMAP215 were added, chromosomes segregated and reconstitution of interphasic MT network was in progress 40 min after Ca^{2+} addition (Figure 6A).

Spindles did not disassemble and chromosomes segregation did not occur 40 min after Ca^{2+} was added to $\sim\Delta$ XMAP215 extracts (Figure 6B). At the same time, in control and XMAP215 add-back samples, MTs started to reorganize into longer, interphasic arrays and sister chromatids segregated. Conversely, addition of an excess EB1 into $\sim\Delta$ XMAP215 extracts did not rescue the phenotype we observed in $\sim\Delta$ XMAP215 extracts (Figure 6B). Together, these data indicate that spindles with substantial lack of MTs at their equator and significantly reduced rate of poleward MT

flux can neither segregate their chromosomes nor disassemble at anaphase onset.

Spindle Assembly Checkpoint in Δ EB1 and $\sim\Delta$ XMAP215 Extracts

The persistence of metaphase-like spindles in Δ EB1 or $\sim\Delta$ XMAP215 extracts after Ca^{2+} addition, suggests that some aspects of mitotic exit could be perturbed in the absence of either of these MAPs. Normally, the onset of anaphase is triggered through activation of the anaphase-promoting complex/cyclosome (APC/C), which targets a large number of mitosis-specific activities for degradation by the proteasome (Morgan, 1999). We wondered whether the APC/C was activated by Ca^{2+} addition in those samples. To test this possibility, we examined whether cyclin B, an important APC/C target, was degraded. Δ EB1 extracts did globally exit mitosis because cyclin B levels decreased after Ca^{2+} addition (Figure 6C). The same happened in extracts partially depleted of XMAP215 (Figure 6D). This demonstrated that the cytoplasm progressed into interphase. These results suggest that the absence of either EB1 or XMAP215 does not lead to a global inhibition of APC/C. Because the APC/C was not inhibited in the global cytoplasm, we suspected that metaphase arrest could be maintained locally

Figure 7. Mad2 localizes to kinetochores in the absence of EB1 or XMAP215. (A and C) Top, recruitment of Mad2 to kinetochores of metaphase-arrested spindles as visualized by immunofluorescence using anti-Mad2 antibody (green). Sperm nuclei are stained blue with Hoechst, and microtubules are red (Cy3-tubulin). Bar, 10 μ m. Bottom, magnification of a portion of the equatorial plate of the spindle displayed above. IF, immunofluorescence. Bar, 5 μ m. (B and D) Percentage of spindles with Mad2 signal on at least one kinetochore. Error bars represent SD ($n = 100$ spindles per condition; average of two independent experiments).

through the activation of the spindle assembly checkpoint (SAC) in the vicinity of the chromosomes.

To test whether defects in kinetochore–MT attachments could locally activate the SAC in Δ EB1 and $\sim\Delta$ XMAP215 extracts, we first monitored by immunofluorescence the recruitment of Mad2, a diffusible component of the checkpoint machinery, onto kinetochores (Chen *et al.*, 1996; Rieder and Maiato, 2004) (Figure 7, A and B). We detected Mad2 on at least one kinetochore in $\sim$ 58% of Δ EB1 spindles, whereas only $\sim$ 13% of control spindles had Mad2 on their kinetochores (Figure 7B). Once EB1 or excess of XMAP215 were added to Δ EB1 extracts, Mad2 was present on kinetochores of only $\sim$ 25% of the spindles (Figure 7B). Moreover, $\sim$ 42% of the spindles had Mad2-positive kinetochores in $\sim\Delta$ XMAP215 extracts, as well as in $\sim\Delta$ XMAP215 extracts to which an excess of EB1 was added (Figure 7, C and D). In the control and in the XMAP215 add-back samples, Mad2 was present on the kinetochores of only $\sim$ 15% of the spindles (Figure 7D). These results indicate that a fraction of the kinetochores is improperly attached to MTs in Δ EB1, $\sim\Delta$ XMAP215, and $\sim\Delta$ XMAP215 + 3x EB1 spindles.

If the SAC was activated around Δ EB1 or $\sim\Delta$ XMAP215 spindles, spindle-associated APC/C substrates should be locally protected from degradation. The kinetochore-associated, mitotic kinesin CENP-E is known to be degraded subsequently to cyclin B after the release of HeLa cells from nocodazole block (Brown *et al.*, 1994). Our experiments in *X. laevis* extracts showed that although the bulk of cyclin B and Xkid was degraded 20 min after anaphase onset, CENP-E levels decreased after 40 min, suggesting that CENP-E is a late APC/C substrate (Supplemental Figure S2, A and B). We tested by immunofluorescence whether CENP-E could be visualized on chromosome spreads of cycled metaphase and anaphase sperm-spindles assembled in Δ EB1 or $\sim\Delta$ XMAP215 extracts. In all metaphase samples, we could

observe paired foci of CENP-E, which corresponded to sister kinetochores (Supplemental Figure S2, C and E). The majority of chromosomes were thread-like and devoid of CENP-E signal in the anaphase control sample, whereas we noticed that chromosomes were mostly clumpy and CENP-E positive in Δ EB1, $\sim\Delta$ XMAP215, and $\sim\Delta$ XMAP215 + 3x EB1 samples. This result indicated that the kinetochore-associated fraction of CENP-E might be protected from APC/C-mediated targeting for proteasomal degradation (Supplemental Figure S2, C–F).

DISCUSSION

In this study, we demonstrated that at physiological levels of EB1 and XMAP215, their interaction is required for proper assembly of *Xenopus* egg extract spindles. The C-EB1 and C-XMAP215 fragments block the interaction between the endogenous EB1 and XMAP215 and cause defects in spindle architecture similar to those generated by the depletion of either EB1 or of $\sim$ 70% XMAP215 (Figures 1–3). Those spindles were $\sim$ 40% shorter than in the control, displayed reduced MT density at their equator and were frequently multipolar. The increase in multipolarity could be explained by the involvement of XMAP215 and EB1 in maintenance of spindle pole integrity because both proteins are known to localize to the spindle poles (Mimori-Kiyosue *et al.*, 2000; Tournebise *et al.*, 2000; Popov *et al.*, 2001, 2002; Rogers *et al.*, 2002; Tirnauer *et al.*, 2002). Alternatively, increased multipolarity could be caused by short and dynamic MTs that form weak antiparallel overlaps at the spindle equators leading to unstable bipolar regimes. Similarly to sperm-spindles, spindles assembled around chromatin beads in the presence of C-EB1 or C-XMAP215 as well as in Δ EB1 or $\sim\Delta$ XMAP215 extracts were $\sim$ 30% shorter than corresponding controls (Supplemental Figure S1). Surprisingly, the MT density at

their equator was not decreased. The drastic reduction of MT density we observe at the equator of sperm-spindles is probably not caused by kinetochore–MT defects, because kinetochore MTs represent only ~5% of MTs in *Xenopus* egg extract spindles (Ohi *et al.*, 2007). Distinct morphologies of spindles assembled in the presence of chromatin beads or sperm could be caused by a potentially different amount of chromatin and its different spatial organization.

We suspect that C-EB1-mediated obstruction of EB1 interaction with other MAPs (e.g., p150^{glued}, CLIP170, CLASPs, or APC) is not the main cause of the observed spindle defects, because their depletions do not lead to the phenotypes observed upon depletion of either EB1 or XMAP215 (Hannak and Heald, 2006b; Niethammer *et al.*, 2007; Akhmanova and Steinmetz, 2008). Also, we exclude the possibility that C-XMAP215 exerts its effect by interfering with the well-known interaction of XMAP215 with TACC3, because TACC3 depletion does not affect spindle length or morphology as C-XMAP215 addition does (Kinoshita *et al.*, 2005) (Figure 3, G–J). Moreover, adding an excess of XMAP215 into extracts containing C-EB1 rescued all the observed spindle assembly defects. Finally, overexpression of EB1 in the presence of C-XMAP215 partially restored decreased spindle length (Figure 3, K and L). These results further confirm that C-XMAP215 and C-EB1 cause spindle destabilization by, respectively, removing endogenous EB1 or XMAP215 from their complex. Interestingly, we also observed that C-XMAP215, particularly in the presence of excess EB1, induces formation of spindles with splayed poles (Figure 3, K–M). Therefore, C-XMAP215 might influence spindle pole focusing, which is an effect we have not observed when we partially depleted endogenous XMAP215 (Figure 1H). This unphysiological role of C-XMAP215 seems to be stimulated in the presence of excess EB1 whose overexpression in the control extracts does not cause unfocusing of spindle poles (data not shown).

We also showed here, through combinatorial add-back experiments, that EB1 and XMAP215 perform nonredundant functions in regulating proper spindle architecture (Figures 1 and 2). XMAP215 could compensate for loss of EB1 when present in excess, whereas an excess amount of EB1, even in partially depleted XMAP215 extracts, could not rescue any of the observed phenotypes, i.e. defects in spindle morphology, reduced rate of poleward MT flux and the absence of chromosome segregation upon anaphase induction. These data indicate that in metaphase *Xenopus* egg extracts, EB1 most likely does not directly influence MT dynamics, but instead enhances XMAP215 activity. Also, in vitro studies suggest that XMAP215 and EB1 regulate MT growth by fundamentally distinct mechanisms. XMAP215 can speed up MT growth in vitro through processive addition of individual tubulin dimers at the MT plus end (Brouhard *et al.*, 2008), whereas proteins of the EB family do not bind to unpolymerized tubulin and often do not strongly affect the MT growth rate in vitro, despite autonomous binding to growing MT ends (Bieling *et al.*, 2007; Niethammer *et al.*, 2007; Slep and Vale, 2007). Several groups showed that EB1 alone marginally promotes polymerization of pure tubulin in vitro, whereas it is a more efficient MT stabilizer in the presence of other MAPs (e.g., p150^{glued} or C-terminal fragment of adenomatous polyposis coli) (Nakamura *et al.*, 2001; Hayashi *et al.*, 2005). In addition, although EB1 binds throughout the cell cycle to MT ends (Kruljac-Letic, unpublished data), it affects MT growth exclusively during mitosis when it interacts with XMAP215 (Tirnauer *et al.*, 2002; Niethammer *et al.*, 2007). XMAP215 localizes to the MTs and centrosomes both in interphase and mitosis (Tour-

nebize *et al.*, 2000; Popov *et al.*, 2001). We showed that the presence of XMAP215 on MTs in Δ EB1 spindles was reduced by ~52% compared with the control. It is possible that EB1 enhances the processivity of XMAP215 (the number of rounds of tubulin addition) (Brouhard *et al.*, 2008), which would result in the increased steady-state amount of XMAP215 on the spindle. Interestingly another MAP, TACC3, was proposed to enhance XMAP215 activity at the centrosome (Kinoshita *et al.*, 2005; Peset *et al.*, 2005), indicating that the spatiotemporal modulation of XMAP215 activity might be an important mechanism for regulating MT growth.

We demonstrated that within the spindle, stimulation of XMAP215's MT-stabilizing activity by EB1 is required to maintain antiparallel MT overlaps at the spindle equator (Figures 2 and 3). This is in agreement with a recent mathematical model of spindle assembly that predicted sparse MT densities at the spindle equator as a consequence of a short average MT length (Burbank *et al.*, 2007). Most MTs at the spindle equator are organized in an antiparallel bundle, and they generate sliding forces required for poleward MT flux. Inhibition of the chromosomal pathway of MT nucleation caused a significant reduction in the density of antiparallel MTs at the spindle equator and led to inhibition of poleward MT flux (Mitchison *et al.*, 2004). Here, we have demonstrated that MT stabilizers EB1 and XMAP215 contribute to MT flux, probably by positively regulating the average MT length and thereby indirectly influencing the ability of MTs to engage into a stable anti-parallel interactions at the spindle equator (Figures 2–5). Alternatively, the role of XMAP215 in poleward MT flux could be more direct. For example, the *C. elegans* homologue of XMAP215 has been isolated from the midzone of anaphase spindles, which is enriched in strongly stabilized overlapping MTs (Skop *et al.*, 2004). Furthermore, it has been speculated that XMAP215 might be involved in cross-linking and thereby stabilizing antiparallel MTs (McNally, 2003). Previous work in *Drosophila* S2 cells indicated that EB1 is not involved in the regulation of poleward MT flux, whereas the XMAP215 homologue (Mps) promoted flux possibly by reducing pauses at MT plus and minus ends (Buster *et al.*, 2007). Taking into account quite distinct poleward MT flux rates between mitotic and meiotic spindles, it would not be surprising if poleward MT flux was regulated differently in various systems (Mitchison, 2005). To summarize, it seems that reduced XMAP215 activity (caused either by its partial depletion or by removing EB1) results in a shortening of spindle MTs and in consequent reduced stability of the antiparallel MT overlaps at the spindle equator. This, in turn, leads to an inhibition of the poleward MT flux and most probably to a decreased robustness of spindle bipolarity (Figures 1–5).

During anaphase in meiotic *Xenopus* egg extracts, chromosome segregation is mainly powered by the poleward MT flux (Desai *et al.*, 1998). Accordingly, we showed that after Ca²⁺ addition, the chromosomes are not segregated in spindles with strongly reduced poleward MT flux, (i.e., in Δ EB1, ~ Δ XMAP215 and ~ Δ XMAP215 + 3x EB1 spindles; Figure 6). Surprisingly, we have also observed that those spindles could not disassemble upon the induction of anaphase (Figures 5 and 6). The global levels of the APC/C substrate cyclin B dramatically decreased after Ca²⁺ addition to these extracts, whereas the kinetochore-associated fraction of CENP-E was still present on the chromosomes 25 min upon Ca²⁺ pulse (Figure 6 and Supplemental Figure S2). Interestingly, in the control sample we could not detect CENP-E on the kinetochores already 25 min after Ca²⁺ addition, although by Western blot we only observed a significant drop

40 min after Ca^{2+} pulse (Supplemental Figure S2). This result suggested that upon anaphase onset the kinetochore-associated CENP-E fraction could be degraded before its cytoplasmic pool. Alternatively, as suggested by Duesbery *et al.* (1997), kinetochore-associated *X. laevis* CENP-E could become masked at metaphase-to-anaphase II transition so that it cannot be recognized by anti-CENP-E antibody. In any case, the presence of CENP-E signal on the kinetochores of ΔEB1 and $\sim\Delta\text{XMAP215}$ chromosomes 25 min after Ca^{2+} pulse and its simultaneous absence from the control chromosomes indicates that depletion of EB1 or XMAP215 causes a delay in anaphase progression.

In fertilized *Xenopus* eggs, the initial embryonic cell divisions take place in the absence of a functional SAC, because the concentration of kinetochores that generate the biochemical signal leading to activation of the checkpoint within the large cytoplasmic volume of the egg is low compared with somatic cells. Nevertheless, *Xenopus* egg extracts are competent to activate SAC, provided that a sufficiently high concentration of sperm nuclei and thus kinetochores is added (Minshull *et al.*, 1994). Zhang *et al.* (2007), by using high sperm concentrations, showed that EB1 depletion arrests *Xenopus* egg extract spindles in metaphase. Our results suggest that even at low sperm concentration in the absence of EB1 or XMAP215, the strong reduction in MT density at the spindle equator prevents normal kinetochore-MT attachments, which might keep the SAC active in the vicinity of chromosomes (Figures 6 and 7). This local activation might be sufficient to allow for a persistence of metaphase-like spindles, whereas the bulk of the extracts progressed into interphase. Alternatively, spindles might shrink and fail to disassemble upon Ca^{2+} addition in ΔEB1 and $\sim\Delta\text{XMAP215}$ extracts because XMAP215 and EB1 may be required for spindle elongation during anaphase. This has been shown for the *S. cerevisiae* XMAP215 homologue Stu2 (Severin *et al.*, 2001). Stu2 mutants arrest in metaphase in a Mad2-dependent manner, whereas mutants for both Stu2 and Mad2 overcome SAC and separate sister chromatids timely, albeit in the absence of spindle elongation. A majority of the anaphase spindles elongated once a mutation for the microtubule destabilizer Kip3 was introduced into the *stu2-mad2* double mutants, suggesting that Stu2 promotes MT growth during anaphase. It is therefore conceivable that XMAP215 and possibly EB1 are required not only for metaphase but also for anaphase MT stabilization in *Xenopus* egg extracts. Clearly, the XMAP215-EB1 interaction allows a precise adjustment of MT dynamics, which is required for the stabilization of antiparallel MT overlaps in the spindle midzone, a key prerequisite for robust spindle bipolarity and proper chromosome segregation.

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REFERENCES

Akhmanova, A., and Steinmetz, M. O. (2008). Tracking the ends: a dynamic protein network controls the fate of microtubule tips. *Nat. Rev. Mol. Cell Biol.* 9, 309–322.

Bieling, P., Kandels-Lewis, S., Telley, I. A., van Dijk, J., Janke, C., and Surrey, T. (2008). CLIP-170 tracks growing microtubule ends by dynamically recognizing composite EB1/tubulin-binding sites. *J. Cell Biol.* 183, 1223–1233.

Bieling, P., Laan, L., Schek, H., Munteanu, E. L., Sandblad, L., Dogterom, M., Brunner, D., and Surrey, T. (2007). Reconstitution of a microtubule plus-end tracking system in vitro. *Nature* 450, 1100–1105.

Brouhard, G. J., Stear, J. H., Noetzel, T. L., Al-Bassam, J., Kinoshita, K., Harrison, S. C., Howard, J., and Hyman, A. A. (2008). XMAP215 is a processive microtubule polymerase. *Cell* 132, 79–88.

Brown, K. D., Coulson, R. M., Yen, T. J., and Cleveland, D. W. (1994). Cyclin-like accumulation and loss of the putative kinetochore motor CENP-E results from coupling continuous synthesis with specific degradation at the end of mitosis. *J. Cell Biol.* 125, 1303–1312.

Burbank, K. S., Mitchison, T. J., and Fisher, D. S. (2007). Slide-and-cluster models for spindle assembly. *Curr. Biol.* 17, 1373–1383.

Buster, D. W., Zhang, D., and Sharp, D. J. (2007). Poleward tubulin flux in spindles: regulation and function in mitotic cells. *Mol. Biol. Cell* 18, 3094–3104.

Carazo-Salas, R. E., Guarguaglini, G., Gruss, O. J., Segref, A., Karsenti, E., and Mattaj, I. W. (1999). Generation of GTP-bound Ran by RCC1 is required for chromatin-induced mitotic spindle formation. *Nature* 400, 178–181.

Carvalho, P., Tirnauer, J. S., and Pellman, D. (2003). Surfing on microtubule ends. *Trends Cell Biol.* 13, 229–237.

Cassimeris, L., and Morabito, J. (2004). TOGp, the human homologue of XMAP215/Dis1, is required for centrosome integrity, spindle pole organization, and bipolar spindle assembly. *Mol. Biol. Cell* 15, 1580–1590.

Castoldi, M., and Popov, A. V. (2003). Purification of brain tubulin through two cycles of polymerization-depolymerization in a high-molarity buffer. *Protein Expr. Purif.* 32, 83–88.

Caudron, M., Bunt, G., Bastiaens, P., and Karsenti, E. (2005). Spatial coordination of spindle assembly by chromosome-mediated signaling gradients. *Science* 309, 1373–1376.

Chen, R. H., Waters, J. C., Salmon, E. D., and Murray, A. W. (1996). Association of spindle assembly checkpoint component XMAP215 with unattached kinetochores. *Science* 274, 242–246.

Desai, A., Maddox, P. S., Mitchison, T. J., and Salmon, E. D. (1998). Anaphase A chromosome movement and poleward spindle microtubule flux occur at similar rates in *Xenopus* extract spindles. *J. Cell Biol.* 141, 703–713.

Duesbery, N. S., Choi, T., Brown, K. D., Wood, K. W., Resau, J., Fukasawa, K., Cleveland, D. W., and Vande Woude, G. F. (1997). CENP-E is an essential kinetochore motor in maturing oocytes and is masked during mos-dependent, cell cycle arrest at metaphase II. *Proc. Natl. Acad. Sci. USA* 94, 9165–9170.

Garcia, M. A., Vardy, L., Koonrugs, N., and Toda, T. (2001). Fission yeast ch-TOG/XMAP215 homologue Alp14 connects mitotic spindles with the kinetochore and is a component of the Mad2-dependent spindle checkpoint. *EMBO J.* 20, 3389–3401.

Goshima, G., Wollman, R., Stuurman, N., Scholey, J. M., and Vale, R. D. (2005). Length control of the metaphase spindle. *Curr. Biol.* 15, 1979–1988.

Hannak, E., and Heald, R. (2006a). Investigating mitotic spindle assembly and function in vitro using *Xenopus laevis* egg extracts. *Nat. Protoc.* 1, 2305–2314.

Hannak, E., and Heald, R. (2006b). Xorbit/CLASP links dynamic microtubules to chromosomes in the *Xenopus* meiotic spindle. *J. Cell Biol.* 172, 19–25.

Harlow, E., and Lane, D. (1999). *Using Antibodies: A Laboratory Manual*, Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Hayashi, I., Wilde, A., Mal, T. K., and Ikura, M. (2005). Structural basis for the activation of microtubule assembly by the EB1 and p150Glued complex. *Mol. Cell* 19, 449–460.

Heald, R., Tournebise, R., Blank, T., Sandaltzopoulos, R., Becker, P., Hyman, A., and Karsenti, E. (1996). Self-organization of microtubules into bipolar spindles around artificial chromosomes in *Xenopus* egg extracts. *Nature* 382, 420–425.

Howard, J., and Hyman, A. A. (2007). Microtubule polymerases and depolymerases. *Curr. Opin. Cell Biol.* 19, 31–35.

Hyman, A., Drechsel, D., Kellogg, D., Salser, S., Sawin, K., Steffen, P., Wordeman, L., and Mitchison, T. (1991). Preparation of modified tubulins. *Methods Enzymol.* 196, 478–485.

Kapoor, T. M., and Mitchison, T. J. (2001). Eg5 is static in bipolar spindles relative to tubulin: evidence for a static spindle matrix. *J. Cell Biol.* 154, 1125–1133.

Karsenti, E., and Vernos, I. (2001). The mitotic spindle: a self-made machine. *Science* 294, 543–547.

Kinoshita, K., Noetzel, T. L., Pelletier, L., Mechtler, K., Drechsel, D. N., Schwager, A., Lee, M., Raff, J. W., and Hyman, A. A. (2005). Aurora A

- phosphorylation of TACC3/maskin is required for centrosome-dependent microtubule assembly in mitosis. *J. Cell Biol.* 170, 1047–1055.
- Kwon, M., and Scholey, J. M. (2004). Spindle mechanics and dynamics during mitosis in *Drosophila*. *Trends Cell Biol.* 14, 194–205.
- Lee, T., Langford, K. J., Askham, J. M., Bruning-Richardson, A., and Morrison, E. E. (2008). MCAK associates with EB1. *Oncogene* 27, 2494–2500.
- Maresca, T. J., and Heald, R. (2006). Methods for studying spindle assembly and chromosome condensation in *Xenopus* egg extracts. *Methods Mol. Biol.* 322, 459–474.
- Matthews, L. R., Carter, P., Thierry-Mieg, D., and Kempfues, K. (1998). ZYG-9, a *Caenorhabditis elegans* protein required for microtubule organization and function, is a component of meiotic and mitotic spindle poles. *J. Cell Biol.* 141, 1159–1168.
- McNally, F. (2003). Microtubule dynamics: new surprises from an old MAP. *Curr. Biol.* 13, R597–599.
- Mimori-Kiyosue, Y., Shiina, N., and Tsukita, S. (2000). The dynamic behavior of the APC-binding protein EB1 on the distal ends of microtubules. *Curr. Biol.* 10, 865–868.
- Minshull, J., Sun, H., Tonks, N. K., and Murray, A. W. (1994). A MAP kinase-dependent spindle assembly checkpoint in *Xenopus* egg extracts. *Cell* 79, 475–486.
- Mitchison, T. J. (2005). Mechanism and function of poleward flux in *Xenopus* extract meiotic spindles. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 360, 623–629.
- Mitchison, T. J., Maddox, P., Groen, A., Cameron, L., Perlman, Z., Ohi, R., Desai, A., Salmon, E. D., and Kapoor, T. M. (2004). Bipolarization and poleward flux correlate during *Xenopus* extract spindle assembly. *Mol. Biol. Cell* 15, 5603–5615.
- Miyamoto, D. T., Perlman, Z. E., Burbank, K. S., Groen, A. C., and Mitchison, T. J. (2004). The kinesin Eg5 drives poleward microtubule flux in *Xenopus laevis* egg extract spindles. *J. Cell Biol.* 167, 813–818.
- Morgan, D. O. (1999). Regulation of the APC and the exit from mitosis. *Nat. Cell Biol.* 1, E47–53.
- Nakamura, M., Zhou, X. Z., and Lu, K. P. (2001). Critical role for the EB1 and APC interaction in the regulation of microtubule polymerization. *Curr. Biol.* 11, 1062–1067.
- Niethammer, P., Kronja, I., Kandels-Lewis, S., Rybina, S., Bastiaens, P., and Karsenti, E. (2007). Discrete states of a protein interaction network govern interphase and mitotic microtubule dynamics. *PLoS Biol.* 5, e29.
- Ohi, R., Burbank, K., Liu, Q., and Mitchison, T. J. (2007). Nonredundant functions of Kinesin-13s during meiotic spindle assembly. *Curr. Biol.* 17, 953–959.
- Peset, I., Seiler, J., Sardon, T., Bejarano, L. A., Rybina, S., and Vernos, I. (2005). Function and regulation of Maskin, a TACC family protein, in microtubule growth during mitosis. *J. Cell Biol.* 170, 1057–1066.
- Popov, A. V., Pozniakovsky, A., Arnal, I., Antony, C., Ashford, A. J., Kinoshita, K., Tournebise, R., Hyman, A. A., and Karsenti, E. (2001). XMAP215 regulates microtubule dynamics through two distinct domains. *EMBO J.* 20, 397–410.
- Popov, A. V., Severin, F., and Karsenti, E. (2002). XMAP215 is required for the microtubule-nucleating activity of centrosomes. *Curr. Biol.* 12, 1326–1330.
- Ribbeck, K., *et al.* (2006). NuSAP, a mitotic RanGTP target that stabilizes and cross-links microtubules. *Mol. Biol. Cell* 17, 2646–2660.
- Rieder, C. L., and Maiato, H. (2004). Stuck in division or passing through: what happens when cells cannot satisfy the spindle assembly checkpoint. *Dev. Cell* 7, 637–651.
- Rogers, S. L., Rogers, G. C., Sharp, D. J., and Vale, R. D. (2002). *Drosophila* EB1 is important for proper assembly, dynamics, and positioning of the mitotic spindle. *J. Cell Biol.* 158, 873–884.
- Sawin, K. E., and Mitchison, T. J. (1991). Poleward microtubule flux mitotic spindles assembled in vitro. *J. Cell Biol.* 112, 941–954.
- Scholey, J. M., Brust-Mascher, I., and Mogilner, A. (2003). Cell division. *Nature* 422, 746–752.
- Severin, F., Habermann, B., Huffaker, T., and Hyman, T. (2001). Stu2 promotes mitotic spindle elongation in anaphase. *J. Cell Biol.* 153, 435–442.
- Skop, A. R., Liu, H., Yates, J., 3rd, Meyer, B. J., and Heald, R. (2004). Dissection of the mammalian midbody proteome reveals conserved cytokinesis mechanisms. *Science* 305, 61–66.
- Slep, K. C., and Vale, R. D. (2007). Structural basis of microtubule plus end tracking by XMAP215, CLIP-170, and EB1. *Mol. Cell* 27, 976–991.
- Srayko, M., Quintin, S., Schwager, A., and Hyman, A. A. (2003). *Caenorhabditis elegans* TAC-1 and ZYG-9 form a complex that is essential for long astral and spindle microtubules. *Curr. Biol.* 13, 1506–1511.
- Tirnauer, J. S., Grego, S., Salmon, E. D., and Mitchison, T. J. (2002). EB1-microtubule interactions in *Xenopus* egg extracts: role of EB1 in microtubule stabilization and mechanisms of targeting to microtubules. *Mol. Biol. Cell* 13, 3614–3626.
- Tournebise, R., Popov, A., Kinoshita, K., Ashford, A. J., Rybina, S., Pozniakovsky, A., Mayer, T. U., Walczak, C. E., Karsenti, E., and Hyman, A. A. (2000). Control of microtubule dynamics by the antagonistic activities of XMAP215 and XKCM1 in *Xenopus* egg extracts. *Nat. Cell Biol.* 2, 13–19.
- Vasquez, R. J., Gard, D. L., and Cassimeris, L. (1999). Phosphorylation by CDK1 regulates XMAP215 function in vitro. *Cell Motil. Cytoskeleton* 43, 310–321.
- Waterman-Storer, C. M., Desai, A., Bulinski, J. C., and Salmon, E. D. (1998). Fluorescent speckle microscopy, a method to visualize the dynamics of protein assemblies in living cells. *Curr. Biol.* 8, 1227–1230.
- Watson, P., and Stephens, D. J. (2006). Microtubule plus-end loading of p150(Glued) is mediated by EB1 and CLIP-170 but is not required for intracellular membrane traffic in mammalian cells. *J. Cell Sci.* 119, 2758–2767.
- Wilde, A., and Zheng, Y. (1999). Stimulation of microtubule aster formation and spindle assembly by the small GTPase Ran. *Science* 284, 1359–1362.
- Wittmann, T., Hyman, A., and Desai, A. (2001). The spindle: a dynamic assembly of microtubules and motors. *Nat. Cell Biol.* 3, E28–34.
- Zhang, J., Ahmad, S., and Mao, Y. (2007). BubR1 and APC/EB1 cooperate to maintain metaphase chromosome alignment. *J. Cell Biol.* 178, 773–784.